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REVIEW

Membrane changes under oxidative stress: the impact of oxidized lipids

Rosangela Itri · Helena C. Junqueira · Omar Mertins · Maurício S. Baptista

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Abstract Studying photosensitized oxidation of unsaturated phospholipids is of importance for understanding the basic processes underlying photodynamic therapy, photoaging and many other biological dysfunctions. In this review we show that the giant unilamellar vesicle, when used as a simplified model of biological membranes, is a powerful tool to investigate how in situ photogenerated oxidative species impact the phospholipid bilayer. The extent of membrane damage can be modulated by choosing a specific photosensitizer (PS) which is activated by light irradiation and can react by either type I and or type II mechanism. We will show that type II PS generates only singlet oxygen which reacts to the phospholipid acyl double bond. The byproduct thus formed is a lipid hydroperoxide which accumulates in the membrane as a function of singlet oxygen production and induces an increase in its area without significantly affecting membrane permeability. The presence of a lipid hydroperoxide can also play an important role in the formation of the lipid domain for mimetic plasma membranes. Lipid hydroperoxides can be also transformed in shortened chain compounds, such as aldehydes and carboxylic acids, in the presence of a PS that reacts via the type I mechanism. The presence of such byproducts may form hydrophilic pores in the membrane for moderate oxidative stress or promote membrane disruption for massive oxidation. Our results provide a new tool to explore membrane response to an oxidative stress and may have implications in biological signaling of redox misbalance.

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R. Itri (✉) · H. C. Junqueira · O. Mertins
Departamento de Física Aplicada, Instituto de Física,
Universidade de São Paulo, São Paulo, Brazil
e-mail: itri@if.usp.br

M. S. Baptista
Departamento de Bioquímica, Instituto de Química,
Universidade de São Paulo, São Paulo, Brazil

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Introduction

It is well known that solar radiation is critical to maintaining life on Earth. However, too much of this radiation is associated with several deleterious effects on living organisms, including damage of the photosynthetic apparatus in plants and loss of homeostasis of cells of the basal layer and dermis, leading to photoaging and skin cancer in humans (Barber and Andersson 1992; Baier et al. 2006; Hsiai and Berliner 2007; Ibuki et al. 2007; Uchoa et al. 2008). Many of the reactions that occur after light absorption are related to the photosensitization phenomenon, which involves the transfer of energy absorbed by a photosensitizer (PS) to neighboring molecules, allowing the PS to return to the ground state and to absorb another photon (Foote 1968).

The photosensitizing reactions can also be used to treat diseases by photodynamic therapy (PDT) (Dougherty et al. 1998; Tardivo and Baptista 2008; Al-Omari 2013). PDT is based on the photooxidation of biological matter and requires the presence of a sensitizer, light irradiation and oxygen in the tissue being treated. The PS absorbs light, thereby generating reactive oxygen species (ROS), such as singlet oxygen ($^2O_1$), hydroxyl radicals (OH•) and the superoxide anion (O_2-•). $^1O_2$ is considered to be the most significant cytotoxic agent involved in PDT (Kochevar et al. 1996; Ermilov et al. 2004). It is locally generated, causing damage to cellular functions and vital structures and, consequently, the direct destruction of the tumor tissue.

Various types of tetrapyrrolic compounds, such as phthalocyanines, chlorins and porphyrins, are currently employed as PSs in PDT (Bonnett 1995; Berg et al. 2005). They have a

high intersystem crossing quantum yield and a long triplet lifetime, which leads to the production of a high singlet oxygen quantum yield. Of note, several protocols have also been developed based on inexpensive PSs by using phenothiazinium derivatives, such as methylene blue (MB; Tardivo et al. 2004, 2006; Tardivo and Baptista 2009; Baptista and Wainwright 2011; Song et al. 2011). The processes related to action mechanisms of PDT have been known about for a long time and have been studied in great detail. However, because of the complexity presented in biological systems, i.e., local concentration of oxygen, PS, light doses, unknown biological targets and unfixed variables, details of how the reactions operate in biological systems are still a matter of conjecture. As a result, the lack of detailed mechanistic knowledge is preventing the full potential exploitation of this methodology for the treatment of various diseases.

The search for new PSs remains focused on seeking compounds that are able to produce singlet oxygen at great efficiency (Allison et al. 2004; Allison and Sibata 2010; Pereira et al. 2010). Recent results have shown that there is a potential to enhance the efficiency of cell death by PDT by increasing the specificity of the reaction instead of increasing the amount of ROS (Engelmann et al. 2007; Pavani et al. 2009; 2012; Oliveira et al. 2011; Deda et al. 2013). For example, a poor PS that generates <0.05 % of triplets and singlet oxygen (crystal violet) but which is localized exclusively and is active in mitochondria is more efficient than a PS which generates a considerable amount of triplets and singlet oxygen (50 %; MB) but which is reduced and consequently inactive in mitochondria at low concentrations (Oliveira et al. 2011). Thus, it would appear more appropriate to seek for a better understanding of the PS action mechanism in order to obtain improved selectivity in the induction of a cell death, rather than generating large amounts of singlet oxygen nonspecifically. Because the main target of PDT action remains the membranes (Thorpe et al. 1995), it is fundamental to understand how membrane structure and properties are affected by photosensitization reactions (Engelmann et al. 2007; Pavani et al. 2009).

Living organisms also have natural PSs that are present in the skin (Wondrak et al. 2006). In spite of the great interest in the photo-protection area by researchers and industry, knowledge of the processes occurring in the skin upon exposure to the sun is still incipient (Halliday et al. 2005; Wondrak et al. 2006). Consequently, sun protection strategies remain incomplete. In the case of the UVB region, light is absorbed directly by the DNA, causing cycloaddition reactions and the formation of mutagenic compounds. In the case of UVA, the effect of sun exposition is due to the photosensitization reactions. The strategies against UVA damage are starting to be implemented, however, there is already conclusive information indicating that visible light also sensitizes natural PSs, such as melanin (Mahmoud et al. 2008; Chiarelli-Neto et al. 2011). The main target of the reactive species generated by the photosensitization reactions are also on membranes.

It is well known that membranes are essential components of living systems, separating the cytoplasm from the external environment, but also surrounding the cell organelles. The matrix of the cell membrane is a phospholipid bilayer that contains proteins and other molecules. Structural changes in the phospholipids promoted by oxidative reactions are known (Girotti 1998), but how their chemical modifications impact the membrane must be unveiled. It has been shown that the intensive peroxidation of phospholipids in membranes composed of phosphatidylethanolamine may promote a loss of order in the system, possibly by the formation of intermediate nonbilayer structures, such as inverted micelles (van Duijn et al. 1984). More recently, reorientation of oxidatively modified acyl chains in monolayers towards the aqueous phase was experimentally characterized by Langmuir balance (Sabatini et al. 2006). Indeed, Molecular dynamic (MD) simulations have provided evidence that oxidized lipid tails with a more polar character migrate towards the water phase, thereby promoting an increase in the mean lipid molecular area, accompanied by a decrease of the lipid bilayer thickness (Wong-Ekkabut et al. 2007; Beranova et al. 2010). As a consequence, lipid packing defects upsurge in the membrane, thus favoring an increase in membrane permeability (Chatterjee and Agarwal 1988 and references therein). Further, MD simulations have also shown that massive oxidation of phosphatidylcholines may promote pores in the membrane (Vernier et al. 2009) and, depending on its extent and molecular features, may destabilize the lipid bilayer, leading to its disruption (Cwiklik and Jungwirth 2010). Interestingly, it has also been shown that the presence of oxidized lipids in living cells enhances the susceptibility of the membrane to electroporation (Vernier et al. 2009). In addition, combining results from fluorescence spectroscopy and MD simulations have demonstrated that oxidized lipids with shortened acyl chain may facilitate lipid flip-flop in liposomes (Volinsky et al. 2011). Therefore, the presence of oxidized lipids in the membrane may cause the loss of lipid asymmetry in the bilayer which, in turn, plays an important role in cell physiology as apoptosis signaling.

