Biophysics of lipid bilayers containing oxidatively modified phospholipids: Insights from fluorescence and EPR experiments and from MD simulations

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List of abbreviations and symbols used:

12-al 1-stearoyl-2-(12-oxo-cis-9-dodecenoyl)-sn-glycero-3-phosphocholine
13-tec 1-palmitoyl-2-(13-hydroperoxy-trans-11,cis-9-octadecadienoyl)-sn-glycero-3-phosphocholine
16-DSPC …
3-DC 3-DoxyICholestane
5-DSPC …
A-ESR angle-resolved electron spin resonance
AFD angle-resolved fluorescence depolarization
BHT butylated hydroxytoluene
DHPE-Bodipy N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (triethylammonium salt)
DLPC 1,2-dilinoleoyl-sn-glycero-3-phosphocholine
DOPC 1,2-dioleoyl-sn-glycero-3-phosphocholine
DPH 1,6-diphenyl-1,3,5-hexatriene
DPPC
FCS fluorescence correlation spectroscopy
FRET Förster resonance energy transfer
GUV giant unilamellar vesicles
HOPLPC 1-palmitoyl-2-(13-hydroxy-9,11-octadecanediienoyl)-sn-glycero-3-phosphocholine
HOSAPC 1-stearoyl-2-(15-hydroxy-5,8,11,13-eicosatetraenoyl)-sn-glycero-3-phosphocholine
HPPLPC 1-palmitoyl-2-(13-hydroperoxy-9,11-octadecanediienoyl)-sn-glycero-3-phosphocholine
Abstract:

This review focuses on the influence of oxidized phosphatidylcholines (oxPCs) on the biophysical properties of model membranes and is limited to fluorescence, EPR, and MD studies. OxPCs are divided into three classes: A) sn-2 hydroxy- or hydroperoxy-dieonyl phosphatidylcholines, B) phosphatidylcholines with oxidized and truncated sn-2 chain with either aldehyde or carboxylic group, and C) in silico studied phosphatidylcholines with truncated sn-1 or both sn-1 and sn-2 chains. It was shown that the presence of the investigated oxPCs in phospholipid model membranes may have the following consequences: 1) decrease of the lipid order, 2) lowering of phase transition temperatures, 3) lateral expansion and thinning of the bilayer, 4) alterations of bilayer hydration profiles, 5) increased lipid mobility, 6) augmented flip-flop, 7) influence on the lateral phase organisation, and 8) promotion of water defects and, under extreme conditions (i.e.
high concentrations of class B and C oxPCs), disintegration of the bilayer. The effects of class A oxPCs appear to be more moderate than those observed or predicted for class B and C. Many of the above mentioned findings are related to the ability of the oxidized chains of certain oxPCs to reorient toward the water phase. Some of the effects appear to be moderated by the presence of cholesterol. Although those biophysical alternations are found at oxPC concentrations higher than the total oxPC concentrations found under physiological conditions, certain organelles may reach such elevated oxPC concentrations locally. It is a challenge for the future to correlate the biophysics of oxidized phospholipids to metabolic studies in order to define the significance of the findings presented herein for pathophysiology.

1. Introduction

Lipid oxidation elicits profound changes in the chemical structure of phospholipid fatty acids [1, 2]. The polar and hydrophilic nature of the oxidized groups suggests scarce compatibility with the hydrophobic inner bilayer [3], and opens the possibility that typical properties such as fluidity and ordering, transition temperature, lateral organization and polarity and permeability might be altered. If true, the detection and the study of these alterations is important in view of their possible impact on the functioning of integral proteins [4], whose optimal activity is often tightly related and critically modulated by the bilayer chemical and physical state [5].

During many decades, and after the proposal of the fluid mosaic model by Singer and Nicholson in 1972 [6], the quest of characterizing the phospholipid bilayer composed by unaltered phospholipids containing unmodified natural fatty acids has prevented the systematic study of the impact of oxidized phospholipids (oxPL) on the bilayer structure and properties. In fact, it has always been a common practice to use antioxidants, such as BHT, and an argon or nitrogen experimental atmosphere in order to prevent formation of undesired oxidized phospholipids in the lipid samples under study. Although avoiding
uncontrolled formation of oxPL is correct experimental practice when studying normal phospholipids, still oxPL deserve consideration in view of their strongly suspected important role in the development/sustaining of pathologies linked to oxidative stress brought about by reactive oxygen species (ROS) [7]. From a historical point of view, it is significant to note that the structure of cardiolipin – the most complicated phospholipid, yet a simple organic molecule - was first established by van Deenen in 1966 [8], considerably later than the first announcement of the structure of the much more complicated molecule of DNA in 1953, demonstrating the slow process of knowledge gain in the field of phospholipids leading to the still existing gap between lipidomics and genomics (and also more –omics). This gap kept growing until few years ago [9] and contributed also to overlooking the study of the chemistry, physics and membrane properties of oxidized phospholipids, although nature has ever since attributed to them pathological and physiological roles that only in this very last decade begin to emerge [7].

A variety of physical or chemical ROS initiators were commonly used (UV- and X-rays, azo-compunds, t-Bu-hydroperoxyde, Fe^{++}/H_2O_2, Cu^{++}) to give rise to lipid oxidation in model or natural membranes, with results strongly dependent on the nature of the membrane and on the radical initiation conditions. Moreover, oxidation was quantified mainly by malondialdehyde determination through the TBARS assay, or by spectrophotometric detection of conjugated dienes at 235 nm wavelength. Both methods were limitative, since malondialdehyde undergoes further oxidation to malonaldehyde [F.M.M., personal observation], which escapes determination by the TBARS assay leading to underestimation of oxidation. Conjugated dienes represent only one of the oxidation cascade steps [10], and does not allow determination of the following oxidized intermediates and final molecules, also leading to lipidoxidation underestimation.

To prevent these drawbacks a reliable strategy was to control the oxidation state of model membranes by mixing pure defined molecular species of oxidized phospholipids with normal phospholipids in known amounts, according to the approach used in earlier studies [11-13].

For this purpose, classic oxidation protocols had eventually to be dropped and substituted with organic synthesis (or biosynthetic) methods in order to get pure oxidized
phosphatidylcholine species instead of the inextricably high variety of oxidized molecules resulting from the use of oxidants [14]. These models do not necessarily reproduce natural membranes oxidatively stressed by exposure to physiologically or pathologically originated ROS. Yet, it must be considered that not even a bilayer reconstituted with the phospholipid extract of a natural membrane will reproduce the original membrane, since membrane lipid topology is irremediably and inevitably cancelled in the lipid blend resulting from the very extraction step. This lipid blend will yield a reconstituted membrane having an overall average lipid composition different from any lipid microdomain present in the original natural membrane.

Nonetheless, results of the characterization of model membranes containing oxidized phospholipids will help understanding the effects of oxidized lipids on biological membranes, though without reference to the amount of oxidized species occurring in a natural membrane. In fact, one must consider that natural membranes are characterized by the presence of lipid microdomains with different phospholipid composition and unsaturation degree. Following ROS attack, fully unsaturated microdomains could well turn into fully oxidized microdomains, though representing a small percentage of the whole lipidome. Furthermore, defining the typical oxidation pattern of an oxidized natural membrane is still a difficult task at the present state of knowledge, since the pathways of oxidative stress are not yet fully known, as is the variety of oxPL formed, depending on the oxidizing agent and the oxidation conditions, or the type of oxidative pathology. To this aim, mass spectrometry studies of oxidative lipidomics in pathological tissues are in progress [15, 16]. Following these considerations, natural lipoperoxidized membrane composition cannot be precisely defined, while characterization of membrane models enriched in the species of interest can give first insights in the general effects of oxidative stress on the bilayer properties.

