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Research Article

Understanding membrane remodelling initiated by photosensitized lipid oxidation

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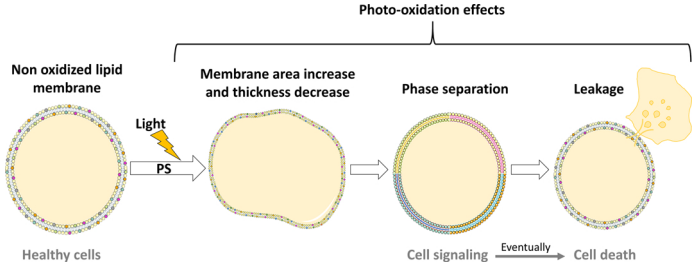
HIGHLIGHTS

- Photosensitization reactions cause membrane oxidation.
- Unsaturated lipids are photooxidation targets.
- Photooxidation causes biophysical and biochemical changes in membrane properties.
- Lipid oxidation has a role in membrane permeability and membrane microdomains.
- Cellular dysfunctions are mediated by lipid oxidation.

GRAPHICAL ABSTRACT



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ABSTRACT

In this review, we describe how photooxidation changes membrane properties that can ultimately lead to permanent membrane damage. Lipid photooxidation occurs in the presence of reactive oxygen species such as singlet oxygen and by direct reactions of lipids with a photosensitizer in the excited state. Indeed, lipid oxidation triggers chemical transformations that can alter lipid packing; change the membrane surface area, thickness and elastic modulus; and induce pore formation and phase separation. Here, we highlight how lipid hydroperoxides promote membrane remodelling and phase separation. Further, we emphasize the alterations caused by truncated oxidized lipids that lead to increased membrane permeability. Finally, the consequences of lipid photooxidation on cell functions are also discussed.

1. Introduction

Life on Earth evolved under the influence of sunlight, which interacts and affects most of the biotic and abiotic processes at the Earth's surface. Through the ages, sunlight has had an impact on various evolving beneficial and essential photobiological processes, such as photosynthesis, biosynthesis of vitamin D, vision and phototaxis (considered a very early form of "vision"). Despite the beneficial roles of light on living systems, photochemical reactions can also result in damage in or lethal consequences for living organisms [1,2]. The light energy absorbed by natural and human-produced chromophores can be transferred to receptor molecules in a process called photosensitization. Through this mechanism, light energy is transformed into chemical reactivity, which activates different chemical mechanisms that do are not functional in the absence of light. Photosensitization usually leads to oxidation (photosensitized oxidation). In turn, it affects some properties of key biomolecules, such as unsaturated lipids, nucleic acids and proteins. These effects can lead to profound consequences, such as mutations, photoaging, and/or the death of organisms [2]. Over time, humans have observed that this spontaneous photoinduced phenomenon can be used as a strategy for the treatment of diverse diseases, currently known as photodynamic therapy (PDT) [3–5].

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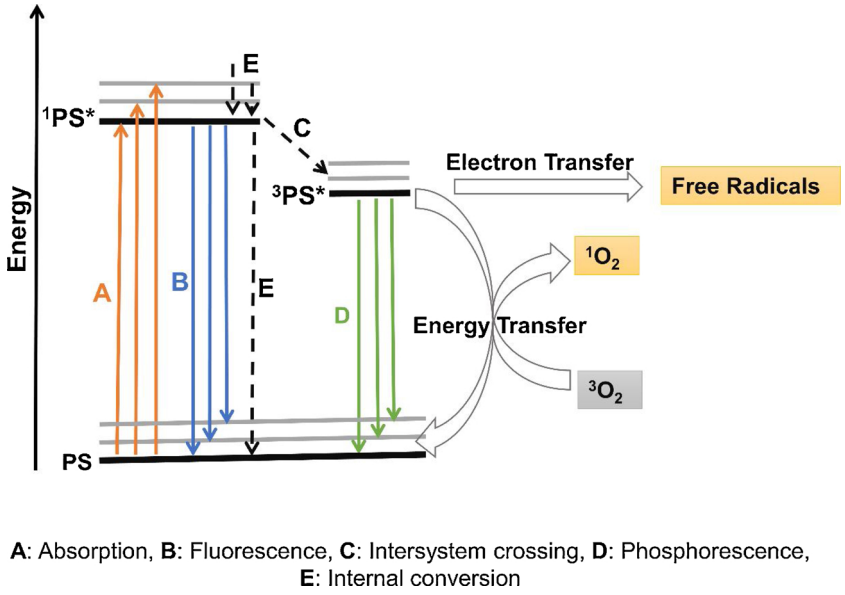

Fig. 1. Representation of the processes involved in photosensitization reactions. PS: photosensitizer at ground state, $^1PS_*$: singlet excited state of the photosensitizer and $^3PS_*$: triplet excited state of the PS, $^3O_2$: molecular oxygen, $^1O_2$: singlet oxygen.

The basic mechanisms that support all the different processes involved in the photooxidation reactions in PDT are intrinsically related to two non-toxic actors, which combine to induce cellular and tissue effects in an oxygen-dependent manner [6,7]. These two main actors are the photosensitizer (PS) undergoing light absorption (on a suitable wavelength – see Fig. 1) and oxygen; together they generate reactive oxygen species (ROS), which can inactivate undesirable cells such as tumour cells or pathogenic microorganisms.

Briefly, the photosensitization reaction is triggered by light absorption of the photosensitizer in its singlet ground state (PS), which causes an electronically excited higher singlet state (^{1}PS*) – Fig. 1, process A. Once in the ^{1}PS* state, the photosensitizer molecule can be converted to its lower triplet excited state by intersystem crossing (ISC), which triggers a multitude of changes in the PS (Fig. 1, process C). Species in the excited triplet state (^{3}PS*) are more easily reduced or oxidized than molecules in the ground state and have a longer lifespan than those in the ^{1}PS* state. Following the generation of ^{3}PS*, the photosensitizer can return to its ground state through two main pathways: (i) by electron transfer (type I mechanism) and (ii) by energy transfer (type II mechanism) [8–11]. In the electron transfer pathway, ^{3}PS* interacts with biological substrates, which results in the formation of radicals and radical ions that subsequently interact with molecular oxygen to give rise to reactive oxygen species (ROS) such as hydroxyl radicals ($OH^{\bullet}$), superoxide ions ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) [8,11,12]. In the energy transfer pathway, the energy from the ^{3}PS* is transferred directly to the molecular oxygen producing singlet oxygen ($^1O_2$) [8,13]. The resulting $^1O_2$ is, in turn, a highly reactive species because of its diamagnetic spin configuration, which favours an electrophilic attack of electron donor molecules [14].

Both mechanisms (electron transfer and energy transfer) may occur simultaneously. The relative importance of one mechanism with respect to the other will depend on, among other factors, the substrate, the distance between the PS and the target as well as the oxygen concentration. The main targets of ^{3}PS* or $^1O_2$ are electron donor molecules, *i.e.*, carbon-carbon unsaturated bonds, amines, sulfides, anions and neutral nucleophiles [11,14–17]. For instance, the empty orbital present in $^1O_2$ enables its addition to double bonds of lipids, proteins and nucleic acids to consequently oxidize them [18–20].

Several types of photosensitizers, such as porphyrins, phthalocyanines, chlorins and phenothiazines, have been currently employed [12]. Even though it initially seems that the efficiency of PDT protocols could be easily controlled by managing the features and concentrations of the PS, light intensity and oxygen availability, the complexity of biological systems hinders such a practical approach. In fact, over the past few years, photosensitization reactions have received much attention in the expectation that they will reveal the main mechanisms of PDT such that better protocols for clinical practice can be defined. Many researchers have driven efforts to reach an "ideal" PS compound that is able to produce singlet oxygen efficiently with minimal photobleaching and to provide good PDT treatment results. However, exploiting the photophysical properties of photosensitizers alone is not enough to guarantee PDT efficiency. For example, *Oliveira et al* demonstrated that, even though methylene blue (MB) generates approximately 50% triplets and $^1O_2$ and crystal violet (CV) produces < 0.05% triplets and $^1O_2$ under photoabsorption, CV is more efficient in killing cancer cells than MB [21]. Both dyes are able to accumulate in mitochondria. However, MB is promptly reduced and unable to compromise mitochondria, thus cell viability is preserved. On the other hand, the highly specific CV localization to mitochondria enables it to cause cell death, even during low ROS production [21].

It is also worth noting that ROS, although very reactive, have short lifetimes in biological media, which implies that these species have a limited diffusion distance within cells (Fig. 2A) [22]. Therefore, their high reactivity and short lifetimes are limiting factors for ROS distribution within cells (Fig. 2). For example, the half-life of H_2O_2 is approximately 1 ms and has a migration distance of ~1 μm [23,24], which is in contrast to the $OH^{\bullet}$ lifetime, which is estimated as 9 ns, resulting in an average diffusion distance of 93 Å [25]. The lifetime of $^1O_2$ in water is approximately 4 μs, which results in a diffusion distance of ~220 nm [22]. Considering that eukaryotic cell diameters are approximately 10–30 μm and the size of the organelles, such as mitochondria, lysosomes and the endoplasmic reticulum, is approximately 500 nm, we can infer that the average diffusion length of ROS is extremely limited on the scale of cell dimensions. Therefore, when ROS are generated near an oxidative target, the cell senses the potential impact. In particular, in the case of membranes (thickness < 10 nm), ROS attack unsaturated phospholipids and sterols as their main oxidative targets (Fig. 2B) [5,26].