Megli et al. (2005) also observed experimentally by electron paramagnetic resonance that phospholipid multilamellar vesicles made of linoleyl polycarbonate (PC) and DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) containing certain percentages of truncated oxidized phospholipids (typically from 5–15 %) are phase-separated. More recently, Volinsky et al. (2012) modeled plasma membrane domain organization using Langmuir monolayers of ternary 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/sphingomyelin/cholesterol and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)/sphingomyelin/cholesterol mixtures. These authors showed that the presence of oxidatively

truncated phosphatidylcholine in the mixtures promotes sphingomyelin/cholesterol-rich domains similar to those observed in plasma membrane rafts (Simons and Ikonen 1997; Brown and London 1998; Silvius 2005), pointing out that phase separation of sphingomyelin/cholesterol was first reported by Estep et al. (1979) by means of high-sensitivity scanning calorimetry experiments. It is worthy of note that changes in the lipid composition of the rafts have been increasingly related with cell signaling processes (Dykstra et al. 2003). 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 (Gajate et al. 2009).

Therefore, to unravel the action mechanisms of oxidized phospholipids in cells it is imperative to follow the effects of oxidation on membranes coupled to molecular changes. It should be stressed that while a sound knowledge of these effects are important for comprehending basic processes underlying photodynamic therapy and photoaging, oxidized lipids are also involved in Alzheimer disease (Markesbery and Carney 1999), in atherosclerotic lesions and in the suppression of mitochondrial function (Chen et al. 2009), among other biological dysfunctions.

The main purpose of this review is to describe the advances that have been made in the field of giant unilamellar vesicles (GUVs) used as a model of phospholipid membranes; such modeling has allowed the physical damage to membranes promoted by in situ oxidative species generation to be explored. We start by describing in detail the photosensitization reactions and the main products of these reactions, as well as the reactions that are known to happen with the phospholipid molecules, thus generating byproducts. We then report the main discoveries that have been realized by using this experimental model. Starting with the development of the model, i.e. visual inspection of photosensitized GUVs by microscopy, we evolve to describe the physical changes that are involved when POPC and DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) membranes are photosensitized (Caetano et al. 2007; Riske et al. 2009; Mertins et al. 2014), noting that these phospholipids contain one and two unsaturated acyl chains, respectively. Moreover, we also show that lipid hydroperoxidation may affect lipid organization within the lipid bilayer composed of ternary mixture of POPC/DPPC/cholesterol, promoting liquid ordered (Lo)–liquid disordered (Ld) phase coexistence (Haluska et al. 2012). These results reinforce the notion that a redox misbalance may have important implications in the rafts organization and, hence, in cell signaling.

Photosensitization reactions

Most of the photosensitization reactions that cause damage in living organisms involve the absorption of light by a PS and the formation of excited triplet species (Foote 1968; Barber and Andersson 1992; Uchoa et al. 2008). Triplets are stronger oxidant and reducing agents than the ground state species and can react with biological targets by electron transfer reactions to form radical species, damaging the structure and function of biomolecules. This is exemplified in Fig. 1 on the electron occupying an excited state orbital (better reducing agent) and/or leaving a vacancy in the ground state (better oxidizing agent). Triplets also efficiently transfer energy to oxygen, form singlet oxygen which is then able to efficiently add to the double bonds of lipids, nucleic acids and proteins, forming peroxides. Therefore, it is possible to separate the mechanisms of photochemical attack to biomolecules into two classes—type I and type II mechanisms (Foote 1968; Tardivo et al. 2005). In the type I mechanism, light energy passes from the excited molecules to biomolecules by electron transfer reactions; this energy can cause the formation of free radicals and, consequently, inflict damage directly to biomolecules; alternatively, damage can be inflicted indirectly through the formation of other reactive oxygen species, such as hydroxil radicals, that will cause the damage. In the type II mechanism, excitation energy is transferred to molecular oxygen, resulting in the formation of singlet oxygen $^1O_2$, which is very electrophilic, being able to cause damage to membranes, proteins and DNA (Fig. 1) (Foote 1968, 1976; Tardivo et al. 2005).

By presenting an empty orbital, $^1O_2$ sets no impediment to react with double bonds. Note that the oxygen molecule in the ground state has two semi-occupied orbitals (Fig. 1) and cannot receive two electrons with opposite spins, as those present in a double bond (Foote 1976; Harding and Goddard 1980; Frimer and Stephenson 1985; Laing 1989; Wilkinson et al. 1995). Thus, the main mechanism of reaction of singlet oxygen is adding to the double bonds of lipids, proteins and nucleic acids. Thereafter, the exact reaction depends on the nature of the double bond. Other reactions can also occur, such as the oxidation of organic sulfide compounds to sulfoxides, which usually occurs by the intermediate persulfoxide species (Clennan 2001). There are three main types of addition reactions (1–3) and an electron transfer reaction (4):

1. The reaction called “ene”, which is characterized by a hydrogen abstraction followed by addition. The double bond shifts to neighboring carbons and a hydroperoxide is produced.
2. Diels–Alder addition, which adds singlet oxygen at the ends of two conjugated double bonds to form an endoperoxide. Such compounds may be stable or unstable.
3. Addition to an activated double bond forming a dioxetane, which usually leads to other decomposition products.
4. Electronic transfer—i.e. singlet oxygen can abstract an electron from many organic compounds (Foote 1976;

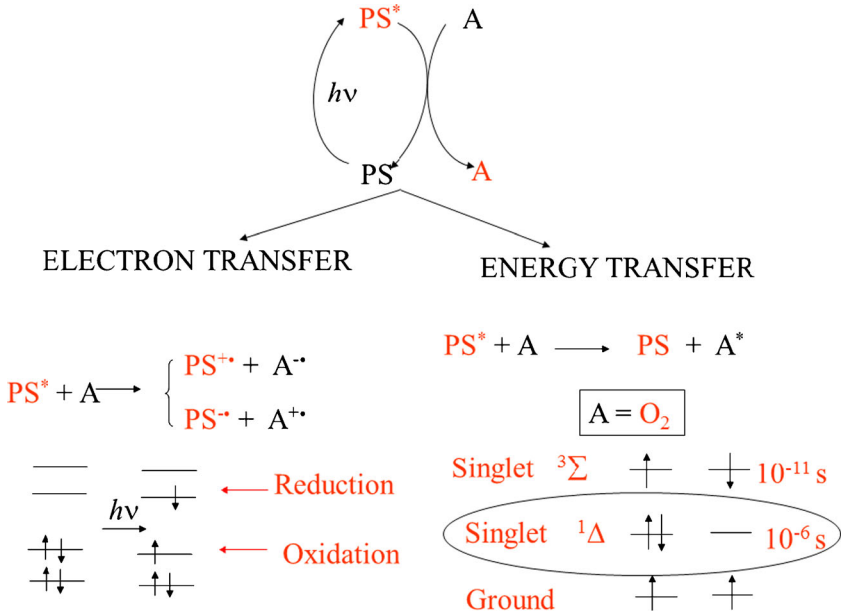

Fig. 1 Main reaction scheme of photosensitization reactions. Photosensitization reactions involve the absorption of electronic energy by a photosensitizer (*PS*) and the formation of excited triplet species (*PS**). Triplets are stronger oxidant and reducing species than the ground state species and can react with biological targets by electron transfer reactions to form radical species that cause damage in biomolecules. Triplets also efficiently transfer energy to oxygen, forming singlet oxygen ($^1O_2$) which is able to efficiently add to the double bonds of lipids, nucleic acids and proteins to form peroxides. The lifetime of the singlet oxygen is given in the *lower right corner* of the figure. *A* Acceptor

Harding and Goddard 1980; Frimer and Stephenson 1985; Laing 1989; Wilkinson et al. 1995; Clennan 2001).