An increasing number of publications dealing with oxidation in well-defined model systems became a motivation to review those contributions. Here, the results of studies in which EPR, various fluorescence methods or computer simulations were used to elucidate the structure and the physical properties of oxidized lipid bilayers, are collected. Interestingly, those studies are up to now limited mostly to oxidation products of the most abundant lipid class of phosphatidylcholines, which we herein refer to as
oxPC. Due to limited scope of the present review, the reader interested in earlier works dealing with in-situ oxidation or experiments performed in vitro and in vivo is referred to the excellent review of Fruhwirth and co-authors [7].

2. EPR, fluorescence and MD methods applied to oxPL studies. What one can learn?

2.1. EPR

Since the dynamic time scale of the inner phospholipid bilayer is of the same order of magnitude of conventional X-band electron paramagnetic resonance spectroscopy (EPR) time scale [17], the EPR spectrum of a magnetically anisotropic paramagnetic probe (the nitroxide) embedded into the bilayer is strongly affected by the fatty acid motional degree. Conversely, this dependence of EPR spectra is telltale of the inner bilayer dynamics that, in turn, strongly depends on the type and the structure of the fatty acids, so that also their physical and chemical modifications are reflected in the EPR spectrum lineshape. As already noted, the polar nature of oxidized fatty acid residues hints at an incompatibility of oxygenated groups with the hydrophobic inner bilayer, thus suggesting that typical bilayer properties, hence the bilayer dynamics, may be altered in a way detectable by spectral variations of a paramagnetic probe EPR spectrum. Therefore, typical bilayer parameters and their variations related to the presence of oxPL can be monitored through the modifications of the spectrum of an EPR probe.

Hereafter only conventional X-band EPR applications will be described, thought, we would like to acknowledge that also W-band EPR (high frequency) [18], and electron spin echo envelope modulation (ESEEM) [19] can be useful to study local polarity and water penetration in oxPL bilayers in more detail.

Fig. 1. EPR lipid markers… 5-DSPC, 16-DSPC, 3-DC – work in progress (Piotr)

2.1.1. Membrane fluidity and gel-to-liquid transition temperature ($T_m$)
Classically, membrane fluidity was first discovered by EPR spectroscopy and found to vary along the bilayer normal with different profiles, at a given temperature, according to the fatty acid chemical nature, as measured by the order parameter $S$ [20]. At a fixed C-position, fluidity also changes with temperature [20], and in many membrane models the trend of the order parameter $S$ features a sharp fluidity increase at the gel-to-liquid transition temperature ($T_m$) typical of the bilayer fatty acid composition [21]. Until phospholipids are only considered, both membrane fluidity and $T_m$ depend on the fatty acid unsaturation degree and chain length. When lipoperoxidation became of concern, the very first idea was that lipid oxidation, known to alter (or abolish) the double bonds arrangement of phospholipid polyunsaturated fatty acids (PUFAs), would alter the bilayer fluidity, and that EPR spectroscopy would be able to detect lipid peroxidation via modification of typical $S$ or $T_m$ values of lipid spin labels, mainly n-doxylstearoyl-phosphatidylcholines (n-DSPC) [10].

2.1.2. Fatty acid ordering

Fatty acid residues into the bilayer also share an average common orientation along the bilayer normal [20]. More precisely, owing to fatty acid increasing segmental motion, fatty acids are pictured to move randomly into an ideal cone whose axis is aligned along the bilayer normal [20]. In case of restricted motion (such as it happens at lower C-positions) the fatty acid chains tend to be oriented along the bilayer normal, so that a geometrically ordered fatty acid ensemble can be envisioned. This picture describes fairly well the structure of an oriented lipid bilayer on a solid support or supported planar bilayer (SPB) [22]. Owing to the axial anisotropy of the nitroxide magnetic parameters $g$ and $A$, two different EPR spectra are observed according to the orientation of SPB normal with respect to the external magnetic field direction, based on the fact that the lipid spin label (5-DSPC) also shares the common orientation (EPR spectral anisotropy). In this way, observation of two distinct EPR spectra upon changing the sample orientation betrays internal bilayer spatial ordering, while a geometrically disordered bilayer will yield orientation-invariant EPR spectra (spectral anisotropy loss). Also in this case, it is expectable that the presence of oxygenated groups, of conjugated dienes, and of cleaved
fatty acids terminating with an aldehyde or carboxylic group, may disturb the fatty acid ordering, hence becoming visible as EPR spectral anisotropy change in spin labelled SPBs [23].

2.1.3. Polarity gradient and water penetration

The polarity profile is also an important feature of phospholipid bilayers, due to water molecules penetrating into the bilayer, and its alteration reveals increased membrane permeability. Water penetration also depends on the phospholipid bilayer composition and is changed, as an example, by the mere presence of cholesterol [24]. The presence of polar groups of oxPL can expectedly draw more water molecules than normal into the bilayer, bringing about local polarity increase. The hyperfine coupling constant of a nitroxide ($A_N$) probe is sensitive to polarity, in the sense that it increases in a polar environment, so that the value of a nitroxide $A_N$ can be used to gauge the bilayer polarity variation at a given C-position by use of n-DSPC [20].

2.1.4. Lateral phase separation

Hydrophobic mismatch originated by different fatty acid chain length often provokes segregation of different phosphatidylcholines in different membrane microdomains [25], giving rise to lateral phase separation. Beyond other techniques, the phenomenon is observable by EPR spectroscopy because the same spin label (particularly 16-DSPC, probing the methyl-terminal part of the bilayer) can yield a more or less fluid response according to its overspread location in both the terminal voids of shorter chains and close to the methyl-terminal of longer chain. In this case the final EPR spectrum appears as the superimposition of two differently motion-restricted EPR spectra, revealing the existence of two differently fluid membrane domains [26, 27]. OxPC-driven formation of membrane microdomains, owing to their polar nature or to different length, can thus be detected by 16-DSPC EPR spectra [28].

2.2. Fluorescence
Similarly to the case of EPR, fluorescence techniques applied to lipid studies require introduction of a fluorophore into the system. This might be achieved by either incorporating an amphiphilic fluorescent dye that aggregate with the lipids due to the hydrophobic effect or covalently attaching a fluorophore to the headgroup or hydrophobic chain of a lipid molecule. The consequences of fluorescent labelling of lipid aggregates are twofold. First, a word of warning, the presence of a fluorophore, which is usually bigger than the free radicals used in EPR, might interfere with the system of interest changing its properties. This should be taken into account when interpreting and when designing fluorescence experiments. A careful choice of fluorescent dye and/or a crosscheck using different fluorescent labels is needed. Moreover, in the view of the anisotropic nature of a lipid bilayer and its highly dynamical character, a precise knowledge on the location of a fluorescent probe is mandatory. On the other hand, a continuously increasing number of fluorescent probes and labels together with the possibility of attaching them at different positions within a lipid bilayer give a great choice of parameters that can be measured at various specific locations. Fluorescent dyes used in the studies reviewed here are depicted in Fig. 2.
Modern fluorescence techniques allow: visualisation of lipid membranes and their lateral structure (including super-resolution techniques), localization and co-localization of molecules, their diffusion, fluidity, distances between them, measurements of physical and chemical properties of the system in the vicinity of the fluorescent probe, and many others. A comprehensive list of fluorescent methods together with important consideration regarding their usage and fluorescence theory is given in [29]. Below, we shortly present those fluorescence methods that have been recently used to study oxidized model lipid membranes.

2.2.1. Fluorescence microscopy
Fluorescence microscopy provides a standard method of lipid bilayer imaging capable of capturing lateral phase organisation. Application of phase selective fluorescence dyes together with confocal scanning microscopy can picture phase domains in SPBs, giant unilamellar vesicles (GUVs), and lipid monolayers at the air/water interface [30]. Only recently, a number of methods have been used to improve the spatial resolution of fluorescence microscopy; previously limited by Abbe’s law of diffraction today can be as good as 20 nm [31].

