Eur Biophys J (2007) 36:499–515
DOI 10.1007/s00249-007-0146-2

REVIEW

Peroxidation of liposomal lipids

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Received: 5 November 2006 / Revised: 20 February 2007 / Accepted: 22 February 2007 / Published online: 23 March 2007

Abstract Free radicals, formed via different mechanisms, induce peroxidation of membrane lipids. This process is of great importance because it modifies the physical properties of the membranes, including its permeability to different solutes and the packing of lipids and proteins in the membranes, which in turn, influences the membranes' function. Accordingly, much research effort has been devoted to the understanding of the factors that govern peroxidation, including the composition and properties of the membranes and the inducer of peroxidation. In view of the complexity of biological membranes, much work was devoted to the latter issues in simplified model systems, mostly lipid vesicles (liposomes). Although peroxidation in model membranes may be very different from peroxidation in biological membranes, the results obtained in model membranes may be used to advance our understanding of issues that cannot be studied in biological membranes. Nonetheless, in spite of the relative simplicity of peroxidation of liposomal lipids, these reactions are still quite complex because they depend in a complex fashion on both the inducer of peroxidation and the composition and physical properties of the liposomes. This complexity is the most likely cause of the apparent contradictions of literature results. The main conclusion of this review is that most, if not all, of the published results (sometimes apparently contradictory) on the peroxidation of liposomal lipids can be understood on the basis of the physico-chemical properties of the liposomes. Specifically: (1) The kinetics of peroxidation induced by an ''external'' generator of free radicals (e.g. AAPH) is governed by the balance between the effects of membrane properties on the rate constants of propagation (k_p) and termination (k_t) of the free radical peroxidation in the relevant membrane domains, i.e. in those domains in which the oxidizable lipids reside. Both these rate constants depend similarly on the packing of lipids in the bilayer, but influence the overall rate in opposite directions. (2) Peroxidation induced by transition metal ions depends on additional factors, including the binding of metal ions to the lipid–water interface and the formation of a metal ions-hydroperoxide complex at the surface. (3) Reducing agents, commonly regarded as ''antioxidants'', may either promote or inhibit peroxidation, depending on the membrane composition, the inducer of oxidation and the membrane/water partitioning. All the published data can be explained in terms of these (quite complex) generalizations. More detailed analysis requires additional experimental investigations.

Keywords Peroxidation · Lipids · Liposomes · Antioxidants · Membranes · Copper · Free radicals

Abbreviations

PC	Phosphatidylcholine
PLPC	Palmitoyllinoleoylphosphatidylcholine
POPC	Palmitoyloleoylphosphatidylcholine
PAPC	Palmitoylarachidonylphosphatidylcholine
DMPC	Dimyristoylphosphatidylcholine
DAPC	Diarachidonylphosphatidylcholine
DPPC	Dipalmitoylphosphatidylcholine
DSPC	Distearoylphosphatidylcholine
PA	Phosphatidic acid

Dedicated to Prof. K. Arnold on the occasion of his 65th birthday.

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PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
LA	Linoleic acid
SM	Sphingomyelin
PUFA	Polyunsaturated fatty acids
AAPH	2,2′-Azo bis (2-amidinopropane) hydrochloride
AMVN	2,2′-Azo bis (2,4-dimethylvaleronitrile)
LOOH	Hydroperoxides
MDA	Malondialdehyde
ROS	Reactive oxygen substances
TBARS	Thiobarbituric acid-reactive substances
TOC	α-Tocopherol, vitamin E
LUV	Large unilamellar vesicles
SUV	Small unilamellar vesicles
MLV	Multilamellar vesicles
SAXS	Small angle X-ray scattering

Introduction

Lipid peroxidation plays a central role in many physiologic and pathologic processes (Mattson 2004; Poon et al. 2004; Schroepfer 2000). Much effort has therefore been devoted to gain understanding of the mechanisms responsible for peroxidation, especially in the context of oxidative stress-related diseases, including cardiovascular and neurodegenerative diseases (Van-Ginkel and Sevanian 1994; Mason et al. 1997; Spiteller 2003). Furthermore, the possible benefits of supplementation of antioxidants raised much attention and many investigations have been conducted with the aim of developing efficient inhibitors of oxidation (Pamplona et al. 2002).

In spite of a large number of investigations devoted to these issues, two major topics remain unresolved, namely quantitative definitions of the terms ''oxidative stress'' and ''antioxidative potency'' of peroxidation inhibitors. Our recent meta-analysis (Dotan et al. 2004) demonstrated that oxidative stress cannot be defined in universal terms, possibly because several types of ''oxidative stress'' exist (Dotan et al. 2004). Similarly, evaluation of the relative antioxidative potency of antioxidants also depends on the assays used for its evaluation.

This lack of a universal quantitative definition of oxidative stress and antioxidative potency, along with the complexity of both the biological systems and the mechanisms of peroxidation, resulted in apparent inconsistencies between the results of different studies. These complications led many investigators to study peroxidation in relatively simple model systems, mostly liposomes made of well defined oxidizable lipid and, in many studies, non-oxidizable lipids, which affect the peroxidation by affecting the membrane properties (Chatterjee and Aagarwal 1988; Mowri et al. 1984). These, as well as other studies on peroxidation of liposomal lipids, yielded interesting results regarding both the dependence of lipid peroxidation on the composition and physical properties of the liposomes, as well as on the inducer of peroxidation and on the presence and concentrations of antioxidants.

Although, these studies yielded much data on the peroxidation of liposomal lipids, many questions remain open and often controversial. In an attempt to explain the apparent inconsistencies between some of the published results, we found it of interest to try to answer several specific questions through a review of the existing data. The present mini-review is far from being comprehensive. It relates, rather selectively (and sometimes rather speculatively), to publications that addressed the dependence of peroxidation on the inducer of oxidation and on the chemical and physical properties of the studied liposomes. These properties also affect the potency of different antioxidants, as briefly discussed below.