Because of the high oxygen concentration at the beginning of the PDT, reaction with oxygen is the most probable route of triple deactivation leading to type II mechanism—i.e. formation of singlet oxygen. Although type II processes are well established and mainly considered in the development of new PDT PSs, there is some evidence that the oxidation of membranes culminating in disruption may need both type I and type II mechanisms (Castano et al. 2004). Evidence for this concept is presented and discussed in this review.

Lipids, including cholesterol and phospholipids with unsaturated chains, are thus important targets of $^1O_2$. Singlet oxygen reacts with unsaturated lipids by the "ene" reaction to form lipid hydroperoxides (LOOH), as shown in Fig. 2 (Chan and Levett 1977; Buettner 1993; Girotti 1998; Korytowski and Girotti 1999). As will be shown below, hydroperoxides can accumulate in large amounts in the membranes, leading to important changes in the properties of the latter (e.g. thickness, area per lipid and two-dimensional organization). There is a possible route of transforming lipid hydroperoxides in other byproducts, including shorter chain aldehydes, which has been called Hock cleavage (Fig. 2). This cleavage starts with the rearrangement of the hydroperoxide, which has a double bond attached, to oxycarbonium ion, which then undergoes nucleophilic attack by water, leading to the cleavage of the C–C bond and the formation of carbonyl compounds (Wang 2010). However, this mechanism has never been proven to occur in phospholipids, although it is known to occur in cholesterol (Brinkhorst et al. 2008). In fact, our results (presented in following sections) are in agreement with the main mechanism being the accumulation of hydroperoxides in the membranes if only singlet oxygen is formed. The presence of autocatalytic peroxidation reactions and the formation of other byproducts will then depend on the presence of metal ions and the generation of other more potent oxygen radicals in the presence of type I reactions.

In the presence of metals, i.e. iron in the ferrous state, the hydroperoxides become highly reactive and may generate other free radicals, i.e. epoxyallylic peroxyl (OLOO), peroxyl radical (LOO•) or alkoxyl (LO•) radicals, that can propagate the peroxidation process by promoting free radical chain reactions (Girotti 1998; Korytowski and Girotti 1999). In the case of the type I mechanism, the reaction of the initial triplet species can form substrate-derived radicals or ROS (anion radical superoxide, hydrogen peroxide and hydroxyl radicals—in the presence of metals) that can lead to peroxidation products similar to those obtained from other ordinary lipid oxidation processes (free radical mechanisms). Abstraction of a hydrogen atom from an unsaturated fatty acid (LH) leads to the formation of a carbon centered lipid radical (L•). This suffers the addition of a molecule of oxygen to form LOO• which is able to react with other fatty acid LH, starting a new chain oxidation leading to the formation of lipid hydroperoxide (LOOH) and another lipid radical (L•). The propagation comprises a new chain oxidation by LOO• and decomposition of the lipid intermediate. It is worth mentioning that the triplet

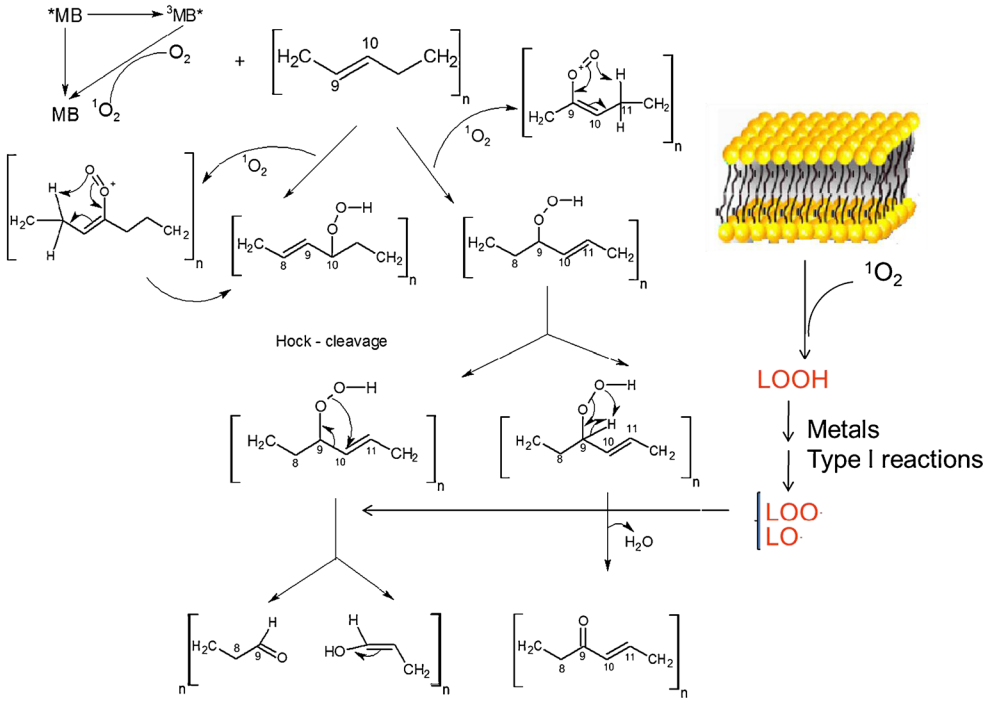

Fig. 2 Generation of $^1O_2$ via photosensitization by a PS and the ene reaction between a lipid double bond and the singlet oxygen to form hydroperoxides at the two carbons that hold the double bond. In this example, the double bond is located between the 9th and 10th carbon in the lipid chain. Subsequent to the formation of the hydroperoxide (*LOOH*), the double bond is kept but shifted in the carbon chain. The hydroperoxides can either accumulate in the membrane or progress to form other radicals [alkoxyl (*LO*•), peroxyl (*LOO*•)] that feed the peroxidation chain reaction in the presence of metals or type I photosensitization reactions. The Hock cleavage mechanism is a possible route to transform lipid hydroperoxide into smaller carbonyl compounds, although this transformation seems to occur only in the presence of PS that reacts by type I mechanism

species can also react with hydroperoxides and with double bonds, leading to radical species that continue the peroxidation chain reaction after the initial attack of singlet oxygen (Gantchev et al. 2003). Cholesterol can also be oxidized by both processes of oxygen free radicals and singlet oxygen (Girotti 1998; Korytowski and Girotti 1999).

Studies of our group have demonstrated that the extent of the lesion in a specific phosphocholine (PC) membrane depends on a combination of $^1O_2$ and free radical formation (see below). For example, LOOH accumulation occurs when the PS generates only $^1O_2$ (as in the case of porphyrins) (Riske et al. 2009). LOOH are a relatively stable species in the absence of metals and light exposition. They can be produced (by photosensitizing lipids in the presence of MB), isolated by high-performance liquid chromatography and kept for future use. In the case of PSs that act by both type I and type II mechanisms (like phenothiazinium derivatives), membrane leakage (Mertins et al. 2014) and breakage occur leading to loss of membrane integrity (Caetano et al. 2007). Therefore, it seems that it is necessary that the PS engages in both type II and subsequently type I reactions to allow more destructive membrane damage, i.e. membrane photodestruction. Furthermore, we will also show that lipid domains can be induced in a membrane containing a ternary mixture of an unsaturated lipid, a saturated lipid and cholesterol by photooxidation (Haluska et al. 2012).

Model membrane: GUVs

The GUV has been extensively employed as a model lipid membrane due to its large dimensions (10–100 μm) being comparable to typical cell sizes, allowing the observation of physical alterations produced in the lipid bilayer in real time by optical microscopy. In this context, there is a great number of studies in the literature showing the response of the membrane interacting with peptides (Ambroggio et al. 2005; Lamazière et al. 2007; Ros et al. 2011), proteins (Kahya et al. 2001; Dezi et al. 2013), alkylphospholipids (Gomide et al. 2013) and photoactive molecules under irradiation (Caetano et al. 2007; Riske et al. 2009; Heuvingh and Bonneau 2009; Diguet et al. 2012; Mertins et al. 2014). Moreover, GUV is a particularly interesting model to use to probe micron-sized lipid domains (Dietrich et al. 2001; Veatch and Keller 2005; Gomide et al. 2013).