2.2.2. Fluorescence correlation spectroscopy

Development of fast correlators and single-photon detection extended application of confocal microscopy allowed analysis of the dynamics of the system of interest. Fluctuations of fluorescence of diluted fluorescent molecules detected from very small confocal volume (~1 μm$^3$), when autocorrelated, gives a direct measure of fluorophore concentration and dynamics. The technique is known as fluorescence correlation spectroscopy (FCS) [32]. In model membranes, it has been mainly used for measuring of the lateral diffusion of fluorescent lipid analogue. For this task, a number of FCS modifications have been made, starting with the so-called Z-scan FCS, which solved the problem of the need of precise focussing of the objective on the 2D sample [33]. Z-scan FCS allows determination of lateral diffusion coefficient of lipids, but also can be used to distinguish between free and hindered diffusion. The method has been applied for SPBs, GUVs, as well as for lipid monolayers.

2.2.3. Fluorescence quenching and Förster resonance energy transfer

Fluorescence quenching and Förster resonance energy transfer (FRET) allow localisation of a fluorophore in relation to the location of its quencher (or FRET partner) molecule. Both techniques, while simple in their principles, are extremely versatile in studying structure of lipid membranes and their interactions with proteins and other compounds. An interesting example of application of fluorescence quenching is a chemical quenching of NBD lipid analogue by dithionite. Since water-soluble dithionite
can not diffuse trough a lipid bilayer, it can quench only the NBD dyes in the outer leaflet (single monolayer) of the bilayer when added to the liposomal suspension. This results in an asymmetrically labelled bilayer, for which the kinetics of lipid flip-flop (hopping of lipid molecule from one leaflet to the other) can be studied, by simply following fluorescence intensity decrease upon NBD-PC flopping from the inner liposome leaflet where it was not affected by dithionite to the outer leaflet, where it is being irreversibly quenched.

2.2.4. **Fluorescence anisotropy**

Membrane fluidity, defined as a reciprocal to lipid structural order parameter $S$, can be derived from fluorescence polarity measurements [34]. The most commonly used fluorescent probes for this purpose are DPH and its derivatives. Located at the hydrophobic backbone of the lipid bilayer DPH is wobbling, which decreases the measured fluorescence anisotropy. The wobbling diffusion rate was shown to be proportional to the local fluidity. An interesting technique that allows measurement of both structural and dynamic properties of oriented planar bilayers using only steady-state instrumentation is the so-called angle-resolved fluorescence depolarization (AFD) method [35]. While, fluidity is very useful measure of local membrane dynamics/packing in the backbone region and especially for gel phase bilayers, for which the anisotropy is high, it is less suitable for probing the headgroup mobility of liquid-crystalline bilayers, for which the method of choice is time-dependent fluorescence shift technique.

2.2.5. **Time-dependent fluorescence shift**

Fluorescence solvent relaxation or time-dependent fluorescence shift (TDFS) is based on the sensitivity of a certain fluorescent polarity probes to the relaxation of their polar surrounding [36]. When applied to lipid membranes, it is capable of probing hydration and mobility at a defined level of the fully hydrated free-standing liquid-crystalline bilayers, which are the most physiologically relevant lipid systems [37]. Time-resolved fluorescence decays measured at different emission wavelengths serve to
reconstruct a time-resolved emission spectra (TRES). Position of TRES, \( \nu(t) \), is shifting in time toward lower energies. It was shown that the total spectral shift, \( \Delta \nu \), is proportional to membrane hydration, and the kinetics of relaxation, often described as mean relaxation time, \( \tau \), is inversely proportional to the local dynamics of the hydrated lipids. The method has proved its usefulness in number of studies being usually more sensitive to small changes in lipid packing than the lateral diffusion measured using FCS. A set of fluorescent polarity probes precisely located at different depths across the lipid bilayer allows probing the whole profiles of the bilayer hydration and mobility, see an example in Fig. 5. It is also relatively easy to link TDFS method with computer simulations, which allows molecular interpretation of the observed changes [38].

2.3. Molecular Dynamic Simulations

Molecular dynamics (MD) simulations employed to lipid membranes provide atomistic-level description of the system. Positions of individual atoms in the system are followed by numerically solving classical equations of motion. Potential energy of interactions is described in a form of force-field, based on both empirical and quantum chemical data. As a matter of fact, given that the chemical and physical parameters of the molecules involved are correct and able to precisely describe their behavior, then those simulations can be considered as a “magnifying glass” with an unprecedented spatial resolution the membrane under scrutiny. MD simulations also include a detailed description of the dynamic of the studied molecules, and almost always the values of the correlation time and of the order parameter calculated by the method at the various C-positions match well those obtained by classic methods (NMR, EPR, fluorescence). Most impressively, also other bilayer features, such as thickness and cross-sectional distribution of atoms along the bilayer normal obtained by MD simulation, closely match the data obtained by X-rays or neutron beam diffraction [39, 40]. Thus, MD simulations may serve as a technique complementary to the experimental fluorescence and EPR methods in which more coarse-grained system properties are obtained. This issue is particularly important in the case of oxidized phospholipid bilayers, as many of the changes with respect to non-oxidized systems originate from the alternation of molecular
configurations due to membrane oxidation. The main effects include conformational changes of oxPL molecules and increased water penetration into the membrane, the latter assisted by formation of new hydrogen bonds. To address these phenomena at an atomistic-level of description, the united-atom force-field based on the Berger's force-field was employed in MD simulations of oxidized membranes [41]. Parametrization for oxPCs lipids was performed by the group of Tieleman in their pioneering MD study of oxidized membrane [40]. In one case, the all-atom force-field was also employed, but not qualitative differences from results obtained using a united-atom force-field were noted [42]. The coarse-grained MARTINI force-field [43] was used in a recent study of the influence of cholesterol on oxidized bilayer allowing for simulations of long-timescale behavior of lipids and estimation of lateral diffusion coefficients in a membrane consisting of 5000 lipid molecules [44].

In the studies presented here the MD results were directly comparable with fluorescence experiments in terms of hydration of the bilayer, lateral diffusivity, and free energy barrier of lipid flip-flop. Indirect comparison included conformational changes of lipids, area per lipid, and membrane thickness. In all cases, at least semi-quantitative agreement was achieved. Some limitations of MD methods, important from the point of view of oxidized membranes, should be noted. First, timescale of the considered processes is limited to hundreds of nanoseconds. This issue can be to some extent overcome by employing free-energy simulation techniques; however, long-time changes in the bilayer structure cannot be fully addressed yet. Second, the size of the simulated membrane is limited to several nanometers. Thus, no phenomena happening at longer length scales can be studied, which includes, for instance, creation of large water defects and pores.

3. Studies on lipid membranes containing chemically defined oxidized phospholipids

Within the recent years several studies appeared focusing on the influence of oxidized phosphatidylcholines on the biophysical properties of model membranes. Chemical structures of oxPCs, which effect on the biophysics of lipid bilayers was investigated, are depicted in Fig. 3. Basically three different types of oxidation products
were studied by EPR, fluorescence or MD simulations: hydroxy- or hydroperoxy-dieonyl phosphatidylcholines (Fig. 3A), truncated phosphatidylcholines (Fig. 3B), and sn-1 oxidized or double oxidized phosphatidylcholines studied in silico (Fig. 3C). We will refer to these three groups as class A, B, and C, respectively.