In this review, we relate mostly to studies on the peroxidation of unilamellar liposomes of different sizes, formed by various methods, to avoid the complex nature of multilamellar liposomes, in which only the outer monolayer of the outermost bilayer is accessible to externally added inducers of oxidation. We attribute special significance to studies in which the liposomes contained more non-oxidizable lipids than oxidizable lipids. In these studies, the oxidizable lipids have relatively small influence on the physical properties of the liposomal bilayers, which enables evaluation of the influence of both the chemical and the physical factors on peroxidation.

For similar reasons, we concentrate on peroxidation studies conducted with liposomes made of pure lipids, whose concentrations in the lipid bilayers, unlike in mixtures of lipids extracted from biological samples, are determined by the researcher. In fact, even the data on liposomes made of synthetic lipids is not always sufficient to answer specific questions regarding the dependence of kinetics of peroxidation on the inducer of peroxidation and on the physico-chemical properties of the liposomes. Variation of the composition, as in extracts of biological specimens, adds another source of uncertainties and possible apparent irreproducibilities.

Polyunsaturated liposomal phospholipids that contain bisallylic hydrogen atoms undergo spontaneous peroxidation by dissolved molecular oxygen. This slow reaction depends on the temperature and on contaminations of the liposomal dispersions by residues of transition metal ions, which catalyze the reaction (Halliwell and Gutteridge 1999). Irradiation of the dispersion also accelerates the

peroxidation (Mishra 2002). These reactions are the major factors in the stability of liposomal preparations used either for drug delivery or for non-medical applications (Stone and Smith 2004; Lasic 1998). In modeling the peroxidation induced by various biologically relevant inducers (e.g. transition metals and enzymes), the most commonly used inducers are copper ions, iron ions (commonly studied in the presence of ascorbic acid), hypochlorite (HOCl), which is the active specie in myeloperoxidase-catalyzed reaction, and ''organic'' radical generators, some of which are water-soluble (e.g. AAPH) and others are lipid-soluble (e.g. AMVN) (Cao et al. 1993; Cao and Prior 1998; Prior and Cao 1999; Chatterjee and Aagarwal 1988; Panasenko et al. 1994).

The mechanisms responsible for the oxidation induced by various inducers (as depicted in Fig. 1) differ considerably. In AAPH-induced peroxidation, free radicals are formed in the solution and attack the liposomes from the external medium. The same may be true for iron-induced peroxidation in the presence of ascorbate because free radicals formed in the solution upon reduction of Fe^{3+} by ascorbate, may be the actual inducer of lipid peroxidation (Halliwell and Gutteridge 1999). By contrast, lipid-soluble generators of free radicals (e.g. AMVN) undergo thermal decomposition in the liposomal bilayer, where the resultant free radicals induce a free radical chain reaction. Both these reactions are quite different from the peroxidation induced by transition metal ions that bind to the liposomal surface, where they form free radicals upon being reduced either by lipid soluble reducing agents such as tocopherol (Yoshida et al. 1994) or by hydroperoxides, as shown for liposomal PUFA by Patel et al. (1997).

Peroxidation of liposomal lipids is affected by many factors, as depicted in Fig. 2. The major difficulty of reviewing the very large number of studies devoted to the effects of these factors on the peroxidation of the liposomal lipids is the interdependencies between the effects of the different factors. Specifically, the major attributes of the various studies are the inducers of peroxidation, the composition and physical properties of the liposomes, with respect to both oxidizable and non-oxidizable lipids, and the antioxidants, when present in the system. Elucidation of the effect of each of these factors requires comparison of the peroxidation in systems that differ only in the specific studied factor. For example, elucidation of the effect of the ratio between a given oxidizable lipid and a given non-oxidizable lipid in the liposomes requires comparison of data on the effects of varying this ratio on the kinetics of peroxidation by a given inducer at the same concentration and temperature. The results obtained in such studies are not necessarily valid for the effect of the above ratio on the peroxidation induced by a different inducer. Similarly, when two inducers exhibit similar kinetics of peroxidation in a given liposomal system, it does not necessarily mean that they will exhibit similar kinetics in a different liposomal system.

Another issue addressed in many studies on the peroxidation of liposomal lipids is the effect of antioxidants on the kinetics of peroxidation (Gutierrez et al. 2003). The major complication in these studies is that the effect of antioxidants depends on both the composition and physical properties of the liposomal system and on the inducer (Gal et al. 2003). Moreover, the dependence of the kinetics of peroxidation on the liposomal composition and physical properties as well as on the inducer and antioxidants also depends on the concentrations of these components in the studied systems. These complex dependencies limit the possibility of reaching generalizations. This is further

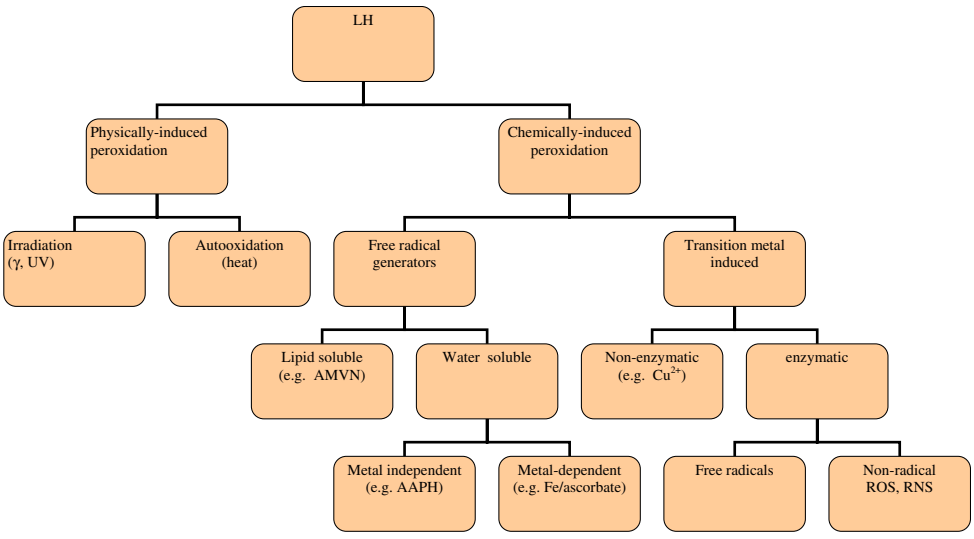

Fig. 1 Classification of the inducers of lipid peroxidation (see text for details)