In summary, the oxidation of biomolecules in the cell environment by photosensitization reactions *in situ* involves the formation of the triplet excited state of PS (^{3}PS*), which may generate reactive oxygen species (ROS) that are able to quickly diffuse within a short-scaled

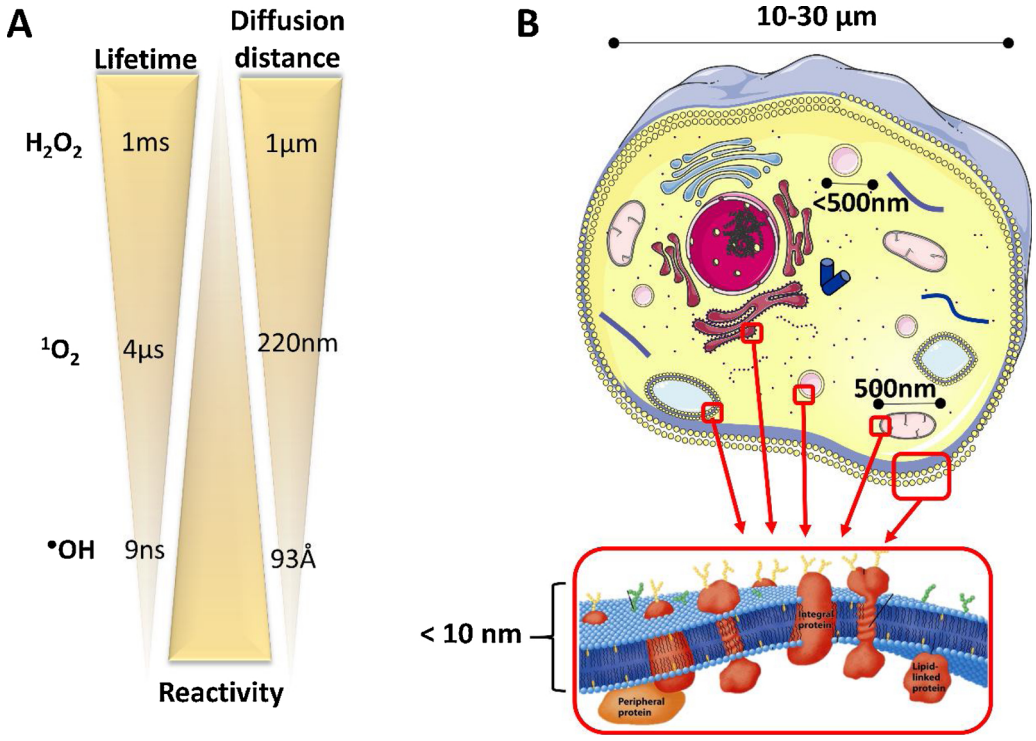

Fig. 2. (A) The relationship between the lifetime, reactivity level and the diffusion distance for common ROS in cells. (B) Illustration of cell, organelles and membrane dimensions.

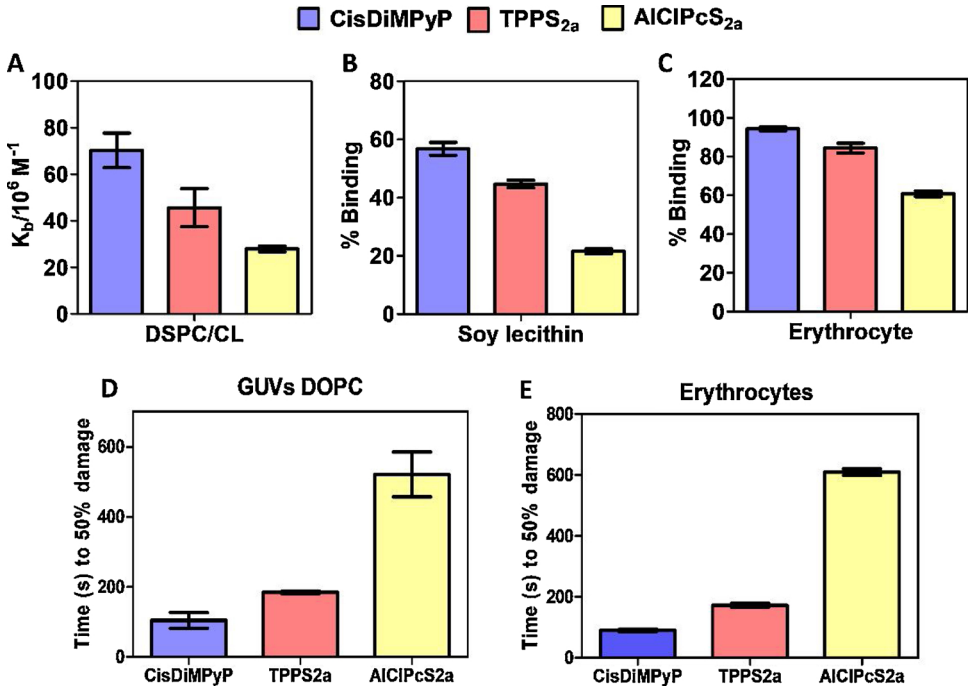

Fig. 3. (A) Binding constant (K_b) to DSPC/CL vesicles (8 µmol of DSPC and 2 µmol CL in 2 mL of 5 mM Tris buffer at pH 7.4). (B) Percentage of binding to soy lecithin vesicles (0.12 mM soy lecithin in 5 mM Tris buffer at pH 7.4). (C) Percentage of binding to erythrocytes (1×10^7 erythrocytes/mm^3 in PBS pH 7.4). (A, B and C) [Photosensitizer] = 7 µM. (D) Time (in seconds) to reach 50% photodamage induced in the GUVs by each photosensitizer. [DOPC] = 2.5 mM, 0.2 M sucrose inside vesicle, 0.2 M glucose outside vesicle. (E) Time (in seconds) to reach 50% photodamage induced in the erythrocyte membranes by each photosensitizer. Bars represent the mean ± SD from at least three independent measurements. CisDiMPyP: *meso*-cis [113](*N*-methyl-4-pyridyl) diphenyl porphyrin dichloride, $TPPS_{2a}$: *meso*-tetraphenylporphine disulfonic acid (adjacent isomer) and $AlClPcS_{2a}$: Al(III) Phthalocyanine chloride disulfonic acid (adjacent isomer). Photosensitizers were purchased from *Frontier Scientific* (Logan, UT), CisDiMPyP catalogue number: D40922, $TPPS_{2a}$ catalogue number: T40637 and $AlClPcS_{2a}$: P40632. Unpublished data (related to $AlPcS_{2a}$) were plotted together with data already published elsewhere [47].

This figure is a derivative of that from another publication [47], adapted with permission from *Springer Nature* under the terms of the Creative Commons Attribution 4.0 International License.

space and react with the target. ^{3}PS* may also react directly with biological substrates [27]. In both cases, it is crucial that the photosensitizer co-localizes or is close to the target, which is expected to be damaged.

By taking into account the importance of PDT action on biological membranes [5,20,28,29], membrane photooxidation is addressed herein, as are its main implications for cellular functions.

2. The importance of the lipid membrane as a target for photooxidation

It is well known that biological membranes are of great importance to maintain cell homeostasis since they encompass and compartmentalize cells and organelles. The major components of all cell membranes are lipids and proteins with small amounts of carbohydrates [30,31].

Most membranes exhibit a lipid-protein ratio of ~1, but the proportion between lipids and proteins may vary considerably depending on the membrane functions [31–33]. Choosing a class of biomolecules, such as lipids or proteins, as the best substrates to optimize the photodynamic effects is not a straightforward task and cannot be generalized. In this way, it is essential to address detailed studies on specific targets.

Some authors have reported that proteins are the main targets for photooxidation within cells due to their high abundance [19,34]. Nevertheless, it should be noted that the lipid bilayer is the basic structural element of membranes and it is organized in such a way that oxidizing relatively few lipids may compromise cell viability and thus lead to homeostasis loss. For example, a small amount of oxidized lipid (~2.5%) is enough to cause a drastic increase in phospholipid bilayer permeability [35]. Further, the presence of oxidized lipids can alter cytochrome *c* binding to the mitochondrial membrane [36]. In addition, most hydrophobic and amphiphilic photosensitizers are taken up by cells and are usually located in cellular membranes due to the similarity in the polar and apolar proportions of both PSs and lipids [37,38].

Certainly, various proteins also interact with some photosensitizers [39]. However, we have previously experienced the affinity of three PSs to protein-free model membranes and erythrocyte cells, for which the membranes are composed of 52% proteins, 40% lipids and 8% carbohydrates [40]. In both systems, the PS-membrane binding constants were CisDiMPyP > $TPPS_{2a}$ > $AlClPcS_{2a}$ (Fig. 3A–C). Additionally, the photoactivity comparison of these 3 PSs in both membranes systems reveals that the rate of permeabilization is inverse to the amount of PS that is incorporated into the membrane (Fig. 3D and E). Further, it was also previously verified that there is a significant correlation between the rate of liposome leakage and the level of erythrocyte haemolysis upon photooxidation [37,41], as shown in Fig. 4. Taken together, these results indicate that PS binding to lipid membranes plays a crucial role in the membrane photoresponse that increases permeability.

In general, stronger PS/membrane binding is correlated not only with faster membrane permeability but also with more effective photoinduced cell death and/or organisms [37,42–44]. For instance, *Pavani and co-workers* demonstrated that a specific PS with membrane binding efficiency of ~60% is able to promote efficient cell death (~ 90%), while another PS with membrane binding efficiency of ~2% promotes death in only 15% of cells [45]. Lipid/membrane oxidation not only is important for PDT but also is the major trigger of cell ageing related to the damage of skin cell membranes by photooxidation reactions induced by UVA and visible light [46]. Additionally, in more generic terms, lipid oxidation seems to be the cause of a variety of ageing-related diseases [47–49].

It is well accepted that PS-membrane binding is driven by the degree of hydrophobicity and/or amphiphilicity of the PS compound

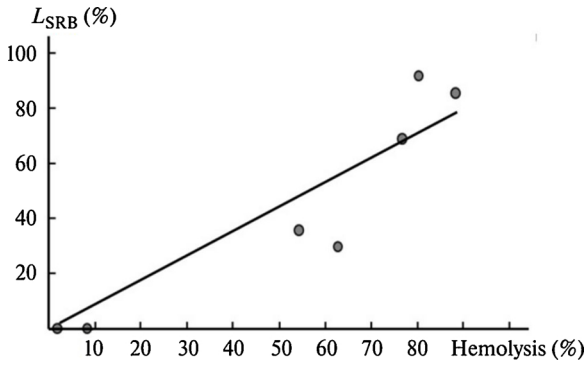

Fig. 4. Linear correlation between haemolysis rate in red blood cells and leakage of sulforhodamine B in LUVs (L_{SRB} %). The results shown are representative of a series of six chlorophyll α derivatives. Reprinted by permission with *Springer Nature*, Russian Chemical Bulletin reference [41]. Copyright © 2018. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[42,50–56]. Usually, the more amphiphilic the photosensitizer, the larger its compatibility with amphiphilic phospholipids of the membrane and, as a consequence, the better the photoactivity may be [37,42,44,54,56,57].

Furthermore, the localization of photosensitizers near the double bond of the alkyl chain favours the photooxidation reaction due to (i) lipid membranes having a higher concentration of oxygen than the surrounding solution, enabling energy transfer from the $^3PS_*$ state to oxygen [38,51,58,59] and (ii) a $^3PS_*$state that can react directly with the biological substrate [27].