![Fig. 3. Chemical structures of oxidized phosphatidylcholines (oxPCs). (A) Hydroxy- (HOSAPC and HOPLPC) and hydroperoxy- (HPSAPC and HPPLPC) phosphatidylcholines. Different cis/trans isomers are possible. 13-tc refers to trans-11,cis-9 isomer of HPPLPC. (B) Truncated (cleaved chain) phosphatidylcholines with aldehyde (12-al, PoxnoPC, and POVPC) and carboxylic (PazePC and PGPC) functional groups. (C) Phosphatidylcholines with sn-1 (ox1-DOPC) and both sn-1 and sn-2 (ox2-DOPC) chains oxidized (in silico studies only). (one more oxPC (9-tc) structure is still missing)](image)

3.1. EPR

A comparative EPR study of the effects exerted on the lipid bilayer by different oxidized PC species, probed with 5-DSPC [14], showed that conjugated dienes oxidized species (Fig. 3A) were unable to disorder the phospholipid bilayer, while extremely oxidized cleaved chain PC (Fig. 3B) were revealed to be responsible for the previously observed strong EPR anisotropy loss induced by Fenton-oxidized phosphatidylcholine. **This study confirmed the need of well defined pure molecular species of oxPC for detailed studies of oxidized bilayers.**

3.2.1. **EPR** studies using 5-DSPC and 16-DSPC spin labels in SPBs and MLVs
More systematic EPR studies were performed by use of four defined oxidized PC molecular species (HPPLPC, HOPLPC, and the truncated lipids: PGPC, and PazePC; see Fig. 3) in hope to attain a better definition of structural changes in MLVs owing to the sharp definition of the oxidized molecules used [10]. This study also indicated that cleaved chain (truncated) PCs (Fig. 3B), especially the $\alpha$-carboxyacyl- species, were able to give rise to micelle formation from the lipid mixtures they were enclosed in, in agreement with a much earlier advanced hypothesis [11]. Therefore, that study was limited to full-chain conjugated dienes PCs, with proven vesicle-forming ability (Fig. 3A). The results of this study clearly indicated that conjugate dienes/DPPC MLVs featured lateral phase separation at room temperature and $T_m$ value lower than that of pure DPPC MLVs. Pure conjugated dienes MLVs resembled more PLPC MLVs and displayed free miscibility with PLPC in mixed MLVs. On the contrary, HPPLPC MLV bilayer appeared to be slightly more rigid, while that of HOPLPC and the polarity profile of MLVs made of the pure conjugate dienes species were similar to those of normal PLPC. Lateral phase separation and $T_m$ decrease were better defined than membrane fluidity variations, while no polarity profile alteration was observed. The latter is linked to water penetration into the bilayer, which is presently under scrutiny by W-band EPR spectroscopy. Generally speaking, those studies firmly established that alterations of phospholipid bilayer structural properties do correlate with the presence of oxidized phospholipid molecules. **In summary, the tendency of class B oxPCs to form micelles was confirmed. For the first time, the lowering of $T_m$ as well as induction of lateral phase separation by class A oxPCs was observed.**

3.2.2. Comparative EPR studies using 5-DSPC and 3-DC spin labels in SPB’s

The inability of conjugated dienes PC to disorder the bilayer fatty acids, as revealed by 5-DSPC in supported lipid bilayers (SPBs), was subjected to deeper study performed by use of a different spin label, 3-DoxylCholestane (3-DC) [45]. This spin label is an $\gamma$-rotor, and allows to probe the bilayer under a different perspective than 5-DSPC (a $\alpha$-rotor). The two spin labels were used comparatively to determine the
disordering capacity of up to all eight oxidized PC molecular species shown in Fig. 3 (class A: HOSAPC, HOPLPC, HPSAPC, and HPPLPC; class B: PoxnoPC, POVPC, PazePC, and PGPC). The response of 3-DC to the presence of truncated (cleaved chain) PCs was a heavy loss of EPR spectral anisotropy, similar to that shown by 5-DSPC. Nonetheless, 3-DC also reported disordering from conjugated dienes PCs, not revealed by 5-DSPC. The different capacity of the two spin labels was attributed more to their different chemistry than to different bilayer structural features. In fact, 5-DSA residue is covalently bound to the PC molecule and takes on a distended asset into the bilayer. 3-DC molecule is reported to orient with its nitroxide group close to the phospholipid polar head region and its hydrophobic frame into the fatty acid chain ensemble, without any strong interactions (the cholesterol -OH group is replaced by the less polar nitroxide ring). Hydroxy- and hydroperoxy-groups of class A oxPCs are reported to come close to the polar head region due to hydrogen bonding and polar interaction, so that they could displace unbound 3-DC and make it move to a more fluid inner bilayer region. As a matter of fact, EPR spectral parameters of this probe in oxidized SPBs favoured more a fairly freely tumbling than a rigid disoriented probe. In support of this explanation, EPR spectra of 5-DSA and of its methyl ester in MLVs were compared. The former, capable of interacting with the bilayer polar region via the free carboxyl group, showed rigid EPR spectra very similar to 5-DSPC’s, while the latter, in which the interaction possibility is suppressed, yielded EPR spectra reporting much higher fluidity, suggesting that displacement of a weakly interacting spin label cannot be excluded. Therefore, results of this study were taken as an indication that 3-DC is useful more for revealing the presence of all perturbing oxidized PC molecules than for gaining information about structural changes. The question about the ordering maintenance of 5-DSPC in the presence of CD-PCs remains unanswered to now. **In summary, although 3-DC is sensitive to the presence of both class A and B oxPCs, it is still uncertain whether it reports variation of the bilayer organisation. However, experiments using 5-DSPC confirm that class B oxPC disorders the lipid bilayer.**

3.2.3. Combining EPR studies with gel chromatography and cryo-electron microscopy
In a recent study we addressed the issue of micelle forming tendency of cleaved chain PCs. Transition of vesicles to micelles upon addition of oxPC is a dramatic event with heavy consequences for the membranes of living cells, ending in membrane local rupture and full breakdown. Between natural phospholipids, micelle forming ability of lyso-phosphatidylcholine has been studied most intensively. Soon after, the lysolecithin antagonist capacity of cholesterol was demonstrated. Since micelle forming capacity of cleaved chain oxPCs (class B), similar to that of lyso-PCs was previously observed, the issue of the antagonist capacity of cholesterol against oxPC membrane disruption was also addressed [46]. The analysis of EPR anisotropy loss of spin labelled oriented bilayers was useful in establishing the increasing bilayer destabilisation with increasing class B oxPC molar ratio, and the reversal of this destabilisation when 40 mol% cholesterol was included in the preparation. Nonetheless, the EPR method was unable to distinguish between the presence of scrambled chains bilayers and formation of micelles solely on the base of EPR anisotropy loss of spin labelled (3-DC or 5-DSPC) oriented bilayers. Correct attribution of chain geometrical disordering was achieved by complementing EPR observations with morphological analysis of lipid suspensions by Sepharose 4B gel chromatography, useful to distinguish between vesicles and micelles. This criterion helped demonstrating that class B oxPCs were able to form micelles in the absence of cholesterol, while when the latter was included, disordered chain bilayers were obtained. Definitive confirmation of oxPC/cholesterol vesicle formation, though internally disordered, was achieved by cryo electron microscopy. In summary, cholesterol was found to reshape class B oxPC micelles into unilamellar structures.

3.2. Fluorescence

3.2.1. Angle-resolved fluorescence depolarization technique (AFD)

Information on structural organization and molecular dynamics within a planar lipid bilayer can be gained by means of angle-resolved fluorescence depolarization technique (AFD). Wratten and co-workers studied bilayers that consisted of class A oxPCs (HPPLPC, or HOPLPC) and phosphatidylcholines (PLPC or DLPC) using angle-
resolved A-ESR and AFD [13]. AFD measurements were performed with DPH introduced in macroscopically oriented planar bilayers containing from 2 to 5 mol% of oxPCs. The analysis of the AFD measurements showed that increasing amounts of oxPCs in the PLPC membranes result in an overall decrease in molecular orientational order as defined by the order parameters of DPH in the bilayers, however, the reorientational dynamics of DPH appeared not to be affected. These results of AFD were supported by those of A-ESR. Despite the fact that DPH and 3-DC used in A-ESR are located differently within the membrane, they gave similar results with respect to membrane order and dynamics. **In summary, DPH fluorescence anisotropy study indicates a decrease in the overall order within phosphatidylcholine bilayers induced by the presence of class A oxPCs.**