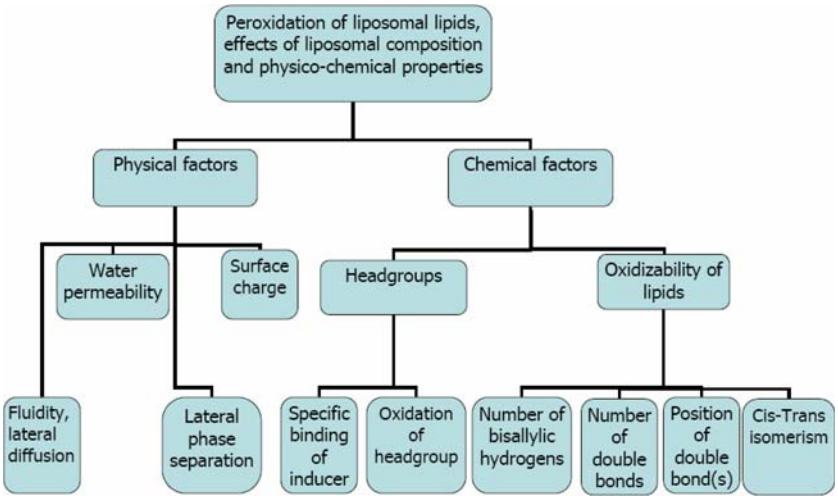

Fig. 2 Physical and chemical factors that affect the peroxidation of liposomal lipids

complicated by the non-monotonic nature of some of such dose-dependencies (Gal et al. 2003), as described below.

The difficulties described above must be kept in mind whenever relating to the effects of the factors that contribute to lipid peroxidation as depicted in Figs. 1 and 2. In the words of the late Efraim Racker regarding a different issue, ''we have to respect small variations that may account for discrepancies in results observed by competent investigators'' (Racker 1979). The reader of the following discussion is advised to adhere to this guideline and also consider the possibility that the methods used for monitoring peroxidation (Halliwell and Gutteridge 1999) can also affect the results, as shown by Gutierrez et al. (2003).

The interrelated dependencies of the kinetics of peroxidation on the liposome composition and on the inducer of peroxidation

Prior to reviewing (and discussing) the dependence of peroxidation on the inducer of peroxidation, on one hand, and on the composition and properties of the liposomes, on the other, it is important to relate to the interrelationships between the effects of these factors. First, we note that peroxidation induced by different inducers may occur via different mechanisms (see Fig. 1) and that these differences are probably responsible for the differences between the kinetic profiles observed for the peroxidation of lipids induced by these inducers. As an example, in many cases, copper-induced peroxidation exhibits an apparent ''lag-phase'' prior to propagation of the free radical chain reaction. Such kinetic profiles can be attributed to product-acceleration, namely to the promotion of peroxidation by the increasingly larger production of free radicals from the continuously accumulated hydroperoxides.

By contrast, we do not expect product-acceleration in AAPH-induced peroxidation, and the experimentally observed monotonic accumulation of oxidation products may be understood in terms of apparent zero order production of free radicals.

In short, both the kinetics of peroxidation and its dependence on the composition of the liposomes depend on the inducer of peroxidation. As an example, the acceleration of copper-induced peroxidation observed upon inclusion of negatively charged phospholipids (either oxidizable or non-oxidizable) may be attributed to increased binding of copper to the liposomal surface (Gal et al. 2003). The lack of effect of negatively charged phospholipids on the kinetics of AAPH-induced oxidation is consistent with this interpretation (Gal et al. 2003). The dependence of peroxidation on the liposomal charge (i.e. on the phospholipids headgroups) is further discussed below. Another example for the interdependence of peroxidation on the composition of liposomes and on the inducer is that liposomal cholesterol does not affect AAPH-induced peroxidation of liposomal PUFA, whereas Cu^{2+}-induced peroxidation of PUFA depends in a complex fashion, on the presence of cholesterol (Schnitzer et al. 2007).

Moreover, the effect of composition on peroxidation depends not only on the inducer but on its concentration as well. As an example, copper-induced peroxidation of liposomal lipids depends on the concentration of copper such that in both liposomes made of PC and liposomes made of PC and PA, increasing the concentration of copper results in a monotonic increase of the rate of oxidation. By contrast, in liposomes made of PC and PS, the dependence of oxidation on copper concentration is non-monotonic such that the rate of oxidation increases upon increasing the concentration of copper, but further increase of the

concentration of copper ions slows down the rate of peroxidation (Gal et al. 2003).

Effect of composition and physico-chemical properties of the liposomes on the peroxidation of liposomal lipids

Scope of the issue

Peroxidation of liposomal lipids is a complex function of the composition of the liposomes. Notably, the composition affects the susceptibility to oxidation in more than one way (Fig. 2). First, the physical properties of the liposomes are governed by their composition, with respect to both oxidizable and non-oxidizable lipids. In turn, the physical properties of the liposomes may affect their susceptibility to peroxidation by various inducers, not necessarily in the same fashion for the different inducers. Non-oxidizable lipids affect peroxidation only through their effect on the physical properties, whereas variation of the composition of oxidizable lipids affect the kinetics of peroxidation by influencing both the physical properties of the liposomes and the chemical properties of the oxidizable lipids, including the number of double bonds in PUFA, the number of bisallylic hydrogen atoms, the position of the double bonds with respect to the liposomal surface, and the geometric isomerism of the double bonds. All these factors affect the physical properties of the liposomes and by that affect the peroxidation.