3. Mechanisms of lipid oxidation: implications for membrane structure

The chemical transformation of lipids that is induced by photosensitization reactions can dramatically disturb membranes through two main pathways: (i) directly by reacting with the excited state of the photosensitizer or (ii) indirectly by reactive oxygen species, for instance, singlet oxygen ($^1O_2$) – see Fig. 5. $^1O_2$ is usually considered the main species involved in lipid photooxidation since it is an electrophilic molecule able to start ene reactions with unsaturated lipids that contain allylic hydrogens, thus producing lipid hydroperoxides (LOOH) [20,60]. The addition of singlet oxygen to the double bonds leads to a shift in the allylic position [61,62]. In turn, new byproducts are formed (Fig. 5). Lipid hydroperoxides (LOOH) are prominent early products of photooxidation and can have several possible fates, depending on whether they are catalysed in the presence of metals or enzymes (*e.g.*, iron or selenoperoxidase), that generate free radicals such as epoxy allylic peroxyl ($OLOO^\bullet$), peroxyl ($LOO^\bullet$) or alkoxyl ($LO^\bullet$), which can propagate the oxidation process [62]. Several different routes lead to the free radical formation of oxidized lipids bearing alcohol, ketone, carboxylic acid and aldehyde [63,64]. For instance, aldehydes can be generated by the Hock cleavage mechanism (transforming LOOH into an aldehyde) or by the β-scission route (converting $LO^\bullet$ to an aldehyde) [65,66], while two $LOO^\bullet$ can yield an alcohol and a ketone by the Russel mechanism [67].

Fig. 5 also shows that the direct contact of $^3PS_*$ with lipids can extract a hydrogen atom from an unsaturated fatty acid (LH) to form a lipid carbon-centred radical ($L^\bullet$). An oxygen molecule can be added to $L^\bullet$ to produce $LOO^\bullet$, which can generate a new chain of oxidation by abstracting another hydrogen from a neighbouring LH, that ends with the formation of LOOH and another lipid radical $L^\bullet$ [63]. As with other mechanisms, direct contact leads to final oxidized byproducts such as alcohols, ketones, carboxylic acids and/or aldehydes [64].

Therefore, the reaction of both LOOH and LH groups with either a radical species or $^3PS_*$ can produce other oxidized byproducts, generally lipids with cleaved tails [27,56,63].

Interestingly, oxidized lipids are able to modify the picture of membranes represented by the classic "*Fluid Mosaic Model*", which was proposed > 45 years ago by Singer and Nicolson [68], to a picture depicted by the "*Lipid Whisker Model*". The latter includes the particular conformation adopted by the oxidized fatty acids projected onto the surface [69–71]. It is in agreement with biophysical findings showing, for example, that the carboxyl group of an oxidized fatty acid moves dynamically towards the water interface [72,73] and that the hydroperoxide group of LOOH is mainly in the polar moiety of the membrane [73,74].

Although the "*Lipid Whisker Model*" complements the "*Fluid Mosaic Model*", it does not yet consider the tendency of oxidized lipids to aggregate and organize into nano- and micro-scale domains [75,76] at the surface of lipid bilayers, as is depicted in the emerging model of self-organized and multi-domain membranes or the "patchwork membranes" representation [77]. These incomplete and evolving models merely indicate that some aspects of the oxidized lipids in biological membranes are still uncertain and need to be explored in depth.

On the other hand, several theoretical and experimental studies make clear that the incorporation of oxidized lipids into biomembranes

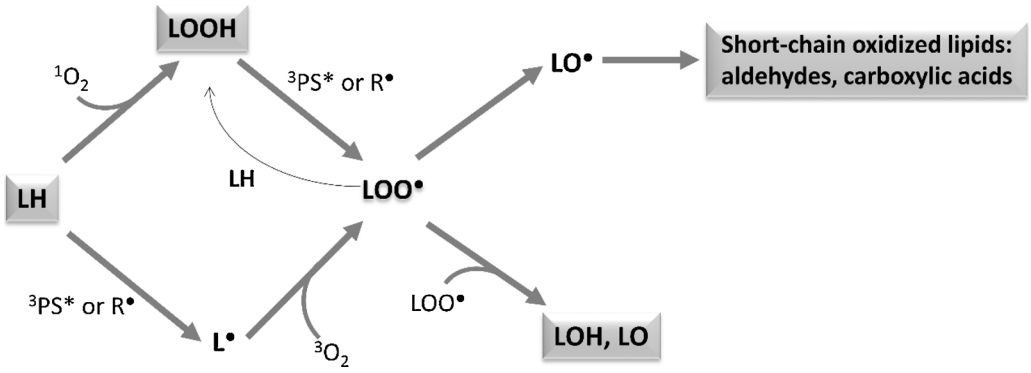

Fig. 5. Possible pathways of photo-induced lipid oxidation. Lipid photo-oxidation triggered by singlet oxygen ($^1O_2$) generated from the triplet excited state of the photosensitizer ($^3PS_*$) to generate LOOH. $R^{\bullet}$: generic radical species, LH: unsaturated fatty acid chain, $L^{\bullet}$: lipid carbon-centred radical, LOOH: lipid hydroperoxide, $LOO^{\bullet}$: peroxyl radical, $LO^{\bullet}$: alkoxyl radical, LOH: alcohol and LO: ketone. Adapted with permission from [27]. Copyright© 2018 American Chemical Society.

promotes diverse structural changes [78,79], including decreased lipid bilayer thickness [73,80], membrane packing [81], membrane fluidity [82], and membrane permeability [83], and an increased hydration layer [84], with the ability to form or disrupt phase separation [76] pore formation [81,85] and eventually disruption [85,86]. Essentially, the structural features, such as the polar head group, acyl chain length and the terminal group of non-shortened and truncated acyl chains govern the main biophysical and biochemical effects sensed by the biomembranes.

Therefore, to briefly explain the lipid chemical alteration caused by photooxidation, we first establish the membrane features associated with new chemical structures that originated from lipid oxidation.

3.1. Lipid hydroperoxide changes the physical properties of the membrane

As mentioned before, lipid hydroperoxide (LOOH) is a byproduct of photooxidation (Fig. 5) [20]. When LOOH accumulates in the membranes, one of the main alterations in membrane structure is an increase in the mean area per lipid and, consequently, an increase in membrane surface area [73,74,87]. In an experimental example, model lipid membranes are represented by a giant vesicle dispersed in a PS-containing membrane (Fig. 6). Initially, the membrane fluctuates under irradiation. Then, the projected area is increased and buds are expelled (white arrow in Fig. 6), leaving the membrane floppy (Fig. 6). Such events take place with no change in membrane permeability, as is shown by the maintained phase contrast of the membrane [87].

The molecular explanation behind the scenario presented in Fig. 6 is a clear example of how lipid oxidation can affect the physical properties of membranes. The increase in membrane surface area is attributed to the addition of the hydroperoxide group to the unsaturated double bond of the acyl chain (Fig. 7). This group is more stable in the polar/apolar interface due to its hydrophilic character and the transient hydrogen bonds it forms with water and lipid carbonyls or phosphate groups [74,88]. As a result, each lipid occupies an increased area, and a decrease in membrane thickness is observed [73,74,82,87,89]. Such alterations were also predicted by simulations of the molecular dynamics [74,89,90].

One efficient method to quantify the excess membrane area resulting from hydroperoxidation is micropipette pulling [82]. In the micropipette technique, a low-tension regimen is applied through a microcapillary glass to aspirate a giant vesicle to its interior and measure the excess area that was hidden in the fluctuating membrane during photosensitization [82]. By the use of this methodology, a total surface area was quantified as an increase of 15% for POPC hydroperoxide and 19% for DOPC hydroperoxide [82]. An increase in the molecular area was also observed for lipid hydroperoxides at the water–air interface by Langmuir monolayer surface pressure. These findings agree with the observations of van den Berg *et al,* who showed that the molecular surface areas of PLPCOOH were increased approximately 50% compared to those of PLPC [91]. Further, membrane manipulation by micropipette aspiration also enables the quantification of the membrane stretching modulus, which is dependent on the proportion of lipid that is converted from the non-oxidized form to the hydroperoxide form [35,82]. Bilayers of non-oxidized lipids such as POPC exhibit stretching moduli (K_A) of approximately 200 $mN.m^{-1}$ and are reduced to 50 $mN.m^{-1}$ for 100% POPCOOH (Fig. 8). Values for K_A from a mixture of POPCOOH:POPC (1:1 M ratio) indicate that the stretching modulus follows a linear variation with the fraction of POPCOOH into the bilayer (see Fig. 8). In other words, the membrane elastic modulus decreases proportionally to the number of hydroperoxide lipids into the membrane [82].

Concomitant with the reduced stretching modulus, of LOOH also promotes a decrease in the membrane thickness. In an interesting experiment, our group recently observed a reduction in POPCOOH membrane thickness of 20%, compared to the non-oxidized POPC bilayer, on the basis of small-angle X-ray scattering (SAXS) data [73]. This reduction occurred because 95% of the hydroperoxide group resides in the polar moiety of the membrane, close to carbonyl and phosphate groups, where it likely forms H-bonds [73].

Notably, we have also shown that the presence of POPC lipid hydroperoxides in the membrane favours lipid de-mixing in a ternary system containing unsaturated and saturated lipids with cholesterol [75,76]. Fig. 9 shows the phase separation observed microscopically for

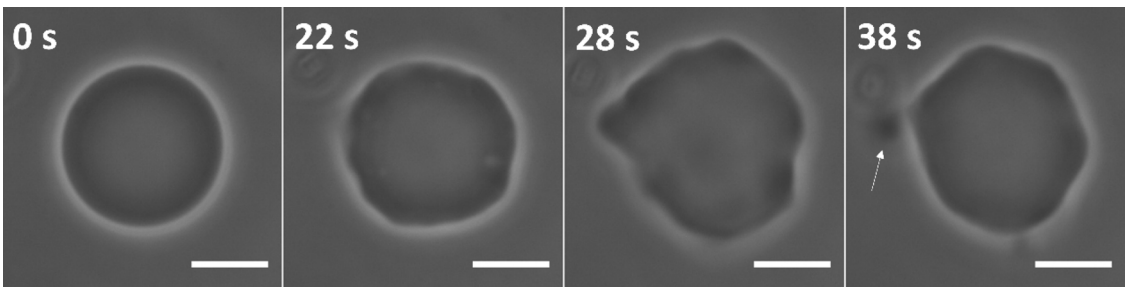

Fig. 6. Morphological changes caused by irradiation of DOPG vesicles dispersed in $TPPS_{2a}$ (0.5 μM) containing a glucose solution. The inner GUV compartment is filled by a sucrose solution. The difference in sugar refractive indexes in the phase contrast model enables the observation by optical microscopy. The irradiation time is shown on top of each snapshot. The scale bar corresponds to 10 μm.