The most common method of GUV formation is known as electroformation, where an alternating electrical field is applied across a buffer-filled chamber bounded by conductive

slides or crossed by metal wires (Angelova and Dimitrov 1986). In particular, GUVs are usually grown in a sucrose solution (0.1–0.2 M) and dispersed in a glucose solution of the same osmolarity, generating a sugar asymmetry between the inner and outer environment of the vesicles. The slight difference in density between the inner and outer solutions allows the vesicles to be easily observed on the bottom slide. Further, the difference in the refractive index between sucrose and glucose solutions provides a better contrast when observing the vesicles with phase contrast mode microscopy.

Of note, growing vesicles in physiological buffers or from lipid mixtures containing charged lipids is still a challenging task by electroformation. More recently, the growing of GUVs composed of anionic lipids has been reported by swelling the lipid precursor films on the top of a dried polyvinylalcohol gel surface (Weinberger et al. 2013).

Physical damage on giant vesicles by singlet oxygen: lipid hydroperoxide formation

Photo-irradiation of PSs such as porphyrins and MB (Fig. 3) generates a high quantum yield of singlet oxygen via the type II mechanism (Fig. 1). In the presence of lipid membranes, singlet oxygen, as a highly reactive oxygen species, promptly reacts with unsaturated acyl chains of lipids, leading to hydroperoxidation (LOOH in Fig. 2). The special feature of the hydroperoxide is that it accumulates in the membranes as a function of singlet oxygen production, in the absence of metals or type I photosensitization reactions. The progression of the lipid peroxidation from the hydroperoxide species to smaller and more oxidized lipid derivatives has not yet been studied in detail.

A first study where hydroperoxide was considered to be a oxidation product was performed by Wong-Ekkabut et al. (2007). Using MD simulations, these authors investigated the effect of lipid peroxidation on the properties of 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphatidyl-choline (PLPC) lipid bilayers. Four main oxidation products of linoleic acid with either a hydroperoxide or an aldehyde group were taken into account. The hydroperoxide lipid tails were created by adding a hydroperoxide group at position C9 or C13 of the linoleate tail with shifting of the double bonds, whereas the aldehyde lipid tails were built starting from linoleic acid and generating 9-oxo-nonanoic acid (9-al) and 12-oxo-9-dodecenoic acid (12-al) (Fig. 1 in Wong-Ekkabut et al. 2007). These oxidized chains replaced the sn-2 linoleate chain. An increase of area per lipid was observed for concentrations ranging from 2.8 to 50 % of each oxidation product. A linear relationship of the area increase as a function of the fraction of oxidized lipid was determined. In addition, the increasing oxidized lipid fraction in the membrane led to higher membrane permeability.

The physical effects caused by the singlet oxygen-generated hydroperoxides on membranes were experimentally addressed by optical observation of GUVs composed of unsaturated lipids. Indeed, experiments with a porphyrin-based PS possessing one unsaturated acyl chain, anchored in GUVs of POPC, were performed by our group (Riske et al. 2009). We synthesized a PS that consisted of a porphyrin molecule attached to two phosphatidylethanolamines, as schematically shown in Fig. 3a (PE-porph). The main spectral characteristics of PE-porph are quite similar to those observed for porphyrin incorporated in model membranes (Riske et al. 2009), with maximum absorption at approximately 400 nm and emission above 600 nm. The quantum yield of $^1O_2$ production was determined to be equal to 0.50 (Riske et al. 2009). In this configuration, singlet oxygen was produced in the water/membrane interface by the anchored porphyrin. The special control of the singlet oxygen generation allowed for some model simplifications that permitted the identification of the initial steps of the photooxidation process.

The GUVs of POPC containing 0.5–10 mol % of PE-porph were irradiated and simultaneously observed under the optical microscope. Visible morphological shape changes occurred in the GUVs as soon as the irradiation started. Figure 4 shows the most prominent morphological changes on the photosensitized GUVs. The lipid membrane from the original spherical vesicles (t=0 s) can be seen to become rapidly floppy accompanied by an increase in the projected area after short periods of irradiation time to such an extent that buds (small vesicles linked to the GUV) are released. The extent of the fluctuations and bud release depends on the amount of PE-porph in the membrane (Riske et al. 2009). Such a photo-induced increase in projected area and fluctuations can be attributed to an increase in the vesicle surface area.

Of note, the irradiation of PE-porph incorporated in GUVs of DMPC, a saturated lipid, did not promote any change in the vesicle (results not shown). Therefore, the observed area increase in POPC GUVs is related to the oxidative reaction of the lipid double bond—i.e. it is driven by the reaction between $^1O_2$ and the POPC double bond, producing POPC-hydroperoxide (POPC-OOH). According to Fig. 2, there are only two different molecules that can be generated, depending on which side of the double bond the reaction occurs. Figure 5 presents the two possibilities. The main hypothesis, which is well accepted in the literature, is that the hydroperoxide group has a more hydrophilic character in comparison to the lipid acyl chain milieu. As a consequence, this group tends to migrate to the bilayer surface (Wong-Ekkabut et al. 2007; Jurkiewicz et al. 2012), promoting an increase in the lipid molecular area as schematized in Fig. 6.

The effect of oxidized POPC molecules on the lipid bilayer was investigated by Khandelia and Mouritsen (2009) using MD simulation. Two oxidized products bearing the carbonyl (PoxnoPC) and carboxyl (PazePC) groups at the end of their

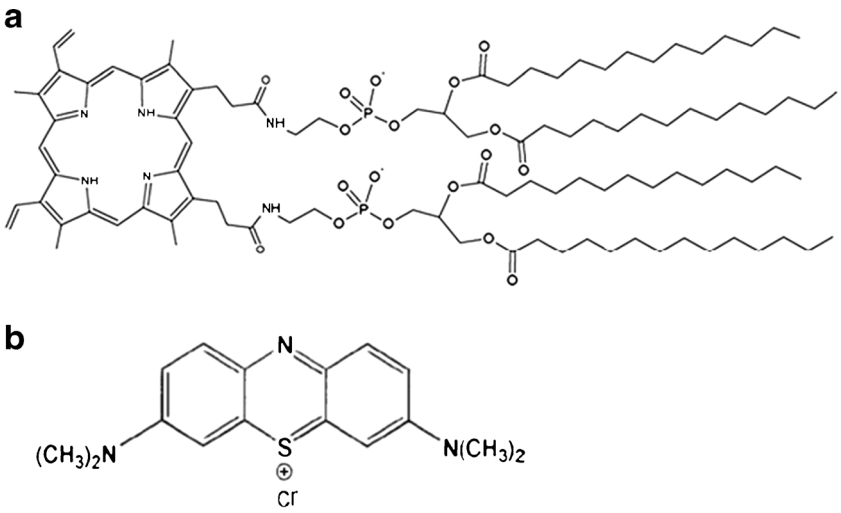

Fig. 3 Molecular structures of porphyrin molecule attached to two phosphatidylethanolamines (PE-porph; **a**) and methylene blue (**b**)

truncated sn-2 chains were studied. Thus, these two POPC oxidized species present a shortened chain due to chain cleavage near the initial position of the unsaturation (Fig. 1 in Khandelia and Mouritsen 2009). The increase in area per lipid was also observed for the aldehyde form (PoxnoPC), whereas a complete acyl chain reversal was observed for the anionic carboxyl form, leading to a smaller area per molecule. Of note, PoxnoPC and PazePC are byproducts originating from the ozone reaction on POPC in the basic or acid subphases, respectively (Lai et al. 1994).