In the following discussion, we first relate theoretically to the possible dependence of peroxidation on the physical properties of the liposomal bilayers. The most complex factor is the packing of lipids in the bilayers. This factor governed by thermodynamic forces, determines all the experimentally detectable physical properties. In homogeneous bilayers (i.e. when no lateral separation occurs), tighter packing (high packing density) results in an increase of some of the measurable packing-related parameters, including the thickness of the bilayers (commonly detected by X-ray scattering and neutron diffraction), the order parameter (commonly detected by NMR and ESR measurements), and the microviscosity of the lipidic milleue (commonly measured by fluorescence anisotropy). Other measurable parameters decrease upon increasing the packing-density, including the surface area per phospholipid headgroup, the fluidity (defined as the reciprocal of the microviscosity) and the rate of the lateral diffusion of the membrane components, which is a decreasing function of the viscosity of the relevant medium (i.e. of the lipid bilayer).

Lipid peroxidation occurs via a sequence of reactions, some of which are monomolecular, other are bimolecular (see below). Assuming that monomolecular steps are much less affected by the physical properties of the bilayer than bimolecular reactions, it follows that as far as the rate of oxidation in homogeneous membranes is concerned, the most relevant experimentally detectable reflection of the packing is the rate of lateral diffusion, through its effect on the rate constants of the bimolecular steps. Accordingly, we interpret the dependence of peroxidation on the composition (and properties) of homogeneous bilayers in terms of the dependence of the rate of peroxidation on the rate of lateral diffusion. Specifically, the rate constant of bimolecular reactions is an increasing function of the rate of lateral diffusion of oxidizable lipids in the bilayers, which means that increased rate of lateral diffusion (in more ''fluid'', less ''rigid'' bilayers) can be expected to enhance both the rate of propagation of free radical chain reactions and the rate of termination of free radicals, which occurs via biradical quenching. These two reactions have opposite effects on the rate of the overall peroxidation (see below). Hence, the overall effect of the rate of lateral diffusion on the peroxidation cannot be unequivocally predicted.

The rate of lateral diffusion within liquid crystalline bilayers of different compositions is likely to be affected by the composition much less than by changes associated with thermal phase transformations. Yet, relatively small changes in the rate of lateral diffusion may alter the rate of peroxidation. The existing data is insufficient to differentiate between the latter effects and alternative factors, such as the solubility of oxygen in the bilayers and the penetration of water into the bilayer (see below).

Following our discussion of the theoretical considerations, we discuss possible implications of these considerations and describe a few experimental results that can be explained (sometimes rather speculatively) in terms of these implications.

Theoretical considerations

The naturally abundant (in membrane) polyunsaturated fatty acid residues (PUFA) are susceptible to peroxidation because they contain bis-allylic hydrogen atom(s), which are relatively easy to abstract by external free radicals. In the presence of molecular oxygen, the primary products of peroxidation are lipid hydroperoxides. These compounds subsequently may undergo thermally induced (and/or transition metal-catalyzed) decomposition, resulting in the production of additional, ''secondary'' free radicals, thus giving rise to further chain peroxidation. Such breakdown of hydroperoxides yields complex mixtures of final, relatively stable products of peroxidation, including unsaturated aldehydes, ketones and alcohols.

Chain peroxidation of oxidizable lipids (LH) occurs upon exposure to external radicals ($X^{\bullet}$) according to the following scheme:

- Abstraction of reactive hydrogen atom from bisallylic positions of PUFA:

$$L - H + X^{\bullet} \rightarrow L^{\bullet} + HX \quad (r1)$$

- Formation of peroxyl-radicals $LO_2^{\bullet}$ via an extremely fast reaction of the lipid-derived radicals $L^{\bullet}$ with molecular, soluble oxygen:

$$L^{\bullet} + O_2 \rightarrow LO_2^{\bullet} \quad (r2)$$

- Formation of lipid hydroperoxides LO_2H, via relatively slow (hence, rate limiting) reaction of peroxyl-radicals with oxidizable lipids LH.

$$L - H + LO_2\bullet \rightarrow LO_2H + L^{\bullet} \quad (r3)$$

The stoichiometry of the latter two reactions of chain peroxidation is:

$$LH + O_2 \rightarrow LO_2H \quad (r4)$$

- Termination of the chain peroxidation via biradical quenching of two peroxyl radicals:

$$LO_2\bullet + LO_2\bullet \rightarrow NRP \quad (r5)$$

Notably, radical peroxidation may be induced either by an ''external'' flux of free radicals $X^{\bullet}$ (e.g. radicals formed upon thermal decomposition of organic generators of free radicals or upon irradiation). Peroxidation may also be induced by free radicals, produced within the system (e.g. upon metal-catalyzed decomposition of preformed hydroperoxides).

The rate of peroxidation (V) is given by Eq. 1 (Halliwell and Gutteridge 1999)

$$V = K_p[LH]\sqrt{\frac{R_i}{2k_t}} \quad (1)$$

In the latter equation, (1) the term $\sqrt{\frac{R_i}{2k_t}}$ relates to the steady-state concentration of free radicals, which is given by the square root of the ratio between the rate of free radical production (R_i) and the rate constant of termination of the chain reaction (k_t). (2) k_p is the rate constant of propagation of the free radical chain reaction, and (3) [LH] is the concentration of oxidizable lipids in the relevant compartments, namely in the bilayer or in those domains of the bilayer in which oxidizable lipids reside, when lateral phase separation occurs.