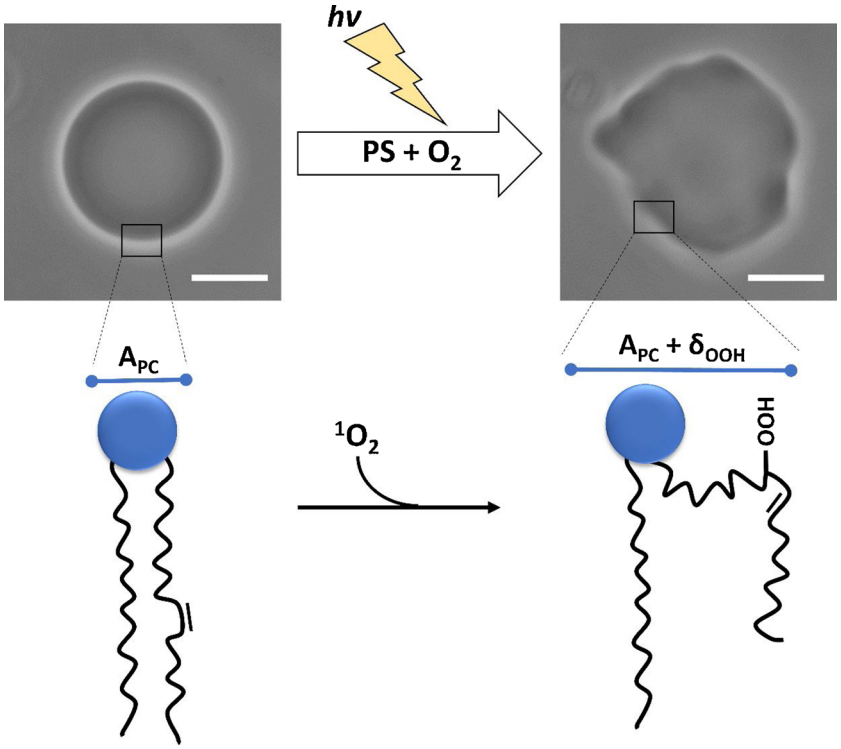

Fig. 7. Membrane morphological transformations induced by lipid hydroperoxide formation. Photoirradiation of vesicles in the presence of a PS generates, for instance, singlet oxygen that rapidly reacts with the double bonds of lipid tails. Such a reaction adds a hydroperoxide group to the alkyl chain (Fig. 5). Such a chemically induced structural change promotes an increase in the area per lipid. Scale bar represents 10 μm.

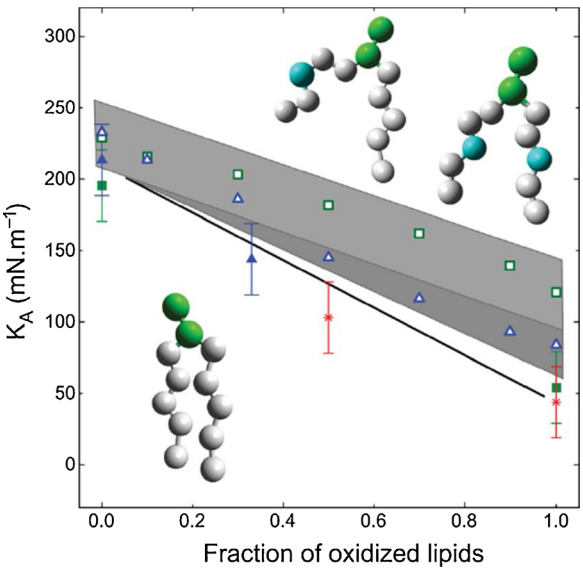

Fig. 8. Correlation between the membrane stretching modulus K_A and the fraction of oxidized lipid for (■) DOPC, (▲) POPC and (*) POPCOOH containing vesicles. The stretching moduli were also obtained through the single-chain mean field (SCMF) theory for POPC (□) and DOPC (Δ). Twelve beads illustrate the cross-grained model used in SCMF in which hydroperoxidation changes the nature of the third bead in one (POPC) or two (DOPC) lipid tails. Reproduced from reference [82] with permission from The Royal Society of Chemistry.

membranes with POPCOOH/DPPC/Chol; in contrast, membranes composed of POPC/DPPC/Chol ternary lipid mixtures are in a homogeneous phase. The presence of phospholipid hydroperoxides in the membrane enabled lipid raft formation. The reader is referred to Section 3.3 for further details on photoinduced lipid reorganization.

It is important to emphasize that the lipid hydroperoxide does not compromise membrane integrity or promote increased permeability, as observed by phase contrast models in Figs. 6 and 7 [76,82,87]. However, it plays an important role in changing the elastic modulus of the membrane and reorganizing the lipid bilayers by favouring phase segregation. Modifying the lipid raft composition and properties through hydroperoxidation may affect, for example, signal transduction that induces cellular processes for the (dis)association of key proteins.

After comprehending the physical alterations caused by the early products formed during photooxidation, *i.e.*, lipid hydroperoxides, we now focus the discussion on the continuity of photosensitization reactions and the consequences of these reactions on membrane permeability (Section 3.2) and lipid rafts (Section 3.3).

3.2. Lipid oxidation alters membrane permeability

The most well-accepted explanation for the correlation between membrane photodamage and photodynamic efficiency at the cellular level [92–95] is based on changes in membrane permeability due to the generation of membrane defects and/or pores *in situ* [96]. Membrane permeation is typically assessed by using a high concentration of a fluorescent dye molecule (*e.g.*, calcein or carboxyfluorescein) that exhibits self-quenching when trapped in the aqueous inner compartment of a liposome. Under photoirradiation, a considerable amount of dye is released from the liposome to the outer aqueous phase and diluted into the much larger volume. As a result, a marked increase in the dye fluorescence is easily detected [97]. Using this methodology, some works have correlated the primary cause of cancer cell death promoted by PDT to increased cell membrane permeability [45,94,95].

In fact, *Tasso and co-workers* reported on a meta-isomer of tetra (pyridyl)porphyrins complexed to $[PtCl(bipy)]^+$ that induces two-fold the phototoxicity in HeLa cells than its *para* isomer. This greater photodynamic efficiency correlates well with the 7-fold faster leaking of carboxyfluorescein (*CF*) from POPC liposomes that is induced by the *meta*-isomer porphyrin compared to that induced by the *para* isomer [94]. Additionally, *Antonenko et al* validated the membrane damaging effect by comparing the efficiency of BACE (chlorin e6 13(1)-N-{2-[N-(1-carba-closo-dodecaboran-1-yl)methyl]aminoethyl}amide-15(2), 17(3)-dimethyl ester) and chlorin e6 in promoting dye leakage from liposomes and their effects on the viability of M-1 sarcoma cells under

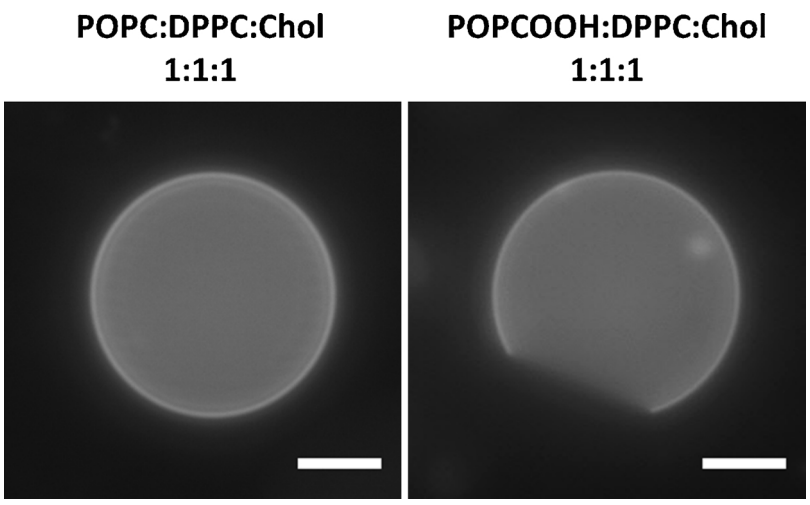

Fig. 9. Fluorescence images of GUVs obtained with a ternary lipid mixture. One GUV has a membrane with non-oxidized lipids (POPC:DPPC:Chol), and the other has a membrane with oxidized lipid (POPCOOH:DPPC:Chol). They have the same relative composition (1:1:1) at 23 °C. The scale bar corresponds to 10 μm.

photoirradiation [95].

In general, faster probe leakage is linked to an increase in PS-membrane interactions [42], irradiation time [98] and the number of unsaturated lipids present in the phospholipid bilayer [80,96,99,100]. Concerning PS-membrane interactions, *Bacellar et al* compared a series of phenothiazinium derivatives with different degrees of amphiphilicity. They demonstrated that the efficiency of *CF* released from liposomes is higher when the PS-membrane binding constant is greater (indicating low aggregation) [42]. It is important to stress that the strong interactions between a PS and components of lipid bilayer, as well as the extent of damage efficacy, do not depend exclusively on the properties of the PS (*i.e.*, degree of hydrophobicity or hydrophilicity), but they also depend on the physical properties of the membrane, which also indicate the impact of photodamage on the membrane [101]. For instance, the added cholesterol or dimyristoyl phosphatidylcholine to the bilayer drives the tetrapyrrole chromophore deeper into the liposome bilayer [92], and the rate of dye leakage through the lipid bilayer increases proportionally to the degree of fatty acid unsaturation [80,96,100].

Even though assays using fluorescent dyes provide important information about the extent and kinetics of the liposome damage, they do not enable the visualization of the physical alterations to the membrane during photosensitization. Hence, many mysterious effects of permeabilization have been revealed by using giant unilamellar vesicles (GUVs) as model membranes. These vesicles can be produced by two different methods: (i) electroformation [102] or (ii) gel-assisted growth [103]. Both typically involve the growth of GUVs in sucrose solution and dilution in glucose solution at the same osmolarity (0.1–0.2 M), thus, establishing a sugar asymmetry inside and outside the vesicles that settles the vesicles to the bottom slides and enabling observation through an inverted optical microscope. The difference in the refractive index between the sucrose and glucose solutions permits monitoring and quantification of the phase contrast intensities as well as the observation of morphological changes taking place in the membrane [80,104,105]. Considering that sugars have a smaller diffusion coefficient in lipid membranes than in water (*e.g.*, the diffusion coefficient of glucose to phosphatidylcholine membranes is 8×10^{-5} μm s^{-1} compared to ~ 40 μm s^{-1} for water [106,107]), they are valuable for observing changes in membrane permeability.