3.2.2. Visualization – microscopy / monolayer studies

The changes of the surface properties of the monolayers of DPPC when mixed with oxidized phospholipids PoxnoPC and PazePC (Fig. 3B) were studied by fluorescence microscopy by Sabatini and co-workers [47]. While analyzing the compression isotherm for pure DPPC film, liquid-expanded to liquid-condensed (LE-LC) phase transition with LE-LC coexistence region is clearly visible. However, isotherms collected for PoxnoPC/DPPC or PazePC/DPPC monolayers show different behaviour of these mixtures upon compression. For increasing fraction of PoxnoPC in DPPC monolayer film, LE-LC coexistence region progressively vanishes, and almost completely disappears at $X_{\text{PoxnoPC}}=0.4$. These results suggest that films with $X_{\text{PoxnoPC}}>0.4$ are predominantly in the LE phase. Similar behaviour is observed for PazePC/DPPC monolayers, but disappearance of LE-LC coexistence region is visible at smaller $X_{\text{PazePC}}$ in the monolayer film. The expansion of the monolayers can be explained by the looping back of sn-2 acyl chain of PoxnoPC or PazePC and accommodating the polar moieties into the vicinity of the lipid headgroup in the air-water interface. More precisely, monolayer expansion was found to be more pronounced for PazePC, which might be explained by presence of the charge, higher affinity for water and larger size of carboxylic moiety with comparison to the aldehyde one of PoxnoPC.
Additionally, it was observed that, in both mixtures, further compression of mixed monolayers caused breaks in the isotherms, showing loss of oxidized lipids from binary phospholipid films into the aqueous phase. Fluorescence microscopy experiments with fluorescent lipid analogue, NBD-PC (2 mol%), which favours the liquid-expanded (LE) phase, were performed to visualize the lateral phase organization. It was shown that DPPC in the LE-LC coexistence region reveals the presence of non-fluorescent solid domains. However, the presence of both PazePC and PoxnoPC in mixed monolayers with DPPC shifts the appearance of LC domains to higher surface pressures. In case of PazePC/DPPC monolayers, smaller number and larger size of the domains can be seen in comparison with PoxnoPC/DPPC film. In summary, for the first time a possible looping-back of the truncated oxidized chain and the so-called extended conformation of class B oxPC was postulated. Fluorescence microscopy showed that oxidation can alter the phase behavior of phosphatidylcholine monolayers.

3.2.3. FRET

To study the conformational dynamics of class B oxPCs (PazePC and PoxnoPC) FRET method was used [48]. The system of interest consisted of pyrene containing phospholipid analogue PPDPC added to oxPC micelles and a water soluble cationic protein, cytochrome c. The analysis of the distance-dependent efficiency of Förster-type resonance energy transfer between those two molecules provided important information on the conformational dynamics of the oxPC. Cytochrome c was found to bind to the PazePC micelles, but only weakly associate with PoxnoPC. Based on those results a model of cytochrome c – oxPC interaction was proposed. According to it, the looping-back truncated chain of PazePC exposes its carboxyl function on the micelle surface were it can readily bind cytochrome c. This is not the case for PoxnoPC, the aldehyde-truncated sn-2 chain of which did not bind to cytochrome c. In summary, the energy transfer between a peripheral membrane protein and fluorescently labeled class B oxPC micelles revealed for the first time an important difference between aldehyde- and carboxy- truncated oxPCs and confirmed the hypothesis that carboxy-
truncated lipids might loop back towards the water phase (see Fig. 4 for illustration).

Fig. 4. Typical configurations of PGPC (A) and POVPC (C) lipids in the POPC membrane. The truncated sn-2 chain of PGPC typically protrudes into the water phase whereas in the sn-2 chain of POVPC resides in the head groups region. Color coding: oxidized lipids - green balls, POPC - black lines, choline groups – small blue balls, phosphate groups – small yellow and red balls. Polar terminal groups of sn-2 chain of OXPCs are depicted as yellow (PGPC) and orange (POVPC) balls.

3.2.4. FCS

To better understand the behavior of class B oxPCs in membranes, well defined model systems were studied by Plochberger and co-workers using FCS [44]. Lateral diffusion of oxPC fluorescent analogue, PGPE-Alexa647 in various SPBs was compared to that of conventional lipid analogue, DHPE-Bodipy. Diffusion constants obtained from line-scan FCS experiments were found to be considerably higher for PGPE-Alexa647 than those for DHPE-Bodipy, which means the oxPC was more mobile than non-oxidized lipid. Insertion of immobile obstacles blocked the diffusion of DHPE-Bodipy while the diffusion of PGPE-Alexa647 was only slightly affected. Moreover, addition of 40 mol% of cholesterol resulted in slowing down and leveling of the diffusion of both probes in
DOPC SPBs. Based on the results of coarse-grained simulations (see chapter 3.3) this effect was ascribed to deeper incorporation of PGPC into DOPC bilayer in the presence of cholesterol.

Lateral diffusion of a fluorescent lipid analogue, Bodipy C12-HPC in oxPC-containing SPBs was also measured by Beranova and co-workers. Z-scan FCS measurements of lateral diffusion of Bodipy C12-HPC in SPBs composed of POPC with 10 mol% of short-chained truncated oxPCs (PGPC or POVPC, Fig. 3B) revealed faster lateral diffusion in both oxPL-containing bilayers. These results were compared with the results from TDFS experiments and MD simulations and are discussed later in the text.

In summary, FCS reports considerably faster diffusion of labeled PGPE. Diffusion of that labeled PGPE is only slightly hindered in the presence of immobile obstacles but slows down and levels with increasing cholesterol content. The latter finding indicates interplay between oxPC and cholesterol, already pointed out in 3.2.3.

The two examined oxPC, on the other hand, lead to slightly faster lipid diffusion probed by labeled DOPC.

3.2.5. Time-resolved fluorescence shift

As mentioned in chapter 2.2.5, time-resolved fluorescence of certain polarity probes gives us a measure of lipid membrane hydration and local mobility. In the work of Beranova and co-workers [49] Laurdan – a fluorescent dye of precisely known location in the phosphocholine bilayer (see Fig. 5) [50, 51] – was used to probe hydration and mobility of hydrated carbonyls of POPC bilayer with 10 mol% of class B oxPCs (POVPC and PGPC). It was shown that both aldehyde and carboxyl at their sn-2 chains (POVPC and PGPC, respectively) considerably disturb the structure of model phosphatidylcholine membrane introducing voids in the hydrocarbon region of the bilayer and polar functional groups (aldehyde and carboxylic) that are looping back toward the aqueous solution. These structural changes profoundly affect the parameters probed by Laurdan. Lipid carbonyls become much more hydrated and mobile. While the effects are apparent for both PGPC and POVPC, the more dramatic effects are observed for the aldehyde-truncated lipid (POVPC). These findings point out that not only chain truncation and
resulting conical geometry of the studied oxPL molecules, but also the chemical character of the truncated chain can alter the mechanistic properties of oxidized lipid bilayer. Thus, the different biological functions that the two lipids have [52] can also be governed through the biophysics of the lipid bilayer apart from the specific receptor recognition. When compared with lateral diffusion measurements performed using FCS, local mobility measured using fluorescent polarity probes proved to be much more sensitive to the presence of truncated oxPLs. This is an advantage of probing membrane properties locally. **In summary, addition of 10 mol% of class B oxPCs strongly increases POPC carbonyl region hydration and local mobility. The effects are stronger for the aldehyde (POVPC) than for carboxylic acid (PGPC).**

### 3.2.6. Polarity profiles

A series of fluorescent polarity probes located at different depths of lipid bilayer allow using the time-dependent fluorescent shift method to probe the whole profile of membrane polarity in the direction perpendicular to its surface. The finding, discussed in chapter 3.2.7, that the addition of PazePC or PoxnoPC largely facilitates flip-flop of phosphatidylserine (PS) in POPC bilayer led to the question of the possible reason for this effect. It is commonly agreed and was also calculated (see chapters 3.3) that the main energetic barrier for the phospholipid molecule moving from one leaflet of the bilayer to another is given by the hydrophobicity of the bilayer interior, through which the polar serine group of PS has to cross. This reasoning motivated the investigation of polarity profiles in POPC bilayer upon addition of PazePC or PoxnoPC. Polarity and mobility changes have been measured at 5 different depths of the bilayer using following fluorescent polarity probes (ordered from the most outside to the most inside locations): Dtmac, Laurdan, Patman, 9-AS, 16-AP [53]. The structures of the probes, their location in the lipid bilayer and the obtained results are shown in Fig. 5. In agreement with the previous studies [49], discussed in chapter 3.2.5, Laurdan and Patman report significantly increased polarity and mobility at the level of lipid carbonyls upon addition of both PazePC and PoxnoPC (the effects of both oxPCs are similar). Polarity and mobility measured for Dtmac and polarity measured for 9-AS and 16-AP show opposite changes
to those of Laurdan and Patman. Environment of Dtica located at the phosphate group is probably affected by the presence of truncated oxidized chains that are looping back toward the aqueous solution. Their presence slightly restricts both mobility and hydration of this region.