In the kinetically simplest case, *when R_i is constant* (e.g. in AAPH-induced peroxidation), and the membrane is homogeneous, the dependence of peroxidation on membrane properties is governed by the two rate constants k_p and k_t as discussed above. For a given composition, both these rate constants are of bimolecular reactions and are therefore expected to increase upon increasing the rate of lateral diffusion of the oxidizable lipids in the bilayers. This, in turn, results in an increase in both the rate constant of propagation (this accelerates the overall peroxidation) and the rate constant of termination (this slows down the peroxidation). Hence, even in this relatively simple case, it is impossible to predict the overall effect of the membrane packing (i.e. fluidity, viscosity, order parameters and other measurable factors) on the rate of peroxidation because k_p and k_t change in the same direction and have opposite effects on the rate of peroxidation.

When phase separation occurs, the concentration of LH in the relevant domains (i.e. those domains in which the oxidizable lipids reside) increases. This may be expected to enhance the rate of peroxidation. Yet, it is impossible to predict (even qualitatively) the effect of the phase separation on the peroxidation, because the rate of lateral diffusion in the relevant domains is different from this rate in homogeneous bilayers and the effect of this rate on the overall rate of peroxidation cannot be predicted, as discussed above.

As a consequence of the above considerations, for any given inducer, the dependencies of peroxidation on the content and concentrations of both oxidizable lipids and non-oxidizable lipids involve at least two factors:

(1) The ''surface concentration'' of the oxidizable lipids in relevant domains, and
(2) The packing (and rate of lateral diffusion) of the oxidizable lipids in the relevant domains of the bilayers.

This of course, complicates the interpretation of the results of experiments aimed at gaining understanding of the ''chemical effects'' of oxidizable lipids, because oxidizable lipids affect the rate of propagation not only by determining the rate of cleavage of bisallylic hydrogen atoms but also by affecting the homogeneity of the bilayers and the lateral diffusion of the oxidizable lipid in the relevant domains.

When the rate of production of free radicals (R_i) is not constant (e.g. in copper-induced peroxidation), the kinetics is more complex. Copper-induced peroxidation involves formation of free radicals at the membrane surface, through interaction of the copper ions with preformed hydroperoxides. According to previous kinetic studies (Maizus 1966; Pinchuk et al. 1998), production of free radicals occurs via the following sequence of events:

(1) Binding of copper ions to the surface of the liposomes, i.e. to the phospholipid headgroups.

(2) Formation of a 1:2 copper to hydroperoxides complex $Cu(LOOH)_2^{2+}$

$$Cu^{2+} + 2LOOH \underset{kd}{\overset{ka}{\rightleftarrows}} Cu(LOOH)_2^{2+} \quad (2)$$

(3) Decomposition of the latter complex, which yields free radicals.

$$Cu(LOOH)_2^{2+} \rightarrow Cu^{2+} + LO^{\bullet} + LO_2^{\bullet} + H_2O \quad (3)$$

Accordingly, the rate of copper-induced production of free radicals, R_i, is given by the equation

$$R_i = k_i\left[Cu(LOOH)_2^{2+}\right] \quad (4)$$

In this equation, k_i is the rate constant of the monomolecular decomposition (Eq. 3) of the complex, which is not likely to depend markedly on the physical properties of the bilayers.

Equation 4 means that the production of free radicals depends on the concentration of the complex, which, in turn, depends on the concentrations of Cu^{2+} and LOOH and on the binding constant K, given by $K = k_a/k_d$. The rate of radical production, being governed by the binding constant K, also depends on the physical properties of the bilayers because the rate constant of the formation of the bimolecular complex (k_a), unlike the rate constant of its dissociation (k_d), depends on the rate of lateral diffusion of LOOH in the lipid bilayers, hence, on the membrane physical properties. This is undoubtedly the case during the initial stage of the reaction, when the concentration of hydroperoxides in the bilayer is relatively low (and therefore rate-limiting). In contrast, when the concentration of hydroperoxides increases (in the course of peroxidation) the concentration of the complex reaches saturation and the rate can be expected to depend only on the concentration of copper and on the two rate constants k_p and k_t (Eq. 1).

Implications of the theoretical considerations

For the sake of simplicity, we first consider a liposome whose bilayer is made mostly of non-oxidizable lipids and contains only a low concentration of oxidizable lipids that are homogeneously distributed in the bilayer. In this case, the physical properties are likely to be determined only by the non-oxidizable lipids. However, both non-oxidizable and oxidizable lipids may induce lateral phase separation in the bilayers. When this occurs (i.e. when two phases co-exist), the oxidizable, poly-unsaturated lipids are likely to be more concentrated in the more fluid domains. This can be expected to result in higher rate constants (both k_p and k_t) and higher concentrations of both LH and LOOH in the peroxidation-relevant domains. The latter effect (surface concentration of oxidizable lipids in the relevant domains) may affect the rate of peroxidation even when the concentration of oxidizable lipids is much lower than the concentrations of non-oxidizable lipids. Yet, in spite of the reservations described above, the most straightforward way of evaluating the dependence of peroxidation on both chemical factors and physical properties of the bilayers is to study liposomes containing a very low concentration of oxidizable lipids. When the oxidizable lipids are homogeneously distributed in the bilayer, the physical properties are essentially determined by the non-oxidizable lipids. Hence, the ''chemical effects'' can be evaluated by comparison of the peroxidation of different oxidizable lipids that reside in the same ''host membrane''. Under this assumption, modification of the physical properties of the bilayer by varying only the non-oxidizable lipids (keeping the oxidizable lipids constant) can be used to evaluate the net effect(s) of the membrane's physical properties on the peroxidation of the oxidizable lipids.

Dependence of lipid peroxidation on the thermotropic phase and on domain formation in the bilayers

The dependence of peroxidation on the lipid phase of liposomal bilayers has been addressed by several investigators, using different methods. Two lines of indirect evidence indicate that peroxidation of liposomal lipids is faster when the oxidizable lipids reside in the relatively rigid gel phase bilayers than in the less tightly-packed liquid crystalline bilayers:

(1) When a polyunsaturated fatty acid (e.g. linoleic acid or arachidonic acid) resides in DPPC liposomes, its peroxidation is more rapid at temperatures below the transition temperature, T_m, i.e. when the bilayer is in a gel phase, than at temperatures above T_m, when the bilayer is in the liquid crystalline phase (Cubillos et al. 2006). Similarly, when PAPC resides in DMPC bilayers, its peroxidation by Fe^{2+}/ascorbate is faster in the gel phase (at 10°C) than in the liquid crystalline phase (at 37°C) of the DMPC bilayers (Mowri et al. 1984).