In fact, our group has reported systematic studies on photosensitization effects in GUVs. As described above, we have shown that photooxidation causes an increase in membrane surface area, usually accompanied by large fluctuations, membrane bud and string formation, and changes in GUV shape (see Fig. 6) [80,87]. Such morphological changes are attributed to hydroperoxide generation (as discussed in detail in Section 3.1; see Fig. 7). However, the integrity of unsaturated unilamellar vesicles is preserved even after the full transformation of POPC into POPCOOH [82,87]. Interestingly, after exhibiting fluctuations and budding, GUV recovers its spherical shape followed by faded phase contrast as a consequence of increased membrane permeability as the photochemical reactions progress [80,100,108]. Such loss in contrast reflects the exchange between the sucrose and glucose solutions. This occurs because during photooxidation, the pores must open, enabling the traffic of sugar molecules of approximately 0.5 nm [109,110]. Eventually, these pores can be visualized at the micron scale before the total collapse of the GUV [86] or not [80], depending on the experimental conditions (*e.g.*, PS concentration and level of oxidative stress). As described by *Caetano et al*, high concentrations (> 25 μM) of methylene blue (MB) in photoirradiated GUVs composed of DOPC cause vesicle disruption and collapse on the substrate [86]. On the other hand, moderate modifications to GUVs under more controlled photooxidation circumstances and at relatively low PS concentrations were reported in other works. In these cases, pore formation with no membrane disruption was detected [56,80,83,111]. In an experiment of GUV photooxidation, the grey level intensity along the GUV equator (Fig. 10A) was monitored over the time it was irradiated. The results indicate sigmoidal decay (Fig. 10B). In this way, one can investigate the permeabilization kinetics that depend on PS concentration and on the degree of lipid unsaturation [80,100]. Essentially, the reaction-diffusion model described by *Mertins et al* indicated a weak tendency of POPC species to aggregate into a pore nucleus, a finding that contrasts with a strong tendency for oxidized DOPC lipids to aggregate in this manner [80]. In other words, the kinetics of pore generation in GUVs also depends on the lipid species, such that, for example, DOPC, which contains two unsaturated alkyl chains, promotes membrane leakage faster than POPC, which has only one unsaturated alkyl chain [80]. Indeed, *Bour et al* also found that morphological alteration, such as area increase and membrane permeabilization speeds, depends on the properties of the unsaturated lipids, such as the number, position and geometry (*cis* or *trans*) of the unsaturation chains [100].

Taken together, these reports indicate that photooxidized by-products, rather than lipid hydroperoxides, can promote membrane pore opening. Indeed, several experimental [27,35,56,86] and theoretical [81,85,112] studies have found a correlation between the presence of oxidized lipids with truncated chains in the lipid bilayer and membrane permeabilization. Oxidized lipids with shortened alkyl chains exhibit different lipid packing than non-oxidized species, thus supporting pore formation in the membrane.

In this context, *Ytzhak and Ehrenberg* have shown that a membrane

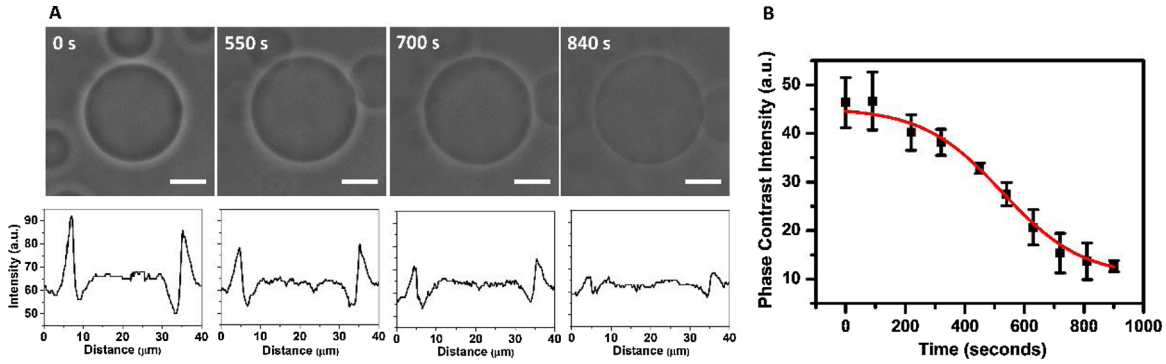

Fig. 10. (A) Optical microscopy images of the giant vesicle (GUV) with phase contrasts created by DOPC and the intensity profiles across the equator of the GUVs during photoactivation of 0.7 μM $AlPcS_{2a}$ dispersed in glucose solution. Scale bar of 10 μm. (B) A typical time evolution of contrast amplitude as a function of time. Each square (■) represents the mean ± SD from eight measurements at the determined time point. The red line represents the best fit to the experimental data by using a Boltzmann equation [83]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

comprised with merely 2% oxidatively shortened lipids (PGPC bearing a carboxylic group and ALDOPC containing aldehyde) has enhanced leakage of K^+ ions, which compromises the ability of the membrane to bear an electric diffusion potential. As the proportion of the oxidized lipids increases, the membrane becomes less stable until it is no longer possible to sustain (16%) an electric potential. Interestingly, the researchers observed a similar level of another type of modified lipid, *i.e.*, lysoPC, did not affect the membrane in terms of ion leakage, which indicates that the impact on the membrane caused by PGPC and ALDOPC is much greater than that caused by lysolecithin [113].

Concomitantly, *Runas et al* demonstrated that small amounts of POxnoPC (truncated oxidized lipid bearing an aldehyde group) can cause a drastic increase in the GUV membrane permeability [35]. Their experimental data indicated three distinct permeability regimes: (i) in the absence of oxidation, a slow rate permeability, 1.5×10^{-6} cm s^{-1}, was measured; in contrast, (ii) permeability was one order of magnitude faster (1.5×10^{-5} cm s^{-1}) when low levels of POxnoPC (between 2.5 and 10%) were present in the lipid bilayer; and (iii) with 12.5% or more oxidized species, the bilayer was permeable to fluorescein-dextran of 40–2000 kDa, suggesting formation of pores wider than ~55 nm [35,114]. It is noteworthy that low levels (~2% mol) of shortened oxidized lipid chains were enough to increase membrane permeability [35,113], whereas other experimental findings indicated that vesicles could not withstand > 18–20 mol% of oxidized lipids without collapsing [35,76,113,115].

In the related cases described above, the oxidation effect on bilayer permeability was examined by replacing defined amounts of the unsaturated lipid with a corresponding oxidized product to mimic the photosensitization process [35,113,114]. Recently, *Bacellar and coworkers* characterized the products formed from photodamaged membranes using a phenothiazinium-based PS [27]. Of note, the authors concluded that the consecutive step following lipid hydroperoxidation takes place *via* alkoxyl radical β-scission in a direct-contact reaction-dependent manner, favouring the formation of aldehydes, which was considered the pivotal reaction indicating membrane permeabilization [27]. In parallel, *Vignoni et al* reported that photooxidation of liposomes using pterin derivatives as PSs led to the formation of hydroperoxide and hydroxyl derivatives, which in turn underwent cleavage to form short-chain secondary products, such as carboxylic acid and aldehydes [56]. All these results confirmed the key role of oxidized truncated alkyl chain lipids in promoting membrane leakage [27,35,56,113,114].

Interestingly, molecular dynamics simulations predict that, in the presence of a significant amount of oxidized lipids, aldehydes and carboxylic groups hanging from the end of truncated chains, which results in a reorientation of the functional groups that affects the polar head region of the lipid bilayer [72,74,81,84,85,116]. As a result of this lipid reorientation, which prevents exposure of the hydrophobic tails, the conical shape inherent to truncated lipids reduces the line tension and counterbalances the modifications to packing to stabilize the micelle-like structure and drive pore opening [72,81,116,117].

It is noteworthy that lipid hydroperoxides do not provoke changes in membrane permeabilization as shortened oxidized chain lipids do. Previous works reported that vesicles are able to maintain sugar asymmetry even after all the lipids are converted hydroperoxides [82,87]. This finding is in agreement simulations of the molecular dynamics that show pore-free membranes containing 100% hydroperoxides [81,112]. It seems that oxidized lipids exhibiting a cylindrical shape do not mediate lipid bilayer leakage. Indeed, simulations revealed that membranes composed of hydroperoxides, alcohols, and ketones exhibit Gibbs free energy barriers for water permeation of approximately 30 kJ.mol$^{-1,}$ which is equal to the that of the non-oxidized species (*i.e.*, POPC, $\Delta G \approx 31 \pm 3$ kJ.mol^{-1}) [27]. On the other hand, truncated lipids with hanging aldehyde groups significantly impact the permeation free energy barrier to water (*i.e.*, ALDOPC, $\Delta G \approx 12 \pm 4$ kJ.mol^{-1}) [27]. This difference in membrane permeabilization capability must be related to the packing parameter ≈ 1 for oxidized lipids, such as hydroperoxides, that favour bilayer structures, against the packing parameter ≈ 0.5 of shortened lipids that tend to form bilayers with pores or micelles [81,117]. Further, *Heuvingh and Bonneau* used GUVs of DOPC and a chlorin PS to observe the effects of asymmetric photooxidation triggering a curvature-associated shape transition and permeabilization [105]. They observed that different shape transitions (*e.g.*, oblate sphere to prolate sphere to buds) typical of membrane curvature modifications accompanied by vesicle permeation [105].

In summary, several reports [76,80,86,87,108] support the idea that GUV remodelling upon photooxidation can be described in two consecutive stages: (i) vesicles exhibit shape destabilization with membrane area increases that correspond to lipid hydroperoxidation (Fig. 11); and (ii) the membrane becomes tense, and then, the membrane permeability rapidly changes. The latter phenomenon is due to pore formation attributed to the generation of cleaved and oxidized secondary products (such as represented in Fig. 11).

It has been shown that the introduction of 30% cholesterol within DPPC and POPG monolayers reduced oxidation at the air-water interface [118]. *Kerdous et al* also showed that the relative permeability of DOPC/cholesterol GUVs containing 30% cholesterol had decreased by approximately 60% under photooxidative stress [104]. A molecular dynamics study indicated that cholesterol-rich membranes (30% cholesterol) do not form pores when 60% of the lipids are oxidized [119]. This failure to produce pores is attributed to cholesterol forming hydrogen bonds with the oxidized lipids to hindering changes to the tilt angles of the interacting lipids and thereby maintains membrane integrity [119]. In this context, cholesterol has been employed to delay the membrane permeation rates of vesicles created with oxidized

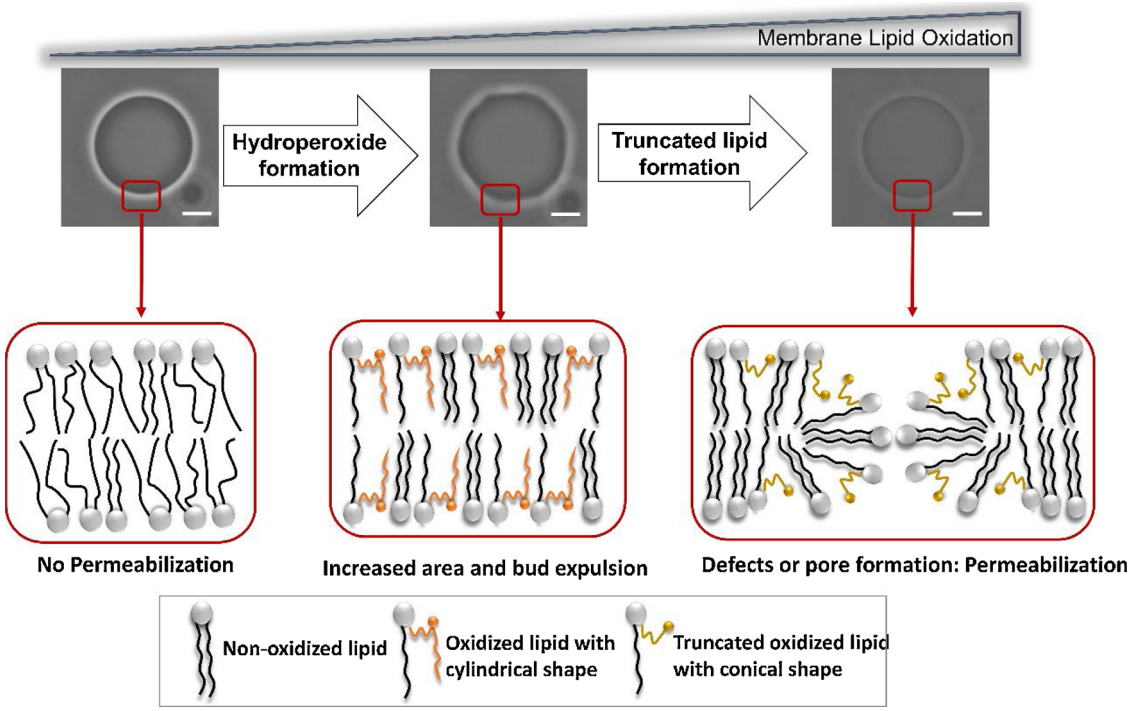

Fig. 11. Sketch illustrating the mechanisms and dynamics of GUVs under photooxidation, highlighting the physical effects caused by membranes that are organized differently because they contain an oxidized lipid of a cylindrical shape or a shortened chain lipid with a conical shape. The scale bar represents 10 μm.

products [114,118]. This delay is likely caused by the oxidized cholesterol products, which are more polar than cholesterol and have a unique spatial distribution that counterbalances the disorder caused by the oxidation of the lipids [112,116,119].