In our case, we have assumed that only hydroperoxide species (POPC-OOH) are formed due to the type II reaction mechanism, with no cleavage of the unsaturated chain (Fig. 5). Therefore, the 9-tc molecule in the MD simulation (Wong-Ekkabut et al. 2007) is the closest oxidation product from the POPC-OOH molecule obtained in our experiment. Extrapolation of the data shown in Wong-Ekkabut et al. to 100 mol % of LOOH (9-tc) in the bilayer leads to a 15 % increase in the lipid molecular area. An attempt to evaluate the increase in photo-induced area in POPC GUVs was also performed by Riske et al. (2009). Figure 7 shows one example of irradiation of a GUV in the presence of an AC-field used to deform the vesicle. As soon as the irradiation starts, the vesicle elongates, assuming a prolate shape with its symmetry axis lying parallel to the field. From the sequence of the images, the time evolution of both semi-axes was extracted and, hence,

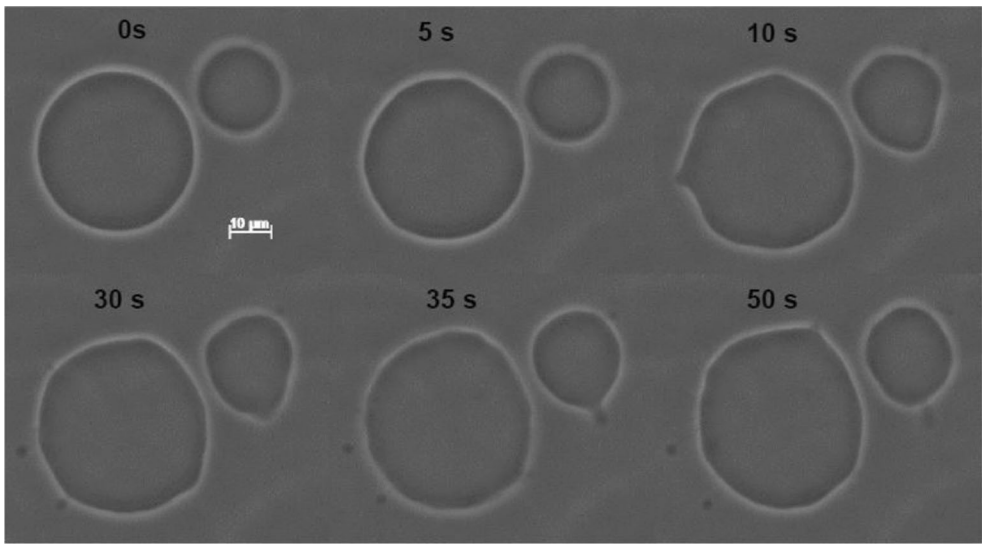

Fig. 4 Effect of irradiation of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) vesicles containing 5 mol% of PE-porph observed in the phase contrast mode. The irradiation time is shown on the *top* of each snapshot. *Scale bar*: 10 μm. Giant unilamellar vesicles (GUVs) were obtained by the electroformation method (Angelova and Dimitrov 1986) as described in Riske et al. (2009). Irradiation of the samples was performed using the HBO 103 W Hg lamp of the microscope (inverted microscope model Axiovert 200; Carl Zeiss AG, Oberkochen, , Germany) with a 400-nm excitation filter

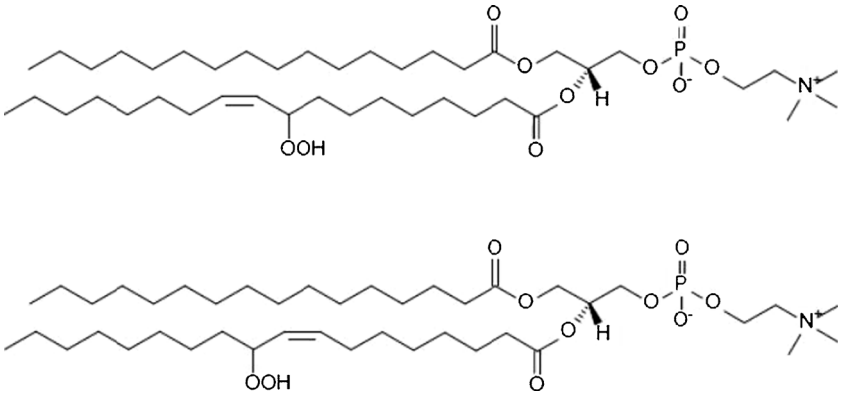

Fig. 5 Products of the singlet oxygen reaction to POPC: $^1O_2$ adds the hydroperoxide group –OOH at either the C9 or C10 position

the excess surface area was calculated. However, the overall quantitative determination of the molecular area changes per POPC induced by hydroperoxidation was prevented by PE-porph aggregation, a consequence of the planar structure of porphyrins.

More recently, we performed experiments of membrane aspiration with pipettes in the low tension regime (Evans and Rawicz 1990) with another PS to avoid aggregation in the membrane. In the micropipette technique a reduced pressure is applied in a microcapillary glass in order to partially suck a giant vesicle to its interior. An adequate constant reduced pressure is related to the membrane tension. As shown in Fig. 8, a POPC giant vesicle dispersed in a solution containing a low amount of MB (Fig. 3b) is aspired in a micropipette and remains stable in the absence of PS irradiation (Fig. 8a). In this case, the excess of area hidden in the membrane fluctuations is unfolded. As soon as singlet oxygen oxidizes the lipids, the excess of membrane surface area partially fills the length of the micropipette—i.e. all the increment in surface area is sucked by the slightly reduced pressure. The experiment strictly allows the calculation of the surface area increase with time until the maximum area increase is achieved (Fig. 8b). Following this procedure, we obtained a total surface area increase of 15 % for POPC GUVs due to POPC hydroperoxidation (Fig. 2), which is in good agreement with the extrapolated data from Wong-Ekkabut et al. (2007) for the 9-tc molecular area. A similar experiment was also recently performed by anchoring a chlorin-based PS on the POPC GUV membrane with similar results (Weber 2012). The similarity between experimental and theoretical observation gives confidence to the concept that the initial steps of the photooxidation result in the accumulation of lipid hydroperoxides in the lipid membrane.

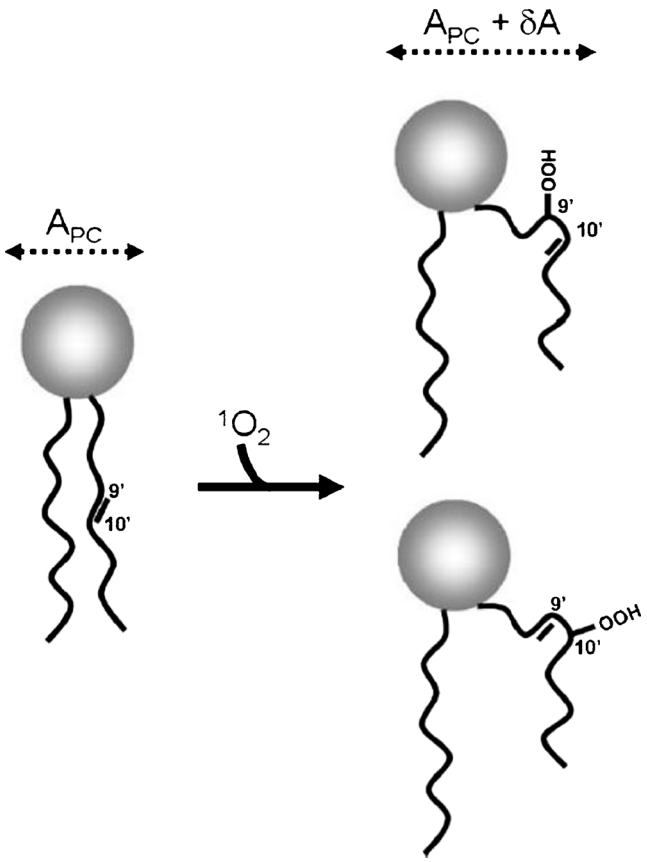

Fig. 6 Schematic representation of the mechanism involved in POPC area increase. Singlet oxygen adds the more hydrophilic group –OOH at either the 9′ or 10′ position, which migrates to the bilayer surface, imposing a kink to the acyl chain, with an accompanying increase in area δA per lipid (reproduced from Riske et al. 2009 with the permission of Elsevier)