(2) Peroxidation of PAPC at a constant temperature (37°C) was faster for PAPC contained in gel phase DSPC bilayers than in liquid crystalline POPC bilayers (Mowri et al. 1984).

In their interpretation of the faster rate of peroxidation in gel phase bilayers, Cubillos et al. (2006) made the reasonable assumption that both the rate constants of

propagation (k_p) and termination (k_t) are lower in the relatively rigid gel phase than in liquid crystalline bilayers. The overall effect of the lipid phase on peroxidation can then be explained if the effect of rigidization-induced decrease of k_t overcomes the effect of rigidization-induced decrease of k_p.

A different interpretation of the relative susceptibility of oxidizable lipids that reside in gel phase bilayers was offered by Mowri et al. (1984) and by McLean and Hagaman (1992). The authors of both these publications proposed that in liposomes made of an oxidizable lipid (e.g. PAPC) and a non-oxidizable (''host'') lipid, relatively ''fluid'', PAPC-rich domains are formed and that propagation within these domains is relatively rapid because the concentration of oxidizable lipids in these domains is high. This interpretation implies that upon addition of oxidizable lipids, gel phase bilayers tend to undergo lateral phase separation into domains of different rigidity with the oxidizable lipids residing in the less rigid domains. This accords with the finding that in gel phase bilayers, relatively rapid peroxidation occurs when the bilayer contains merely 5 mol% PAPC, as compared to 10 mol% in liquid-crystalline bilayers (Mowri et al. 1984).

In conclusion, the existing data is insufficient to conclude whether the apparent higher susceptibility of oxidizable lipids in gel phase bilayers to oxidation is due to phase separation or merely due to the relative effect of the rate of lateral diffusion on k_p and k_t.

Chemical affectors of peroxidation

The generally accepted paradigm is that the stability of liposomal lipids against peroxidation decreases upon increasing the degree of unsaturation. In organic solvents, the stability of PUFA residues of phosphatydylcholine towards AAPH-induced peroxidation is a decreasing function of unsaturation (Araseki et al. 2002), as intuitively expected. By contrast, the oxidizability of PUFA esters that reside in emulsion particles in aqueous media exhibited an opposite trend (Araseki et al. 2002; Kobayashi et al. 2003). Most intriguingly, the degree of unsaturation had little effect (if any) on the oxidizability of liposomal PUFA (Araseki et al. 2002).

Another interesting finding of the latter study is that the arachidonic acid residues of PAPC are less susceptible to peroxidation in PAPC liposomes than in liposomes made of a 1:1 mixture of DPPC and DAPC. The authors attributed this finding to differences in the physical properties of the different bilayers, without specifying this difference. We think that in liquid crystalline bilayers made of PAPC, the PAPC is homogenously distributed, whereas in a 1:1 mixture of DPPC and DAPC, a lateral phase separation occurs, resulting in the formation of DAPC-poor gel phase domains and DAPC-rich liquid crystalline domains. As a consequence, the concentration of oxidizable lipids in the ''relevant domains'' of the bilayer increases, which results in enhanced rate of peroxidation. The existing data is insufficient to conclude whether the higher oxidizability is indeed due to lateral phase separation or due to the effect of the difference in the rate of lateral diffusion. Based on data of many other investigations, we think that phase separation is more likely to be responsible for the higher susceptibility of the liposomal lipids to peroxidation.

A different explanation for the higher oxidizability of arachidonic residues in the DAPC/DPPC mixed bilayers relates to the observation that in tightly packed membranes the water permeability is relatively low (Kobayashi et al. 2003) and the bilayer contains less water, particularly in the vicinity of the double bond regions of the PUFA. This effect, in turn, may result in reduced susceptibility of the lipids to peroxidation because water around the bisallylic positions reduces the homolytic cleavage of the bisallylic C-H bonds, as in emulsified lipids in aqueous media (Kobayashi et al. 2003). Notably, the latter hypothesis is inconsistent with the interpretation offered by Samuni et al. (2000) to their observation that cholesterol protects egg yolk liposomes from storage damages by ''drying'' the bilayers (see below).

The slight effect of the degree of unsaturation on the susceptibility of liposomal lipids to peroxidation also contradicts (at least apparently) the results of Samuni and Barenholz (1997) that degradation of liposomal lipids induced by gamma-irradiation increases upon increasing the degree of unsaturation of the acyl chains. The latter intuitively expected results are also consistent with the results of the investigation of Vossen et al. (1995), on the susceptibility of lipids extracted from the endothelial cells of human umbilical vein. Exposure of liposomes made of these lipids to H_2O_2 in the presence of copper ions resulted in peroxidation of all the oxidizable lipids. The susceptibility of the various PUFA was directly proportional to the number of double bonds.

Another example of ambiguity regarding the difference between the sensitivity of different liposomal phospholipids to peroxidation is that geometric isomers of a given PUFA differ considerably from each other (Sargis and Subbaiah 2003). Specifically, although trans isomers have been associated with various diseases, the cis–cis isomer of PLPC is much more sensitive to peroxidation than the trans–trans isomer (Sargis and Subbaiah 2003). In an attempt to gain understanding of this finding, Sargis and Subbaiah (2003), compared the oxidizability of two liposomal systems of similar fluidities (and most likely of similar rate of lateral diffusion), each containing one of the two isomers. The results of this elegant study (Sargis and Subbaiah 2003) revealed that trans double bonds are

inherently resistant to oxidation, namely that the difference between the sensitivity of the two geometric isomers is not merely a result of their effect on the packing of lipids in the bilayers. Yet, an alternative possibility that cannot be ruled out is that ''phase separation'' occurs in the bilayer of one or both these liposomes, such that the oxidizable fatty acids reside in phases of different physical properties.