3.3. The impact of lipid photooxidation on membrane domains

The arrangement of lipid composition on plasma and organelle membranes is of paramount importance not only to control molecular transport phenomena and related outer compartments but also to regulate cell function [120,121]. Different functions are driven by the diverse combination of lipids and the organization of various cell types, tissues and organelles. Consequently, the correct distribution of membrane components influences several cellular processes directly. The existence of different lipid species in the lipid bilayers may promote lateral segregation into membrane microdomains. These microdomains are formed because distinct lipids tend to segregate due to their particular physicochemical properties [122–124]. In the case of lipid rafts (Fig. 12), liquid-ordered phases (Lo) are dispersed in liquid-disordered phases (Ld) and operate as platforms that can include or exclude protein receptors (*e.g.*, GPI-anchored proteins and kinases) responsible for stimuli-induced signal transduction, membrane trafficking, and metabolic regulation that affects cellular homeostasis [125]. Lipid rafts are thought to be a crucial step in the evolution of cellular complexity [124,126].

The presence and exact nature of raft domains in live cells remain unclear due to the lack of tools that would enable their direct detection through observation. Most studies on rafts in cells have postulated that they are small (< 200 nm) and very dynamic with short lifetimes and variable sizes [127–129]. Basically, the membrane raft concept has been supported by mimetic model membranes, which provide clear evidence of the Lo–Ld phase separation (Fig. 12) [124]. The same findings are reported for investigations on the role of oxidized lipids during lipid phase segregation in which artificial membranes made of purified lipids systems have been employed [76,130–132].

More than 10 years ago, *Ayuyan and Cohen* reported observing photoinduced lipid rafts on model membranes on the basis of morphological alterations in GUVs composed of ternary lipid mixtures of cholesterol, unsaturated and saturated lipids that they visualized with fluorescent probes [133]. Fluorescent lipid probes have been widely used to study phases in model membranes, for which diagrams have been created and through which membrane behaviour in cells have been predicted. To avoid misinterpretation, the authors emphasized caution in performing these experiments, suggesting the use of antioxidants (*e.g.*, tocopherol that is native to the cell membrane) to prevent artefacts [133]. Using a fluorescent probe, *Zhao et al* also observed large domains that were derived from the coalescence of smaller pre-existing lipid domains in a ternary lipid system [134]. They suggested that this artefact could be attenuated by reducing the dye concentration and the illumination intensity [134]. For example, by decreasing the concentration of TR-DHPE (Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt), they observed that the domain artefact formed with kinetics that were 10-fold slower [134].

On the other hand, *Staneva and co-workers* reported that photoinduced domain formation in GUVs can be purposely used as a method to mimic rapid lipid raft formation processes in artificial membranes, thereby enabling the dynamics and phase morphologies of the generated liquid ordered (Lo) microdomain to be determined [130]. They suggested that the photoinduced Lo domain forms rapidly through spinodal decomposition rather than nucleation processes. Further, ganglioside GM1 (an essential component directly involved in several raft-associated cellular processes) intrinsically destabilizes the Lo phase due to its relatively wear interaction with other lipids [130]. Recently, the same group demonstrated the formation and dynamics of β-amyloid polymerization on raft-mimicking Lo domains photogenerated in GUVs composed of PC/SM/Chol/GM1 [135].

In an interesting experiment, our group intentionally employed a porphyrin anchored in a lipid (PE-porphyrin) as a photosensitizer to study the changes in the phase behaviour caused by photooxidation of GUVs. Of thirteen different lipid ternary mixtures composed of POPC/DPPC/cholesterol, only particular compositions induced phase coexistence under irradiation [75]. We speculated that the boundary line was displaced vertically towards the higher cholesterol content with

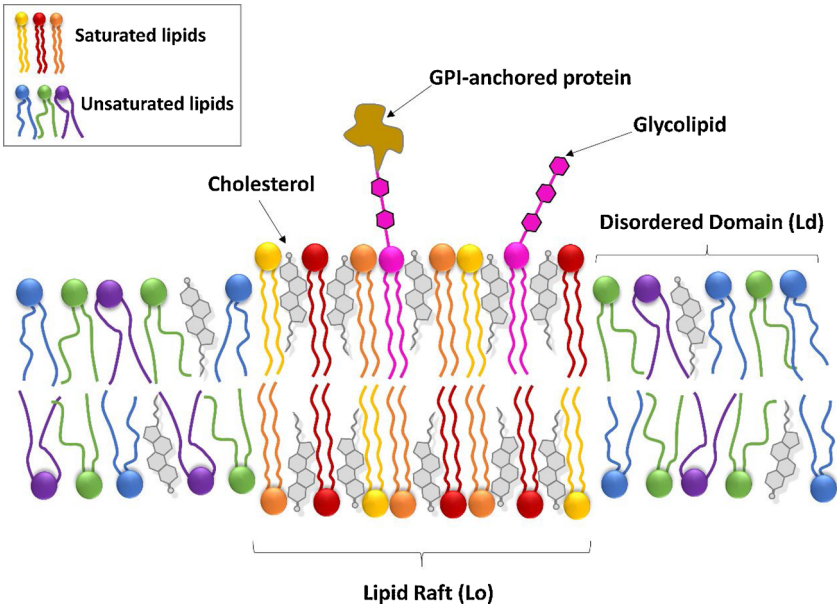

Fig. 12. Scheme illustrating a lipid raft. Lo: liquid ordered; Ld: liquid disordered.

respect to the ternary phase diagram of POPC/DPPC/cholesterol mixtures in the absence of oxidized species. The explanation for this phenomenon, as indicated in the phase diagram, concerns the type of oxidized lipid generated in this system. Owing to the presence of the porphyrin photosensitizer anchored on the membrane led to singlet oxygen production under irradiation, and $^1O_2$ reacts with the unsaturation of lipids (*i.e.*, phospholipid and cholesterol), we hypothesized that the generated POPC hydroperoxide (POPCOOH) is a main promoter of lipid lateral diffusion [75]. In fact, in subsequent studies, we were able to prove that POPCOOH was involved in phase separation in several lipid mixtures, as indicated on the phase diagram depicted in Fig. 13 [76]. By replacing POPC with POPCOOH (Fig. 13), we defined three distinct regions [76]: (i) a homogeneous phase, (ii) a liquid disordered – liquid ordered (Ld–Lo) coexistence phase and (iii) a fluid gel phase (Fig. 13B); these findings stand in contrast to those based on non-oxidized lipids, for which only a homogeneous phase is observed on the phase diagram (Fig. 13A).

Recently, *Wnetrzak et al* studied the influence of the oxidized cholesterol derivative (7α-Chol-OH) on sphingomyelin/POPC mixtures by Langmuir monolayer surface pressure, Brewster angle microscopy and theoretical calculations. Their results showed that interactions in the sphingomyelin/7α-Chol-OH system are twice as strong as those of a sphingomyelin/cholesterol regime, and they attribute this result to the increased packing density and higher stability of the model lipid raft caused by the presence of 7α-Chol-OH [131].

Other authors also indicated the potential consequence of oxidative stress in membrane microdomain segregation mechanisms by using monolayer compression isotherms and Brewster angle microscopy [131,136]. Monolayer experiments revealed that oxidized lipid phases are defined by a significant increase in average molecular area and reduced surface potential [137,138]. For instance, PazePC (a phospholipid carrying a carboxylic acid group on a shortened oxidized chain) occupies > 35% more molecular area its non-oxidized counterpart (POPC) [136]. Therefore, a gradual exchange of POPC for its oxidation product (PazePC) in the PC/SM/cholesterol membrane can stabilize phase separation and prevent the miscibility transition in the monolayer system [136,138]. In addition, *Megli and co-workers* also recognized that oxidized lipids induce phase separation in more rigid DPPC-rich membranes by electron paramagnetic resonance [139].

Even though different research groups have reported that photosensitization induces lipid microdomains, the detailed explanations of this mechanism are controversial and poorly understood. *Ayuyan et al* suggested that phases separated due to sphingomyelin peroxide products formed during photosensitization [133]. *Zhao and co-workers* suggested that lipid oligomers might be formed by free radical-induced double bond dimerization [134], while our results indicated that phase coexistence was linked to the generation of lipid hydroperoxides [75].

In all these cases, the oxidation reactions were performed *in situ* on GUVs within the field of the fluorescence microscope, limiting the ability to discern the factors that govern this phenomenon. In this context, we recently described a direct experimental approach that enables us to mimic the photooxidation process by replacing a controlled amount of unsaturated phospholipid with a corresponding amount of oxidized product [76]. Indeed, our data demonstrated the role of two types of oxidized lipids on membrane microdomains: (i) an oxidized lipid with a cylindrical shape due to the hydroperoxide group (*i.e.*, POPCOOH) is strongly related to the formation of microdomains and does not affect membrane permeation, and (ii) a lipid with an oxidized shortened chain, such as PazePC, which has a conical shape that contributes to lipid mixing and membrane permeabilization (Fig. 14) [76].

Two main aspects of the evidence that lipid hydroperoxides contributes to phase segregation were considered: (i) the larger mean molecular area occupied in the bilayer compared to that of the non-oxidized species (POPCOOH ~78 Å > POPC ~65 Å) [73] and (ii) the tail lengths that are shorter, rendering them 20% thinner [73], such that they can potentially induce phase segregation to minimize the exposure of hydrophobic tails to the aqueous media. In the case of truncated lipids, the lower packing parameter inherent to chains supports the contention that oxidation may disrupt domains and contributes to membrane permeabilization [76].