For both 9-AS and 16-AP located roughly at 9\textsuperscript{th} and 16\textsuperscript{th} carbons of the lipid hydrocarbon chains, polarity is noticeably decreased. Unfortunately due to complex photophysics of those dyes precise calculation of relaxation time has not been possible and thus no information on probable mobility alterations can be given. The overall changes in membrane polarity profiles in oxidized bilayers, relative to polarity profiles obtained for pure POPC, have a sinusoidal shape (see Fig. 5). An explanation for the decreased polarity of the membrane interior probed by 9-AS and 16-AP might be the thinning of the bilayer upon addition of oxPL observed in MD simulations [53]. This might cause restriction of mobility of the fluorophores buried in tighter packed hydrocarbon chains (higher total density in the center of oxidized bilayer as seen in Fig. 4B in [53]). Altogether, even the polarity of the membrane interior is not increased, water penetrates much deeper (increased hydration at the carbonyls) and membrane becomes thinner, which all lead to the dramatic increase of the observed PS flip-flop. In summary, presence of class B oxPCs significantly changes hydration and thus polarity of the bilayer, which likely contributes to the faster PS flip-flop in oxidized model lipid membranes.
Fig. 5. Relative polarity/mobility profiles of oxPC-containing POPC bilayer. TDFS parameters relative integrated relaxation time (A) and differences in spectral shift (B), normalized to the values obtained for pure POPC. Structures of polarity probes are shown at their positions known from literature (see the text). For the sake of clarity of presentation the position of Laurdan has been shifted by 0.1 nm outward from Patman.

3.2.7. Dithionite quenching

Maintaining lipid bilayer compositional asymmetry is known to be important for cell physiology and a failure in fulfilling this requirement can lead to cell death by triggering the so-called programmed cell death (apoptosis). One of the distinctive signs of cell malfunction that can be recognized by the appropriate receptors resulting in cell apoptosis is a presence of phosphatidylserine (PS) molecules in the outer leaflet of the cellular membrane. In the healthy cell PS is kept in the inner leaflet of the bilayer as a result of equilibrium between the action of ATP-dependent substrate-specific transporters
on the one side and the flip-flop – an entropy-driven process of lipid randomization (cross bilayer diffusion of lipids) on the other. It was proposed that the flip-flop might be facilitated by the action of a protein called scramblase [54], which has not been identified so far.

The recent fluorescence quenching studies have shown that the presence of class B truncated oxPCs (namely PazePC and PoxnoPC) can radically raise the flip-flop rate in a model membrane [53]. The kinetics of flip-flop of the NBD labelled PS has been followed using a dithionite-quenching assay [55]. Dithionite is water-soluble but do not permeate through lipid membrane and when added to liposomal suspension chemically quenches NBD-labeled lipids only in the outer leaflet of the bilayer. The measured attenuations in the extent of decrease in fluorescence upon the external addition of dithionite have shown that the presence 14 mol% of PazePC or 16 mol% of PoxnoPC in POPC bilayer reduces the PS flip-flop half-time from weeks to hours. These results demonstrate that a passive biophysical mechanism might be sufficient for the loss of lipid compositional asymmetry, i.e. a scramblase protein might not be needed. **In summary, class B truncated oxPCs increase lipid flip-flop rate.**

Fig. 6. Four stages of the POPS lipid (green balls) flip-flop (progressing from left to right) across the POPC (black lines) membrane in the presence of PoxnoPC oxidized lipids (blue balls). Water is presented as red balls. Water defects in the vicinity of POPS head group are typically present in the initial stages of the flip-flop process while the transient transmembrane pore is formed as the POPS head group penetrates the hydrocarbon region.
3.3. Molecular dynamics simulations

Molecular dynamics studies of oxidized phospholipid bilayers were initiated by Wong-ekkabut and co-workers [40]. Properties of a PLPC (1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphatidylcholine) membrane with varying amount of different oxidation products were investigated. As the products, four oxidized phospholipids derived from the following fatty acids were considered and used as a replacement of sn-2 chain in PLPC: 9-hydroperoxy-trans-10,cis-12-octadecadienoic acid (thus, forrmig HPPLC lipid), 13-hydroperoxy-trans-11, cis-octadecadienoic acid (13-tc), 9-oxo-nonanoic acid (PoxnoPC), and 12-oxo-cis-9-dodecenoic acid (12-al). These acids are products of linoleic acid peroxidation, based on a previous theoretical study [56]. Thus, four oxidation products, with either aldehyde or hydroperoxide group at the terminated sn-2 chain, and with two different localization of polar moieties in the oxidized chain were accounted for. Oxidation ratios between 2.8 and 50% were considered.

The main effect observed for oxidized bilayer was the bending of oxidized tails toward the water phase. Polar groups of oxidized chains formed hydrogen bonds, predominantly with water and, to a lesser extent, with lipid headgroups. This reorientation was stronger for lipids containing hydroperoxide groups due to higher hydrophilicity of hydroperoxide with respect to that of aldehyde group. The changes in conformation of oxidized chains led to increased area per lipid (APL) by up to 10% in the case of 50% of oxidation. APL increased monotonously with the increasing content of oxidized lipids. Conformational changes in the oxidized membrane caused reduction of deuterium order parameter of lipids with respect to pure-PLPC membrane; this effect was stronger in the presence of aldehyde-terminated lipids than the acid-terminated ones. Changes in electron density were also observed, namely, an increased density in the membrane interior and a decreased density in the headgroup region, accompanied by a shift of the headgroup peaks toward the membrane interior. Membrane thickness was generally reduced with increasing oxidation ratio. No demixing of lipids was observed within the simulation timescale and lateral diffusivity was not affected by oxidation. Creation of water defects, which were larger than in the non-oxidized membrane, was observed due to reorientation of oxidized chains. A free-energy barrier of water
permeation estimated employing the PMF method was reduced by up to 10 kcal/mol (from ~29 kcal/mol) in the case of 50% oxidized bilayer. The main conclusion given in the paper is that the cell membrane damage which accompanies oxidation is caused by increase of membrane permeability for water. This result is in accord with early experimental studies of the leakage of oxidized liposomal membranes [57, 58].