Our experimental results clearly demonstrate that membrane oxidative stress promoted by singlet oxygen produces lipid hydroperoxides which, in turn, contribute to an increase in membrane surface area. Noteworthy, the initial sugar asymmetry, which is evidenced by the presence of the phase contrast ring in the GUV images (Figs. 4, 7), was kept during the PE-porph irradiation process and, therefore, the pores which would allow the traffic of sugars through the membranes were not formed. According to the simulations of Wong-Ekkabut et al. (2007), the 9-tc molecule is the oxidized product with the smallest increased simulated membrane permeation, although it has been shown that membranes containing up to 50 % of 13-tc are prone to electroporation (Vernier et al. 2009). Thus, photooxidation of PE-porph in the vicinity of the lipid interface promoted the exclusive formation of POPC-OOH that

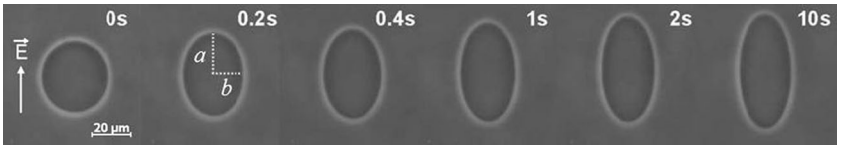

Fig. 7 Irradiation of a GUV containing 3 mol% of PE-porph in the presence of an AC-field (10 V, 1 MHz), which induces prolate deformation. The electric field direction is indicated on the *left* and the irradiation time is indicated at the *top* of each snapshot. *Scale bar*: 20 µm (reproduced from Riske et al. 2009 with the permission of Elsevier)

accumulated in the membrane, leading to an increase in bilayer area while maintaining vesicle integrity. In this way, no reaction via the triplet state of the porphyrin-based PS and the chain double bond occurred, which could propagate the peroxidation chain reaction (Fig. 2).

According to the literature, membrane permeation upon oxidation depends on the features of the byproducts that are produced (Wong-Ekkabut et al. 2007; Vernier et al. 2009; Lis et al. 2011; Conte et al. 2013). When oxidation is very strong, namely, above a certain threshold, membrane integrity can be compromised, and vesicle leakage (Heuvingh and Bonneau 2009; Lis et al. 2011; Mertins et al. 2014) and destruction have been reported (Caetano et al. 2007; Cwiklik and Jungwirth 2010; Diguet et al. 2012). This point will be discussed in detail in the following section.

Lipid hydroperoxide and photoinduced lipid domains

It is well known that polyunsaturated fatty acids, which are prone to be oxidized by singlet oxygen, seem to play important roles in the structure and formation of the membrane rafts (Schley et al. 2007). Changes in the lipid composition of the rafts have been increasingly related with cell signaling processes (Dykstra et al. 2003). It has also been shown that in some cases oxidized species may stimulate signaling within the cell (Suzuki et al. 1997; Girotti 1998; Chiaramonte et al. 2001; Vaughn and Deshmukh 2008).

GUVs have been extensively used to investigate the phase behavior of lipids and its relation to various aspects of lipid rafts (Dietrich et al. 2001; Veatch and Keller 2005; Feigenson 2009). These studies have been important in contributing to our comprehension 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 in which there is a tendency of the lipids to either be completely mixed or to coexist in separate lipid phases. In fact, the formation of lipid domains has been observed by photo-stimulating fluorescent lipid probes in GUVs made of ternary mixtures of lipids (Ayuyan and Cohen 2006; Zhao et al. 2007). Some authors caution that lipid peroxidation can be a serious artifact in the study of GUV domains, suggesting ways in which this effect can be decreased, such as by using anti-oxidant agents (Morales-Penningston et al. 2010). Ayuyan and Cohen suggested that domains are generated due to the formation of peroxidation products, specifically of sphingomelin (SM), by Haber–Weiss and Russel mechanisms of oxidation reactions. Zhao et al. (2007) suggested that large photoinduced domains arise from the coalescence of smaller pre-existent lipid clusters that grow under oxidation.

Therefore, it is important to understand whether the photoinduced processes change the organization of the lipids in a membrane as well as the mechanisms of these changes. Thus, to get a better insight of how oxidized lipids may affect the distribution of lipids in a ternary mixture, GUVs composed of POPC, DPPC, cholesterol and 1 mol% of PE-porph (Fig. 3a)

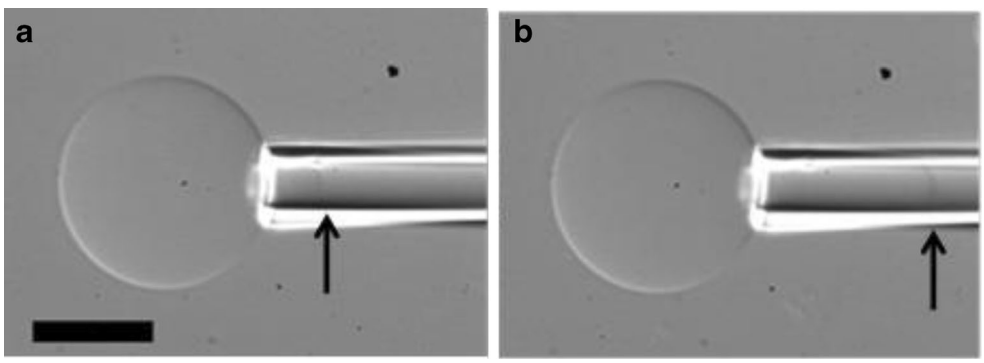

Fig. 8 Differential interface contrast microcopy images of the time-running sequence of a POPC giant vesicle dispersed in a methylene blue solution (30 µM) under photo-irradiation (665 nm) and aspired in a micropipette under reduced pressure corresponding to a membrane tension of 0.3 mN/m. **a** The vesicle remains stable without irradiation (0 s), **b** membrane surface area increases inside the micropipette under the same constant pressure as a result of formation of the POPC-hydroperoxide (POPC-OOH) (80 s). *Arrows* indicate the maximum extension of the membrane into the micropipette. *Scale bar*: 10 µm (for both snapshots)

were investigated (Haluska et al. 2012). As described above, PE-porph allows the photooxidative reaction to induce in situ formation of lipid hydroperoxides. Thirteen different ternary mixtures chosen from the POPC:DPPC:cholesterol phase diagram (fig. 2 in Haluska et al. 2012) were studied, with the cholesterol content varying from 9 to 23 % in terms of membrane composition. All samples exhibited homogeneous fluorescence distribution under low-light NBD-probe fluorescence. The effects of the irradiation (400 nm) on all three-component samples led initially to increases in both fluctuations and apparent surface area, with significant morphological changes for samples with a greater amount of POPC, similar to the photo-response of the POPC membrane containing PE-porph (Fig. 4). However, some vesicles with particular compositions (POPC:DPPC molar ratio of 1:2, 1:1 and 2:1, and cholesterol amount of >9 %) displayed many small fluorophore-rich domains which grew with irradiation time (Haluska et al. 2012). Figure 9 shows a typical example of such an evolution. We observed that the growing domains have a round shape, indicating Ld–Lo phase coexistence (Dietrich et al. 2001; Morales-Penningston et al. 2010). The oxidative side effect caused by irradiation of the fluorescent probe is minute in comparison to the extensive lipid hydroperoxide formation caused by porphyrin. The explanation for this is that the main relaxation from the excited state in the probe occurs via fluorescent emission and not by intersystem crossing to the triplet state, as is the case for PS molecules.

Therefore, by controlling the membrane composition we were able to identify a region in the POPC:DPPC:cholesterol phase diagram where Lo–Ld coexistence emerges from initially homogeneous mixtures. Of note, cholesterol in the phospholipid bilayer also represents another target of singlet oxygen. As a consequence, cholesterol hydroperoxides must also be formed concomitantly to POPC-OOH during the photooxidative process. However, due to the initial increase in membrane area observed, we attributed the formation of the lipid domain mainly to in situ generated POPC-hydroperoxide (Haluska et al. 2012). In any case, the molecular changes induced by hydroperoxidation transform the POPC:DPPC:cholesterol ternary diagram into a new phase diagram of the mixture POPC (POPC-hydroperoxide):DPPC:cholesterol (cholesterol hydroperoxide). The combined effect of both POPC and cholesterol photooxidation 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 higher cholesterol content with respect to the ternary diagram of POPC:DPPC:cholesterol mixtures in the absence of oxidized species (Haluska et al. 2012). This study also demonstrated that cholesterol oxidation exclusively is not sufficient to have an observable effect on membrane mechanics.