In line with the recent discovery that oxidized lipids can disrupt membrane microdomains as well as form it, *Matsufuji et al* synthesized oxidized ceramide derivatives by oxidizing the OH group of ceramide to generate a carboxylic acid (PCerCOOH) or methylester (PCerCOOMe) and evaluated how these oxidative derivatives affected the physical properties of membrane [132]. They found that phase separation largely disappears in GUVs composed of oxidized ceramide bearing carboxylic acid with POPC or with palmitoylsphingomyelin. In contrast, oxidized ceramide-containing methylester groups (PCerCOOMe) was not able to destabilize membrane immiscibility. This effect was attributed to the unprotonated form of carboxylic acid ($PCerCOO^-$) at neutral pH, which hinders gel phase formation *via* electrostatic

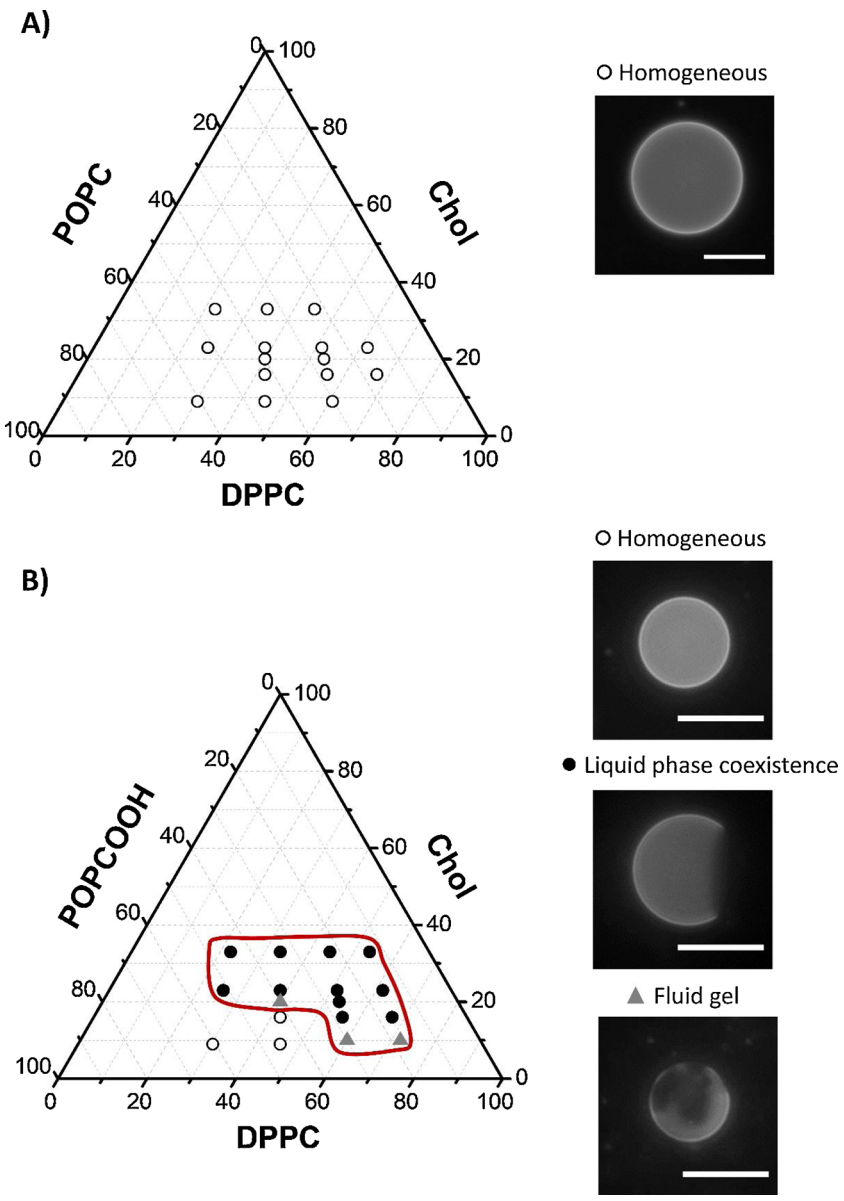

Fig. 13. Phase diagram established from microscopic observations of GUVs (A) POPC/DPPC/Cholesterol and (B) POPCOOH/DPPC/cholesterol, both containing 0.2% rhodamine-DOPE. White circles (○) represent GUVs showing uniform fluorescence; black symbols (●) refer to GUVs exhibiting Ld/Lo phase coexistence; and grey triangles (▲) represent the samples displaying fluid/gel coexistence. The scale bar corresponds to 20 μm. Reprinted from [76], Copyright© 2019, with permission of Elsevier.

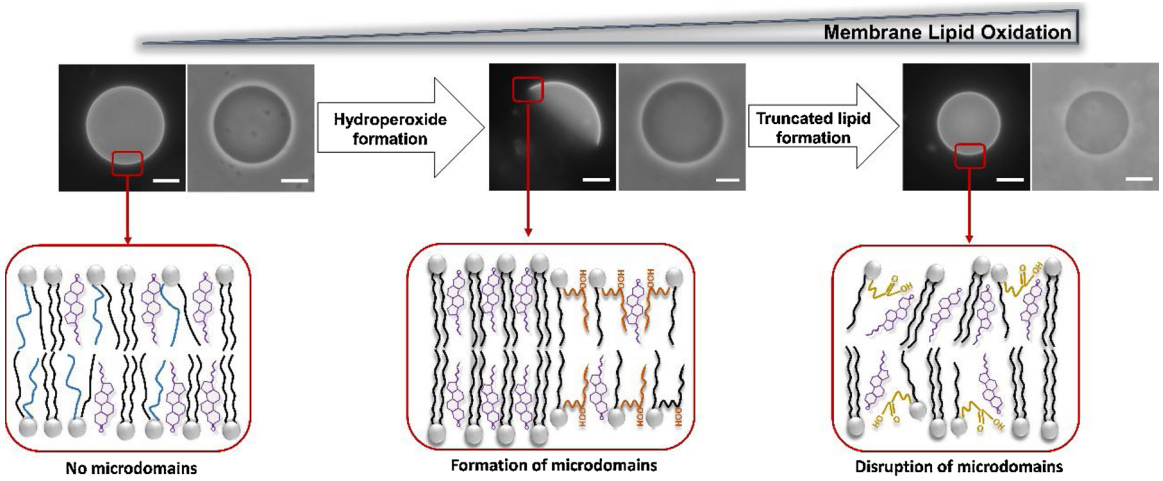

Fig. 14. Proposed mechanism showing dynamic formation and disruption of microdomains under photooxidation. Scale bar represents 10 μm.

intermolecular repulsion [132].

In agreement with findings in model membranes, *Brameshuber and co-workers* determined the ability of oxidized lipid species to disrupt GPI nanoplatforms in the plasma membrane of Chinese hamster ovary (CHO) cells using single-molecule microscopy [140]. They incubated CHO cells expressing mGFP-GPI (monomeric GFP anchored *via* GPI) with 10 μM of oxidized lipid (POVPC or PGPC) and observed a significant reduction in the mGFP-GPI dimer fraction compared to that of the control. In other words, > 73% of all detected nanoplatforms had disintegrated upon addition of an oxidized lipid carrying a carboxylic group (PGPC) or an oxidized lipid carrying an aldehyde group on the truncated chain (POVPC) [140]. As a consequence, any signalling that involves GPI proteins might be disrupted by the presence of oxidized lipids, which decreases the raft-type associations of signalling pathway proteins [140].

In summary, in this section, we presented several critical aspects of lipid oxidation, such as its ability to destabilize the "patchwork membrane", that have recently emerged and contribute to the understanding of cellular dysfunctions other than those that cause membrane permeabilization. For example, lipid rafts can maintain important proteins of signalling cellular process [125], and oxidized lipids (*e.g.*, lipid hydroperoxide, oxidized forms of cholesterol) not only promote membrane permeabilization but also can perturb cell function by influencing the lateral arrangement of lipids in the membrane [76,131]. In turn, these lipid functions may be responsible for cell death [140,141] or pathological diseases [135,142–144].

4. Implications of lipid oxidation on cellular functions

As explained above, lipid photooxidation damages membrane structure, modifying its physical properties. Since lipids are responsible for maintaining the integrity of cellular membranes, extensive photooxidation of lipids directly affects the biophysical membrane properties such as the level of hydration, bilayer thickness, packing, volume occupied per lipid, phase separation and content loss [78,79]. As a consequence, the changes to the cell biochemistry and the affected cellular functions may trigger cell death. For instance, enzymes can be activated by alterations in membrane structure [145]. It has been shown that phospholipase A2 (PLA_2) activity is increased in vesicles containing oxidized soybean phosphatidylcholine compared that the activity in non-oxidized phospholipid membranes [145] because PLA_2 does not act on a tightly packed lipid bilayer [146], but the lipid oxidation alters the membrane packing, thus enabling PLA_2 to act [82]. Considering that PLA_2 is critically involved in the cellular signalling that leads to apoptotic cell death [147], lipid oxidation indirectly causes biochemical changes that activate cell death. Indeed, regarding PDT signalling, it was reported that photooxidation initiates apoptosis *via* PLA_2 and PLC (phospholipase C) enzymes due to the breakdown of membrane phospholipids [148]. The activation of these enzymes produces second messenger molecules important to early steps of a signalling cascade that culminates in DNA fragmentation and apoptotic cell death [148].

It should be noted that all cellular responses caused by lipid oxidation must be determined by the way in which the PS reaches the target membrane. PS can be translocated to the plasma membrane or to internal membranes, including mitochondria, lysosomes, endoplasmic reticulum and Golgi apparatus. The localization of PS in a specific membrane depends on its chemical nature (hydrophobicity, type and number of charges, polarity and asymmetry), the incubation period of cells with PS and the cell type [44,57,149,150]. Although no straightforward pattern or rule dictates the trafficking of PS to specific organelles, some studies indicate that lipophilicity and charge strongly influence PS localization in the cell [44,111,150–153]. For example, lipophilic PSs bearing positive charges usually accumulate in the mitochondrial membrane due to electrostatic attraction generated through its negative electrochemical potential (which is six-fold greater than that of the plasma membrane) [111,152,154,155]. Moreover, PS location can also vary depending on the incubation time. In general, a short incubation time (0.5–1 h) guides PS to the plasma membrane, while a long incubation period (> 4 h) enables PS to reach internal cell membranes (*i.e.*, those of mitochondria, lysosomes, and/or endoplasmic reticulum) [156–158].