In the following study, Khandelia and Mouritsen investigated properties of POPC bilayer with up to 25% of oxidized lipids, either PoxnoPC or PazePC [42]. These two lipids were chosen as stable products of lipid oxidation under physiological conditions [59, 60]. A complete reversal of orientation of the anionic PazePC chains, without a loss of bilayer integrity for up to 25% content of PazePC was observed, with the reversal being more pronounced in higher PazePC content. The carboxylic groups were able to extent up to 1 nm into the water phase beyond the average position phosphorous atoms. This was a first direct observation of the so-called “extended lipid configuration” suggested previously by Kinnunen [61]. For neutral PoxnoPC, the chain reversal, although not complete, was also observed. An incomplete reversal of aldehyde-terminated chains caused stronger perturbation of bilayer structure than in the case of PazePC. Note, however, that for comparisons between the membrane with neutral PoxnoPC and anionic PazePC the presence of neutralizing sodium counterions in the latter system must be accounted for as a factor introducing extra stabilization of the bilayer. Alternation of electron density upon oxidation was observed, with the density increased in the center of the bilayer and its maxima shifted toward the center. For PazePC a small increase of the density in the aqueous region due to the sn-2 chain reversal was present. APL for PoxnoPC increased, whereas for PazePC a reduction of APL was observed, the latter resulting from the presence of sodium ions in the system (sodium cations strongly bind carbonyl groups of lipids causing the reduction of APL [62]. The decrease of membrane thickness was observed in both systems, being stronger for PoxnoPC. The deuterium order parameter of lipids was reduced in oxidized bilayers with respect to PLPC membrane. No significant alternation of lateral diffusion was detected. Oxidation lowered the dipole potential of bilayers, although no significant change in N-P vectors (from phosphorus to nitrogen in the headgroup) distribution was found. Generally, alternations of membrane properties due to oxidation described by
Khandelia and Mouritsen correspond with those found by Wong-ekkabut et al. The main difference between these two studies is the complete reversal of orientation of the anionic chains observed in the oxidized lipids considered by Khandelia and Mouritsen, whereas anionic-terminated lipid chains were not accounted for in the earlier work by Wong-ekkabut et al.

The mechanism of membrane damage due to lipid oxidation was studied by two of the present authors for massively oxidized bilayers [63]. Namely, a 100% oxidation of a DOPC membrane was modeled by having DOPC molecules exchanged with oxidation products. Four pairs of DOPC oxidation products, with either both or only the sn-1 acyl chain terminated with aldehyde were considered (ox1-DOPC and ox2-DOPC). Additionally, the presence of short-chain products (either aldehydes or hydrocarbon) was accounted for. In the systems with both acyl chains oxidized the membrane was unstable due to the presence of large water defects which lead to significant water permeability across the bilayer. As a result, the bilayer structure was destroyed in ~10 ns leading to formation of micelle-like structures. In the case of systems with only one acyl chain oxidized, the bilayer structure was not destroyed in the simulation timescale (up to 100 ns). Reorientation of oxidized chains toward the water phase was observed; however, not all chains were reoriented since they were terminated by uncharged aldehyde groups, which is in accord with previous computational results for aldehyde-terminated oxidized lipids [40, 42]. Short-chain products were incorporated in the stable bilayers. In the stable systems, the membrane thickness decreased in the membrane containing the aldehyde short-chain product and increased in the system with the hydrocarbon short-chain product, the latter due to the hydrophobic character of the oxidation product molecules which resided predominantly near the bilayer center and thus prevented membrane shrinkage. Thus, the thickness of the oxidized bilayer was shown to be dependent on the type of short-chain products, which goes beyond the conclusions of earlier studies where short-chain products were omitted and solely the reduction of membrane thickness was observed upon oxidation [40, 42]. APL in the stable systems was increased by up to 32%, mainly due to the enhanced formation of water defects in the bilayer. Increased water permeability with respect to the not oxidized bilayer was observed in all considered membranes. Moreover, in the stable oxidized bilayers transient transmembrane water
pores were formed, with the pore opening and closing observed in tens of nanosecond timescale. This is in accord with both computational [40] and experimental [57] results regarding an increase of permeability in oxidized lipid membranes. Destroying of membrane upon oxidation was shown to be caused by formation of water defects and increased water permeability, which is in accord with a postulate given by Wong-ekkabud and co-workers [40].

Increased hydration of oxidized membranes was further studied in the joined experimental and theoretical study by Beranova and co-workers [49]. POPC membrane with either 10% of PGPC (anionic) or POVPC (neutral) was studied. Reorientation of oxidized sn-2 chains was observed, being almost complete for anionic chains of PGPC (see Fig. 4). APL of POPC+POVPC membrane was close to that of pure-POPC, whereas a decrease of APL was noted for POPC+PGPC bilayer. In the latter case, the reduction of APL was caused by both reorientation of oxidized chains toward the water phase and the presence of sodium cations. MD results were in accord with SR experiment regarding the increased lipid mobility, both local and lateral, in oxidized membranes. It was concluded that, the reversal of oxidized chains leads to increased water penetration, and thus increased lipid mobility in the region of carbonyl groups, probed in SR experiments. The mobility was higher for POPC+POVPC membrane due to incomplete reorientation of chains which caused creation of a significant number water defects. The increase of lateral diffusivity was higher for the POPC+PGPC system due to the lack of lateral interaction, which limited lateral movements in the case of POPC+POVPC.

Water permeability of oxidized membranes was directly addressed in the study of Lis and co-workers [64] with computer simulations being combined with scattering stopped flow experimental measurements. Properties of bilayers composed of DOPC with pairs of oxidation products (ox1-DOPC, i.e., a lipid with one oxidized chain terminated by an aldehyde group, and a short-chain aldehyde) were investigated as a function of oxidation ratio. A monotonous increase of APL with increasing oxidation ratio (from ~3% for 15% oxidation, to ~50% for 90% oxidation), as well as reduction of bilayer thickness were observed. Both character and extent of water permeation were shown to strongly depend on the oxidation ratio. At 15-30% oxidation only individual water molecules spontaneously permeated across the bilayer. At 55-66% oxidation water
clusters, originating from water defects in the headgroup region, formed and were transferred across the bilayer. At 66% oxidation transient water chains formed and spread across the membrane. At 75-90% oxidation the transmembrane water pores were created in the tens of nanoseconds timescale. For oxidation ratios ≥75% the flux of water across the bilayer was independent of the oxidation ratio but the time needed for pore opening was increasing with membrane oxidation.

The influence of cholesterol on lateral diffusivity of oxidized lipids was addressed in coarse-grained MD simulations of a DOPC bilayer with 1% of PGPC and up to 50% of cholesterol [44]. Structural properties of the system without cholesterol were in agreement with atomistic-level simulations. In the absence of cholesterol the lateral diffusion of PGPC was 1.4-fold faster than that of DOPC. This effect was rationalized by a relatively weak incorporation of PGPC in the DOPC membrane. The presence of cholesterol facilitated the incorporation of PGPC in the DOPC matrix diminishing the differences between the lateral diffusion of PGPC and DOPC at 40% cholesterol content, in accord with FCS experimental findings. In the work of Volinsky et al. the influence of lipid membrane oxidation on lipid transmembrane transfer (flip-flop) was studied [53]. By means of MD simulations employing the potential of mean-force technique a free-energy barrier for the flip-flop of anionic POPS lipid in oxidized POPC bilayer was estimated. Oxidation of the membrane (POPC +10% PoxnoPC) resulted in an approximately 20% reduction of the free-energy barrier (from 120 to 100 kJ/mol). The role of oxidized lipids was mainly in increasing the hydrophilicity of the membrane interior which facilitates the transfer of hydrated anionic PS headgroup across the hydrophobic acyl chain region. The aldehyde groups of PoxnoPC interact with water and thus stabilize trans-membrane pore formed during PS transfer (see Fig. 6). Both experimental and simulation results thus show that lipid oxidation leads to a sizable speed up of scrambling of PS between the two leaflets of the PC membrane.

4. Summary

The results of the individual EPR, fluorescence and MD studies are already summarised at the end of every subsection in part 3. To give an overview the specific
effects of the individual experimentally available oxPCs on the biophysics of a bilayer are presented in table 1.