The effect of phospholipids headgroups

Peroxidation of phospholipid headgroups is rare. Yet, the headgroups of the phospholipids affect the peroxidation of the PUFA residues of the liposomal phospholipids. As discussed above, the actual effects of the headgroup on peroxidation depend on the inducer of oxidation. Thus, negatively-charged bilayers bind more transition metal ions than uncharged liposomes and by that promote the peroxidation induced by these ions, without affecting the peroxidation induced by free radical generators (Gal et al. 2003; Dacaranhe and Terao 2001).

Beyond surface charge, the phospholipid headgroups may also affect other bilayer properties, including the packing of the lipid chains, the thermotropic phase behavior and the isothermal lateral phase separation of the bilayer into discrete domains of different compositions and different physical properties and by that affect the peroxidation of liposomal lipids. The effect of the headgroups on the isothermal phase separation of the bilayers is likely to contribute to the rather confusing results obtained by Montfoort et al. (1987). These authors studied the effects of PE, SM, lyso-PC and cholesterol on the Fe^{2+}/ascorbate-induced peroxidation of PAPC in liposomes made of PAPC, PS (5 mol%) and one of the above phospholipids. The main observations of the latter study were that:

(a) PE (25%) inhibited peroxidation
(b) SM (a known ''rigidifier'' of bilayers) inhibited peroxidation in a concentration-dependent fashion, exhibiting a maximal inhibitory effect at 10 mol% and smaller effects at higher concentrations.
(c) Lyso-PC (a known ''fluidizer'' of bilayers) inhibited peroxidation at low concentrations (up to 1.5 mol%) but accelerated peroxidation at higher concentrations.
(d) Cholesterol (an effective ''rigidifier'' of liquid-crystalline bilayers) inhibited peroxidation at relatively low concentrations (up to 20 mol%) more that at higher concentrations.

These complex results cannot be explained as being merely due to surface charge or due to the packing of lipids in the membrane or to both these factors. In the absence of a more specific explanation, we have to assume that the complexity of these results is, at least partially, due to phase separation in the studied liposomes.

Another example of the complex dependence of peroxidation on the phospholipid headgroup is the protective effect of docosahexaenoyl PE, that resides in the external layer of liposomes against AAPH-induced peroxidation of liposomal docosahexaenoic acid (Kubo et al. 2003). Peroxidation induced by other inducers may be affected differently, depending on the site of production of free radicals with respect to the oxidizable site of the PUFA.

Of special interest are the unique effects of phosphatidylserines (PS). Using egg PC liposomes, Dacaranhe and Terao (2001) discovered that liposomes containing PS are more stable against Fe^{2+}/ascorbate-induced peroxidation than liposomes containing either PA or PG or PE. No such effect of PS was observed against AAPH-induced peroxidation. This unique effect of PS on transition metal-induced peroxidation cannot be explained by enhanced binding of metal ions because both PA and PG exhibited no such effect. The alternative explanation, raised by the authors, was that PS suppresses the decomposition of hydroperoxides (which are the primary products of peroxidation) and by that exerts its unusual effect. This hypothetical explanation has yet to be investigated. The effect of PS on the potency of antioxidants is also quite unique, as described below.

The biological relevance of the results described above is quite questionable because they differ considerably from the results obtained for liposomes that were made of the membrane lipids of red blood cells upon exposure to hemoglobin (Szebeni and Toth 1986). Unlike in other liposomal systems, both PA and PS inhibited the peroxidation of the latter liposomes, whereas both PE and PI promoted peroxidation.

In conclusion, in view of the complexity of the possible effects of the headgroups on the peroxidation of liposomal lipids, the available data is insufficient to enable any generalization. Further studies are required.

Effects of cholesterol

Inclusion of cholesterol in phospholipid bilayers alters the membrane properties in a complex fashion. At relatively low cholesterol/PC ratios, the cholesterol slightly alters the packing of phospholipids in bilayers, causing ''rigidization'' of liquid crystalline bilayers and ''fluidization'' of gel phase bilayers (Ohvo-Rekila et al. 2002; Yeagle 1985; Franks 1976; De Kruijff et al. 1976; Engelman and Rothman 1972). As a consequence, addition of cholesterol to liquid-crystalline bilayers slows down the lateral diffusion of all the bilayer components, whereas introduction of cholesterol into gel-phase bilayer enhances the lateral diffusion. These cholesterol-induced alterations of the bilayer properties are only valid as long as the cholesterol and phospholipids are mixed homogeneously. This is the case

up to a limiting maximum of cholesterol/phospholipid molar ratio in the bilayer. For a given phospholipid, this maximum depends on the temperature and on the lipid phase that exists in the absence of cholesterol at the given temperature. At higher cholesterol/phospholipid ratios, a lateral phase separation occurs, resulting in cholesterol-rich, relatively rigid domains (patches) and cholesterol-poor, more fluid continuum (Ohvo-Rekila et al. 2002). Upon further increase of the cholesterol content, cholesterol-rich domains may become the continuum, in which relatively fluid, cholesterol-poor lateral domains exist (Ohvo-Rekila et al. 2002).

In short, cholesterol rigidifies liquid crystalline bilayers, fluidizes gel phase bilayers and/or induces lateral isothermal phase separation of the lipids in the bilayer. This complex behavior complicates the interpretation of the effect of cholesterol on the structure of lipid bilayers of different compositions. Furthermore, the complex dependence of peroxidation on the composition, structure and physical properties of the bilayers further complicates the interpretation of the effects of cholesterol on lipid peroxidation. These complexities may explain the apparent inconsistencies between the reported results on the peroxidation of liposomal cholesterol, on one hand, and on the effect of cholesterol on the peroxidation of the liposomal PUFA, on the other.