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. In the case of hydroperoxidation, increase in the average area per lipid could increase the incompatibility between the different lipids 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 could be the case of POPC-hydroperoxide, increases the lateral tension and, hence, the line tension in the raft boundaries (Akimov et al. 2007). 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 (Akimov et al. 2007).

With a view of better comprehending the role of POPC-OOH (POPC-hydroperoxide) in the phase diagram, we have recently established a new phase diagram replacing POPC by its hydroperoxide counterpart, i.e., a ternary phase diagram of POPC-OOH:DPPC:cholesterol up to cholesterol content of 33 mol %. Interestingly, by replacing POPC by its hydroperoxide we were able to reproduce part of the photo-

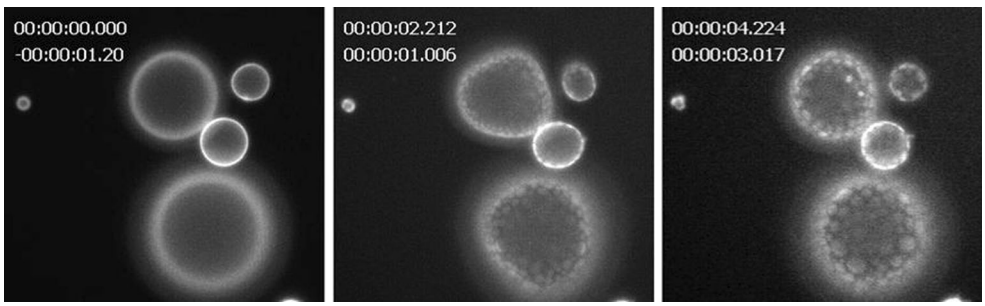

Fig. 9 Images obtained with fluorescence microscopy (inverted microscope Axiovert 200, Carl Zeiss) of the sample composed of POPC:1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) 1:2 and cholesterol content of 23 % with 1 mol% of PE-porph (0.1 mol % NBD-containing phospholipid bilayer) under irradiation (400 nm). *Top left number on each image* Time. The beginning of irradiation is set at time 0. In the sequence of images taken from 0 to approx. 4 s, GUVs respond to light exposure displaying lipid demixing that ends up at a liquid ordered (Lo)–liquid disordered (Ld) phase coexistence

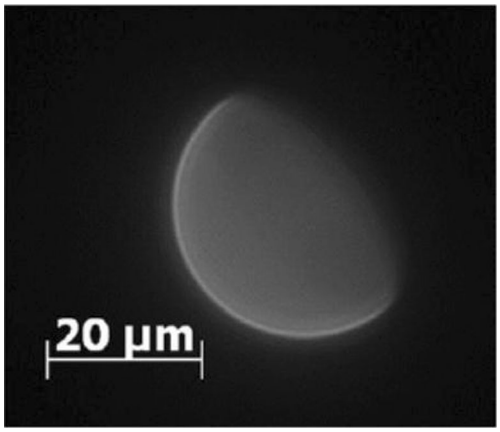

Fig. 10 Image obtained with fluorescence microscopy (inverted microscope Axiovert 200, Carl Zeiss) of the sample composed of POPC-OOH:DPPC 1:2 and cholesterol content of 33 % (0.1 mol % rhodamine-containing phospholipid bilayer) displaying Lo–Ld phase separation

induced POPC:DPPC:cholesterol phase diagram (Fig. 2 reported by Haluska et al. 2012) for a higher amount of DPPC. Figure 10 shows one example emphasizing that POPC-hydroperoxide is able to impact the organization of membrane lipids.

Phase separation in binary systems has been previously reported with a modest addition of truncated oxidized lipids (5–15 mol %) on lipid bilayers (Megli et al. 2005). This effect was attributed to changes in lipid tail chemical structure that may facilitate lipid lateral diffusion (Beranova et al. 2010). More recently, lipid phase separation has also been observed in monolayers composed of ternary mixtures of POPC:sphingomyelin:cholesterol upon addition of a certain percentage of PazePC (Volinsky et al. 2012). A surface analysis of a series of mixtures in which POPC was gradually exchanged with PazePC revealed a linear increase in the miscibility transition pressure with an increase in the content of PazePC (Fig. 5 in Volinsky et al. 2012). According to these data, approx. 20 mol % of the POPC should be exchanged to its oxidized analog to induce lipid mixing at 32 mN/m, i.e., at a surface pressure that is considered to prevail in cellular bilayer membranes. Therefore, the model membrane must always present lipid phase separation when massive oxidation takes place. Such an appearance of lipid domains has been attributed to a hydrophobic mismatch imposed by the truncated oxidized lipid PazePC (Volinsky et al. 2012).

What is interesting is that both POPC-OOH in bilayers and PazePC in monolayers show the ability to modulate membrane biophysical parameters in such a way that they influence the two-phase coexistence. Therefore, it seems that oxidized lipids must, indeed, play an important role in modifying the properties of the Ld phase, which in turn affects Ld–Lo phase separation. However, the exact mechanism that drives the lipid separation has not yet been elucidated and represents a challenge for future work.

It is however interesting to stress that the phase separation response to oxidative stress opens a number of important pathways for biophysical studies. For example, it could be hypothesized that domain formation after oxidation might facilitate oxidized lipid detection by signaling for recovery or elimination by the cell. Moreover, changes in the biophysical properties of the bilayer have been demonstrated to trigger signaling pathways (Cremesti et al. 2002). As a consequence, selective (dis)association of essential proteins within lipid raft scaffolds may take place in response to oxidative stress promoted in the plasma membrane.

Membrane permeability and integrity: chain cleavage

Photodamage on biological membranes promoted by PS may have different levels and characteristics depending on the PS features. According, the photosensitization of PS as porphyrin produces a high quantum yield of singlet oxygen via the type II mechanism (Fig. 1), thus generating lipid hydroperoxides in the membrane (Fig. 2). On the other hand, another PS, such as MB, has the possibility to produce a high $^1O_2$ quantum yield via the type II mechanism (Fig. 1) as well as radical species via the type I mechanism (Fig. 1) (Junqueira et al. 2002), eventually promoting acyl chain cleavage (Fig. 2). Of course, chain cleavage opens the possibility of generating several truncated byproducts, such as ketones, aldehydes or carboxylic acids.

In order to better understand how the cleaved lipids impact on the photooxidized membrane, a closer analysis of the damage promoted by irradiation of MB on GUVs composed of DOPC is now considered. Figure 11 displays morphological changes on the lipid membrane of DOPC giant vesicles dispersed in 10 µM of MB aqueous solutions and submitted to continuous photo-irradiation (665 nm). Note that only singlet oxygen produced in the vicinity of the phospholipid bilayer (around 100 nm) is able to interact with the membrane due to its short life time (on the order of 4 µs) in aqueous solution (Caetano et al. 2007). Small adsorption of MB to PC-based membrane is evidenced by a weak MB fluorescence signal in the membrane contour (Mertins et al. 2014). So, a certain amount of $^1O_2$ is also produced in the membrane through the type II mechanism.