<table>
<thead>
<tr>
<th>oxPC</th>
<th>effect on biophysical properties of model lipid membrane</th>
</tr>
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<tbody>
<tr>
<td>HOSAPC</td>
<td>loss of 3-DC EPR anisotropy in SPB [45]</td>
</tr>
<tr>
<td>HOPLPC</td>
<td>fully miscible with PLPC (similar polarity profile) [10] 25 mol% lowers T_m and causes phase separation of DPPC MLVs at room temperature [10] loss of 3-DC EPR anisotropy in SPB [45] strong reordering of 3-DC in SPB at 40 mol% cholesterol both pure and in PLPC mixtures [46] 2-5mol% decreases orientational order but does not affects reorientational dynamics in PLPC SPBs [13]</td>
</tr>
<tr>
<td>HPSAPC</td>
<td>loss of 3-DC EPR anisotropy in SPB [45]</td>
</tr>
<tr>
<td>HPPLPC</td>
<td>fully miscible with PLPC (similar polarity profile) [10] 25 mol% lowers T_m and causes phase separation of DPPC MLVs at room temperature [10] loss of 3-DC EPR anisotropy [45] 2-5mol% decreases orientational order but does not affects reorientational dynamics in PLPC SPBs [13]</td>
</tr>
<tr>
<td></td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>(13-tc – a trans-11,cis-9 isomer of HPPLPC) 2.8-50 mol% in PLPC, sn-2 chains loop back toward water and form H-bond with water and lipid headgroups, the reorientation is stronger than for aldehydes (PoxnoPC and 12-al), APL increases, thickness reduces, order parameter decreases, water defects are promoted resulting in increased water permeability [40]</td>
</tr>
<tr>
<td>12-al</td>
<td>2.8-50 mol% in PLPC, sn-2 chains loop back toward water and form H-bond with water and lipid headgroups, the reorientation is less pronounced than for hydroperoxides (???, and ??), APL increases, thickness reduces, order parameter decreases, water defects are promoted resulting in increased water permeability [40]</td>
</tr>
<tr>
<td>PoxnoPC</td>
<td>25 mol% with DPPC forms MLVs, pure compound forms micelles, heavy loss of EPR anisotropy in PLPC SPB [45] 10, 20, 40 mol% gradually expands DPPC monolayers and abolishes LE-LC phase coexistence, at higher laterall pressures lost into the subphase, shifts LC domains to higher pressures, lowers surface potential, truncated aldehyde sn-2 chain likely reverses toward water subphase [47] micelles of pure compound do not bind to cytochrom c (weak association was observed) [48] 10 and 16 mol% change polarity and mobility profiles in POPC LUVs, and accelerates POPS flip-flop; PoxnoPC stabilizes transmembrane water pores during PS headgroup flip-flop locally increasing the polarity of membrane interior [53] 2.8-50 mol% in PLPC, sn-2 chains loop back toward water and form H-bond with water and lipid headgroups, the reorientation is less pronounced than for hydroperoxides (???, and ??), APL increases, thickness reduces, order parameter decreases, water defects are promoted resulting in increased water permeability [40] 1-25 mol% in POPC, partial reversal of sn-2 chains, APL increases, thinning, reduced order parameter [42]</td>
</tr>
<tr>
<td>POVPC</td>
<td>25 mol% with DPPC forms MLVs, pure compound forms micelles, heavy loss of EPR anisotropy in PLPC SPB [45] 10 mol% makes POPC carbonyl region considerably more hydrated and more mobile, slightly increases lateral lipid diffusivity, truncated chains prefer orientation parallel to the bilayer surface, APL close to that of pure POPC bilayer [49]</td>
</tr>
</tbody>
</table>
| PazePC    | heavy loss of EPR anisotropy in SPBs (in what lipid? micelles?) [10] [14] [45] 10, 20, 40 mol% gradually expands DPPC monolayers and abolishes LE-LC phase coexistence (both the effects are stronger than those for PoxnoPC), at higher laterall pressures lost into the subphase, shifts LC domains to higher pressures, lowers surface potential, truncated acidic sn-2 chain likely reverses toward water subphase [47] micelles of pure compound bind to cytochrom c [48] 10 and 14 mol% change polarity and mobility profiles in POPC LUVs, (MD here), and accelerates...
POPS flip-flop the effects are stronger than those of PoxnoPC [53]

1-25 mol% in POPC, complete reversal of sn-2 chains, APL decreases (due to the presence of sodium cations), thinning, reduced order parameter [42]

PGPC

- recovery of bilayer structure upon addition of cholesterol [45]
- cholesterol prevents micelle formation at higher PGPC amount in PLPC [46]

1 mol% PGPC in DOPC and 0-50% cholesterol, reorientation of sn-2 chains towards water phase, headgroups of PGPC shifter toward the water phase and its lateral diffusion faster than DOPC, these two later effects diminished in the presence of cholesterol [44]

10 mol% makes POPC carbonyl region more hydrated and more mobile, increases lateral lipid diffusivity, truncated chains loops back into water, APL decreased (due to presence of sodium cations) [49]

ox1-DOPC

- 100 mol% ox1-DOPC bilayer, looping back of oxidized chains toward water phase, large water defects present including transmembrane pores, APL increased, thickness depends on the hydrophobicity of short-chain oxidation products present in the bilayer (decreases for hydrophilic products, increases for hydrophobic) [63]
- 3-90 mol% ox1-DOPC in DOPC, APL increases, thickness decreases, water permeation increases, below 75 mol% of oxidation water transferred as single molecules or small clusters, starting from 75 mol% water pores formed, pore opening depends on oxidation ratio [64]

ox2-DOPC

- 100% ox2-DOPC bilayer not-stable, strong water penetration and destroying of the bilayer structure at nanosecond timescale [63]

5. Future perspectives of oxPL research

The main interest in the study of oxidative lipidomics stems from increasing knowledge of the relationships existing between pathologies and oxidative stress. To date, oxidative stress is increasingly considered an important, if not fundamental, aspect of many pathologies. Oxidative damage to living cells is being actively studied under the genomics and proteomics profile, that is at the level of functional biomolecules. Yet, though considered mainly under the structural aspect, the phospholipid bilayer also plays an important functional role [Kinnunen]. A cell membrane (or that of an organelle) should be considered as the crossroad of all omics, in which their integration and proper functioning is directed and modulated by phospholipids. Oxidative modification of these molecules is strongly suspected to impair their role, suggesting that the study and the characterization of OxPL and of lipoperoxidized membranes may allow new clues to how oxidative stress is involved in many diseases. Inasmuch the study of oxidative lipidomics is expected to shed light in pathology, then the development of these studies can be considered worthy.

In our opinion, this development should occur along three main routes: mass spectrometry lipidomics, oxidized membrane biophysics, and oxidized (phospho)lipids metabolism.
In fact, owing to the overwhelming amount of OxPL species, their chemical and structural characterization is the indispensable basis for oxidative lipidomics studies. ESI-MS appears as an invaluable tool to this task, as shown in many instances [rev. by Domingues, Spickett], useful also to build a data bank of standard MS spectra of OxPL for their nanoscale detection in pathologic lipidomes.

Biophysics of membranes containing OxPL species which is in the focus of this review is expected to define how they affect the structure, the properties and functioning of a biomembrane in which OxPL grow after the ROS attack. In addition to pure lipid membrane models, reconstituted models encompassing also functional proteins are devised in order to study how OxPL affect lipid-protein relationship, not only from the point of view of enzyme functioning but also of protein folding and functional assembly into the bilayer. Such studies may help establishing the extent of OxPL involvement in diseases at the molecular level in the membrane.

An impulse to metabolic studies of OxPL and of oxidized fatty acids should result in more information about how these molecules are related to the rest of a living cell functioning. In fact, deacylation-reacylation of OxPL should yield the basis for oxidative stress repair, as well as catabolism of oxidized fatty acids. A naturally oxidized membrane should be observable through a time window in between the antioxidant defence mechanisms (SOD, glutathione reductase and peroxidase, ander other anti-ROS enzymes) and repair systems. When oxidative diseases are considered, knowledge of these mechanisms would help to understand how much OxPL contribute to the overall evolution of an oxidatively diseased cell in pathologic organs.

References