Considering that PS can act in membranes in different categories and that each subcellular membrane induces a distinct cellular response due to the diversity of its lipid composition, we subdivided this section to address the biological photooxidative consequences accordingly to the membrane features specific to the plasma, mitochondrial and lysosomal membranes. It is important to emphasize that PS does not always or solely localize in one type of membrane [29,159,160], but it can be incorporated more commonly in more than one target on the basis of the physical features, chemical aspects and concentration levels of the PS.

4.1. Consequences of plasma membrane photooxidation

Considering that the plasma membrane of eukaryotic cells contains > 30% of unsaturated lipids [161–163], especially phospholipids, and oxidation can generate shortened chain lipids that favour pore formation, it is expected that plasma membrane photosensitization promotes loss of barrier functions and allows the exchange of intracellular and extracellular cell contents. In fact, several works describe dramatic changes in plasma membrane morphology accompanied by cytoplasmic leakage, depletion of intracellular ATP and cell shrinkage under photosensitization [28,156–158,164–166].

Therefore, concerning the plasma membrane as a PS target, the main mechanism of cell death has been attributed to necrosis-like death [156–158,164–166]. As an advantage, cells die very rapidly (over 24 h after PDT treatment). However, surviving cells may grow quite normally after a long period of time following PDT (~ 120 h) [156]. Necrosis leads to the release of intracellular DAMPs (damage-associated molecular patterns), which can trigger the inflammation process [167,168]. Indeed, *Greenberg et al* demonstrated that truncated oxidized lipids enable direct physical access to the cell surface macrophage CD36 receptor since the hydrophilic part of oxidized lipid is projected to the bilayer surface [69]. This ability of macrophages specifically recognizes oxidized lipids on the cell membrane surface plays an important role in phagocytic engulfment and removal of apoptotic and senescent cells [69]. It seems that truncated oxidized lipids favour the inflammation process either promoting membrane pores responsible for release of the intracellular DAMPs or facilitating the access of macrophage receptors.

It must be noted, however, that necrosis is not the only possible scenario for plasma membrane oxidation. The formation of shortened chain lipids during oxidation not only leads to membrane permeabilization but can also provoke lipid raft disassociation [76,140]. In this context, *Brameshuber et al* demonstrated that the disarrangement of nanoplatforms in the plasma membrane of Chinese hamster ovary (CHO) cells, caused by the addition of POVPC, was correlated with the increased signal of the apoptosis marker annexin V, suggesting apoptosis as the cell death mechanism [140]. Of note, oxidatively truncated phospholipids can also be internalized to intracellular compartments through TMEM30a (β-subunit transmembrane protein 30A) [169], allowing its interaction with pro-apoptotic protein Bid, which promotes mitochondrial membrane depolarization and initiates the intrinsic pathway to apoptotic cell death [170].

In the absence of reducing agents and/or catalytic metal ions, lipid hydroperoxides (LOOH) can accumulate during photosensitization and perturb both plasma membrane structure and function due to their increased hydrophilicity [171]. Therefore, the generation of the primary products of lipid photooxidation, such as lipid hydroperoxides, may act as signalling molecules [62]. Some authors describe that relatively mild (sublethal) oxidative injury activates enzymes (*e.g.*, phospholipid peroxidase glutathione 4 - GPX4) to remove lipid hydroperoxides from the plasma membrane [172–174]. However, overwhelming lipid hydroperoxide accumulation in the presence of iron and

under inactivated or suppressed GPX4 results in regulated cell death recently recognized as ferroptosis [173–176]. Because singlet oxygen triggers lipid hydroperoxide production into cell membranes, it is possible that a similar situation might occur in the PDT scenario. Moreover, model membrane systems reveal that LOOH favours lipid raft formation [75,76], which could be another indication that LOOH probably acts as an early mediator of the signalling process to cell death instead of causing plasma membrane disruption.

4.2. Consequences of mitochondrial membrane photooxidation

Taking into account that mitochondria work as cell batteries that govern energy generation by ATP synthesis, thus involving an important role as subcellular organelles, several authors have focused on mitochondria as targets for PDT [21,160,177–180]. Among the lipids in mitochondria, highly unsaturated cardiolipin (CL) compromises > 20% by weight of the total lipid components of its organelle [181]. Cardiolipin interacts with some specific proteins, such as cytochrome *c*, due to the negative charge of phosphate groups. CL photooxidation thus affects its conformation, changing its affinity for membrane proteins, which results in apoptosis cell death [182–184].

In fact, *Shidoji et al* demonstrated by NMR spectroscopy that CLOOH species binds to cytochrome *c* to a lower extent than to non-oxidized CL [185]. Specifically, when 1 or 2 acyl CL groups are hydroperoxides and the remaining 1 or 2 moieties are not, the difference in the NMR shift signal with respect to the intact CL is less pronounced than that of CL trihydroperoxides. This suggests that at least 2 out of 4 acyl groups on CL are essential for binding to cytochrome *c* [185]. In addition, *Kawai and co-workers* determined that the presence of PazePC (a truncated oxidized lipid bearing carboxylic acid) increases the dissociation constant between cytochrome *c* and mimetic membranes of mitochondria, while the presence of POPCOOH diminishes this same dissociation constant [36].

Considering that one of the main apoptosis events is cytochrome *c* detachment from mitochondria, these findings provide evidence for the role of CL oxidation during photosensitization of the mitochondrial membrane. In fact, the decrease in cytochrome *c* association with the inner mitochondrial membrane causes an increase in the pool of free cytochrome *c* available for release into the cytoplasm through pores formed in a process dependent on protein Bax and Bak [183,186]. Moreover, CL oxidation also enhances pore formation in the bilayer, favouring cytochrome *c* release to the cytoplasm [183].

To test the hypothesis that CL is a key upstream target in mitochondria-dependent PDT-induced apoptosis, *Kriska et al* investigated the correlation between damaged mitochondrial membrane and ALA in tumour cells containing a mitochondrial GPX4-overexpressing clone (7G4) and in cells displaying a vector control clone (VC) [184]. In the protocols in which the PS localized in mitochondria, VC cells died *via* apoptosis. In contrast, a significant decrease in cytochrome *c* release, caspase-3 activation and chromatin condensation in 7G4 cells was observed [184]. These results were attributed to the transfected clone (7G4) overexpressing glutathione peroxidase (GPX4) in the mitochondria and detoxifying cells of the CLOOH formed during PDT. In fact, CLOOH accumulated ~70% slower in the 7G4 cells than in the VC cells, a finding that is consistent with GPX4-catalysed LOOH reduction and detoxification [184].

Recently, *Takano and co-workers* described a linked donor-accepting molecule designed to oxidize selective lipids in mitochondria through photoinduction of electron transfer reactions [187]. Among the studied molecules, the most hydrophobic compounds localized in high numbers to the mitochondrial membrane with a remarkable ability to oxidize lipids, mainly cardiolipin. Enhanced levels of CL oxidation in the mitochondria of HeLa cells, assessed by using Mito-PeDPP (*i.e.*, a fluorescent reagent for detecting mitochondrial lipid peroxidation), were correlated with high levels of annexin V and propidium iodide, thus indicating efficient cell death *via* apoptosis [187].

4.3. Consequences of lysosomal membrane photooxidation

Lysosomes are membrane-bound organelles carrying approximately 50 different hydrolases. These hydrolases are responsible for digesting molecules from extracellular media (in the case of endocytosis and phagocytosis) or degradation and recycling aged and damaged intracellular contents (in the case of autophagy) [188]. Lysosomes are unique in that they contain bis(monoacylglycero)phosphate (BMP), also known as lysobisphosphatidic acid (LBPA). BMP is a negatively charged phospholipid that comprises approximately 15% of the total phospholipid composition of lysosomes and late endosomes [189]. The unusual structure and stereochemistry of BMP are thought to play important roles in lysosomes, including their structural integrity, endosome maturation, and lipid and protein sorting and trafficking [190]. In PDT protocols similar to that used to study cardiolipin in mitochondrial membranes, BMP was found to have unsaturated acyl chains that can be easily oxidized during photosensitization. Emerging experimental evidence suggests that changes in lysosomal functions may be the "Achille's heel" of cancer cells, because they can affect the signalling pathways that involve the permeabilization of the lysosomal membrane and the release of enzymes into the cytosol, leading to cell death [159,191,192].

One of the consequences of photodamaging lysosomal membranes is the release of cathepsins into the cytosol that can cleave BID into t-BID, which in turn triggers the release of cytochrome *c,* stimulating caspase activation *via* aggregation of Apaf-1. Cathepsins released because of lysosomal membrane permeabilization (LMP) promote the degradation of anti-apoptotic Bcl-2. Both situations, *i.e.*, conversion of BID to t-BID and degradation of Bcl-2, result in apoptotic cell death [193–196]. Lysosomal membrane impairment is also associated with the loss of autophagic functions since it plays an important role in the degradation and recycling of damaged or aged intracellular biomolecules [111,159,197]. Recently, our group showed that a nanomolar dose of PS can accumulate in lysosome membranes, transforming life-beneficial autophagic activity into a destructive process that enhances the efficiency of photodynamically promoted cell death [111]. In this context, targeting lysosomes may be a relevant strategy for eliminating cancer cells, which create a stress environment that differs from that generated by normal cells to trigger the autophagic [198,199]. Since this strategy has emerged, the development of PSs that specifically target lysosomes has been considered of great importance [200,201].

Recently, double-targeting protocols that take advantage of lysosomal membrane permeability (LMP) and include other organelles have emerged as a promising methodology to improve PDT efficiency [159,192,202]. For example, *Kessel et al* demonstrated that induction of low levels of lysosomal photodamage (caused by chlorin NPe6) followed by induction of a low levels of mitochondrial photosensitization (by a benzoporphyrin derivative) significantly enhanced the efficacy of PDT in murine hepatoma cells [192]. However, the opposite sequence of damage induction (*i.e.*, mitochondrial damage prior to lysosomal impairment) was less efficient [192]. Interestingly, by using only one PS (*i.e.*, dimethyl methylene blue, DMMB) localized in both organelles (lysosomes and mitochondria), we were able to observe a 200-fold enhancement of cell death compared to a condition in which ROS are generated with nonspecific intracellular targets. The increase in cell death efficiency is explained by the activation of mitophagy (due to mitochondrial damage) and the inhibition of autophagy (due to lysosomal damage). Cells undergo a delayed but very efficient death when autophagy is suppressed [159].

5. Conclusion

The scenario of membranes depicted in the classic "*Fluid Mosaic Model*" has been changing in recent years. For example, the "*Lipid Whisker Model*" includes the effects, particularly impairments to structures and functions,that oxidized lipids may induce. Nevertheless, the

latest model does not take into account the tendency of oxidized lipids to disturb the “patchwork membrane”. Thus, a deeper comprehension of membrane lipid properties and dynamics is opening up entirely new routes to discovery, particularly with regard to the effects of lipid oxidation. We hope to encourage future research and expect that this review creates a better and deeper understanding of photoinduced membrane remodelling at the molecular and cellular levels.

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