As noted in Fig. 11, original spherical vesicles remain physically stable as far as there is no irradiation. However, the DOPC vesicle becomes soft and noticeably floppy after a short period of MB irradiation (image corresponding to 150 s of irradiation in Fig. 11). Such a behavior is attributable to the increase in membrane area followed by bud release (Mertins et al. 2014). Thus, this first stage of membrane photodamage is related to the formation of DOPC hydroperoxide due to $^1O_2$ attack to the acyl chain double bonds, similar to POPC hydroperoxidation produced by porphyrin photosensitization (Riske et al. 2009). Therefore, the accumulation of DOPC

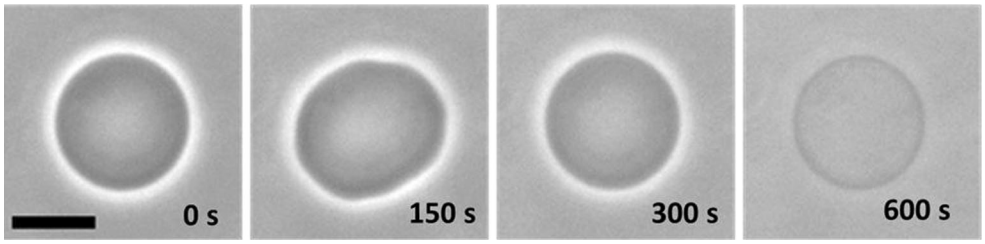

Fig. 11 Phase contrast microcopy images of a time running sequence of a DOPC giant vesicle in a low concentration of methylene blue (10 μM) under photo-irradiation (665 nm). The vesicle remains stable without irradiation (0 s), after which the membrane flickers as a result of phopholipid photodamage (150 s); thereafter, membrane tension increases (300 s) and finally the phase contrast slowly fades, reflecting the increase in membrane permeability. *Scale bar*: 15 μm

hydroperoxide in the membrane leads to an increase in the membrane surface area as a response to the hydrophilic character of the -OOH group (Wong-Ekkabut et al. 2007).

Following MB irradiation different scenarios are, however, observed: eventual transient micro holes are noted on the membrane for a few seconds without disruption of the vesicles (Mertins et al. 2014), GUV recovers its spherical shape and the membrane fluctuates less than before irradiation (image corresponding to 300 s of irradiation in Fig. 11), followed by a continuous decrease of the vesicle optical phase contrast (600 s in Fig. 11). Such a loss of contrast reveals the traffic of fluids from the core of the vesicle to the external environment and vice versa. Indeed, the loss of contrast denotes that both internal and external solutions are being homogenized. In this way, pores of at least 1 nm in diameter must be formed in the lipid bilayer that allow sugar traffic. As such, pore formation on the membrane is also one of the prominent physical consequences of DOPC peroxidation promoted by the photo-irradiation of low amount of MB (up to 40 μM in the aqueous solution according to Mertins et al. 2014). The speed of membrane contrast loss is dependent on the concentration of MB in the outer vesicles solution being faster for higher amounts of MB. This means that there is a threshold concentration of oxidative species necessary to promote a pore in the membrane which, by turn, increases membrane permeability.

Cwiklik and Jungwirth (2010) studied pore formation in oxidized DOPC bilayers by numerical simulations. Four couples of oxidative products were investigated with distinct cleavage locations and chain removal. According to their results, products with only one oxidized chain lead to either transient or stable pore formation. As such, cleavage of one of unsaturated chains of DOPC must occur under photooxidation in the experimental GUV results (Fig. 11). Therefore, our results demonstrate that it is necessary that the PS engages in both type II and subsequently type I reactions to allow pore formation and, hence, membrane leakage. The kind of shortened oxidized DOPC that is formed, such as aldehyde or carboxylic acid, for example, is still under investigation by our group.

Heuvingh and Bonneau (2009) reported the oxidation of GUVs made of DOPC by a chlorin PS at a concentration of 50 μM. In these experiments, vesicle permeation was also evidenced by phase contrast and showed an increase upon oxidation. Again, the type I reaction was hypothesized, governed by chain propagation. The last experiments of these authors focused on introducing cholesterol within the DOPC bilayers (Kerdous et al. 2011); the results showed that with increased amount of cholesterol, the effect of membrane permeation can be lowered.

Of note, similar membrane contrast loss has been also observed for POPC GUVs dispersed in a low amount of MB aqueous solution. Nevertheless, at the same MB concentration, the irradiation time necessary to provoke membrane leakage is longer for POPC than that found for DOPC membranes (Mertins et al. 2014). Ytzhak et al. (2013) showed that as the degree of fatty acid unsaturation increased, the photosensitized passage of the small molecules through the lipid bilayer increased. Further experiments are required to

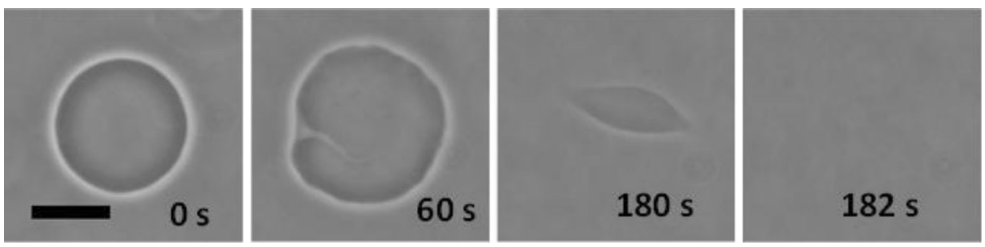

Fig. 12 Phase contrast microcopy images of a time running sequence of a DOPC giant vesicle in a high concentration of methylene blue (50 μM) under photo-irradiation (665 nm). The vesicle remains stable without irradiation (0 s), following which the membrane is largely perturbed as a result of phospholipid photodamage (60 s), as denoted by the upsurge of large invaginations and/or buds release; thereafter, the photooxidation evolves to membrane disruption and consequently to the vesicle burst (180 s). Ultimately, it is no longer possible to identify the lipids by optical microscopy (182 s). *Scale bar*: 15 μm

understand the role of phospholipid molecular features in the photo-response of the membrane.

Finally, when a relatively high concentration of MB (>50 μM) is present in the DOPC giant vesicles solution, the physical damage promoted on the membrane bears a stronger effect and membrane integrity is compromised (Caetano et al. 2007). Figure 12 shows an example of GUV dispersed in 50 μM of MB aqueous solution upon continuous irradiation (665 nm). As one can see, after a short period of irradiation, membrane disruption is observed, followed by vesicle collapse. Cutting of the lipid chains at the initial unsaturation location forming nonanoic acids was proposed (Caetano et al. 2007) as the most probable scenario. Cwiklik and Jungwirth (2010) also showed that byproducts where both chains are cleaved more strongly perturb the bilayer, which is rapidly destroyed.

To conclude, in this review we present the recent advances that have been made using GUVs as model phospholipid membranes, which are allowing us to better explore the membrane photo-response to in situ generation of oxidative species. Light absorption by a PS generates excited triplet states to produce ROS. Two mechanisms of ROS reaction are possible: type I, where the triplet state can generate radical species that are conducted to the peroxidation chain reaction, and type II, where the triplet transfers its energy directly to molecular oxygen, forming singlet oxygen $^1O_2$. The extent of the lesion caused in the lipid membrane seems to depend on the combination of $^1O_2$ and free radical formation. We have presented evidence that when the PS generates just singlet oxygen there is only the accumulation of lipid hydroperoxide in the membrane. As a consequence, the increase in membrane area is a fingerprint of the photooxidation, with no noticeable change in membrane permeability. On the other hand, in the case of a PS that acts by both type I and type II mechanisms, both membrane leakage and integrity can be compromised. The membrane instability seems to be associated to the formation of cleaved-acyl chains. A possible route that transforms lipid hydroperoxide in shortened oxidized molecules may occur in the presence of a PS that reacts by type I mechanism. We also show that oxidized lipids as hydroperoxides can influence the lipid mixing in a mimetic plasma membrane, thereby promoting Ld–Lo phase coexistence. Interestingly, such an effect opens a field of important pathways for further biophysical studies. Finally, photo-controlled leakage of membranes can also pave the way for novel strategies of light-induced drug delivery (Pashkovskaya et al. 2010; Uda et al. 2010; Diguet et al. 2012).

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Human and animal Studies This article does not contain any studies with human or animal subjects performed by the any of the authors.

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