Prior to relating specifically to the peroxidation of liposomal cholesterol, it is important to first note that several studies demonstrated that the peroxidation of liposomal cholesterol is much slower than peroxidation of liposomal PUFA (Barclay et al. 1990; Barclay 1993). In fact, under our experimental conditions (Schnitzer et al. 2007), cholesterol appears to be resistant to copper-induced peroxidation, independent of the presence of oxidizable lipids, such as PLPC, in the liposomes. These results accord with the previous results obtained for AAPH-induced peroxidation of lipids in both liposomes and organic solutions (Barclay et al. 1990; Barclay 1993). In our studies (Schnitzer et al. 2007), the AAPH-induced peroxidation of liposomal cholesterol was apparently independent of the presence (and content) of PUFA. Similarly, AAPH-induced peroxidation of PLPC was only slightly affected (if at all) by cholesterol.

By contrast, Cu^{2+}-induced peroxidation of liposomal PUFA was markedly affected by cholesterol. The following examples demonstrate how the rate of peroxidation depends on the addition of cholesterol and how the observed effects can be explained in terms of the considerations discussed above:

(1) Inclusion of cholesterol in DLPC vesicles significantly enhanced lipid peroxidation (Jacob and Mason 2005). The author's interpretation of this finding was based on the results of McLean and Hagaman (1992), who showed (see above) that peroxidation increases upon increasing the rigidity of the packing of lipids within the bilayers. Accordingly, the acceleration observed upon addition of cholesterol was explained as being due to the rigidifying effect of cholesterol on the packing of lipids in the liquid crystalline bilayers. In the words of Jacob and Mason (2005), ''cholesterol increases the lateral packing of the phospholipids acyl chains, facilitating the propagation of free radical through the lipid bilayers''. This interpretation is consistent with the results of several other investigations that claimed that reducing the rate of lateral diffusion enhances the peroxidation (e.g. Mowri et al. 1984; Cervato et al. 1988; Viani et al. 1990). Yet, the available data is insufficient to rule out the alternative explanation based on the implications of a cholesterol-induced lateral phase separation (e.g. Jacob and Mason 2005).

(2) AAPH-induced peroxidation of pirinaric acid contained in DPPC liposomes was inhibited by inclusion of cholesterol (35 mol%) in the DPPC bilayers (Girao et al. 1999). This can be due to the fluidizing effect of cholesterol on the gel phase bilayers of DPPC, in agreement with the paradigm that lipid peroxidation increases upon increasing the rigidity of the bilayer (see above). Nonetheless, the latter generalizations refer to the balance between the contributions of k_t and k_p to the overall rate of peroxidation. This balance is likely to depend on the actual packing of lipids in the liposomes.

(3) Fe^{2+}/ascorbate-induced peroxidation of liposomal PAPC (10 mol%) in gel phase bilayers made of DSPC was relatively rapid at 37°C (Mowri et al. 1984), namely much below the phase transition temperature (T_m) of DSPC. As discussed above, this observation can be attributed to a high surface concentration of PAPC in PAPC-rich, DSPC-poor liquid crystalline domains (Mowri et al. 1984). Addition of cholesterol (about 5 mol%) slowed down the peroxidation, which the authors attributed to elimination of the lateral phase separation and the consequent surface dilution of the oxidizable lipids (Mowri et al. 1984).

(4) Our recent kinetic study of the effect of cholesterol on the peroxidation of PLPC in liposomes made of POPC and PLPC (with or without cholesterol), revealed that AAPH-induced peroxidation of PLPC is essentially independent of the presence of cholesterol in the bilayers (Schnitzer et al. 2007). In contrast, copper-induced peroxidation was affected in a complex fashion, such that in cholesterol-containing

liposomes the initial rate of peroxidation was lower than in the absence of cholesterol but the maximal rate was higher than in the absence of cholesterol. This complex behavior can be understood in terms of the theoretical approach described above, as follows:

(a) The rate of peroxidation is a function of the concentration of the $Cu(LOOH)_2^{2+}$ complex (Eq. 4).
(b) For a given copper concentration, the concentration of the latter complex depends on the concentration of LOOH and on the binding constant K. In turn, K may be expected to be lowered by introduction of cholesterol (see above).
(c) The observed cholesterol-induced reduction of the rate of the initial steps of peroxidation may be a result of the reduced concentration of the complex caused by the lower value of the binding constant, K.
(d) In contrast, at later stages, when the concentration of LOOH is high, the concentration of the complex is limited by the concentration of copper. Hence, under these conditions the cholesterol-induced reduction of the binding constant K cannot be expected to reduce the concentration of the complex. As a consequence, the rate of radical production R_i is not likely to be affected by cholesterol. Under these conditions, the effect of cholesterol on the maximal rate depends only on the cholesterol-induced variation of the rate constants k_t and k_p and the observed enhancement of the overall maximal rate is probably an outcome of the balance between the effects of cholesterol-induced reduction of both k_t and k_p (see above).

(5) UV radiation-induced peroxidation of liposomal PUFA, made either of synthetic lipids or of the mixture of lipids extracted from the stratum corneum, revealed that in both these liposomal systems, inclusion of cholesterol in the bilayer results in an increase in the level of hydroperoxides and a decrease in the level of MDA (Schonfelder et al. 1999). This may indicate that cholesterol inhibits the decomposition of the hydroperoxides formed as a first step of the peroxidation. This again may result from the balance between the effects of cholesterol on k_t and k_p.

Evaluation of the antioxidative potency of various antioxidants using liposomes

The possibility of gaining biologically relevant information on the potency of antioxidants in model membranes cannot be taken for granted. The biological relevance of the potency of antioxidants to protect liposomal PUFA against oxidation depends on the choice of both the model membrane and the inducer of peroxidation, because the effects of various antioxidants depend on both these factors (Gutierres et al. 2003).

At the present time, no experimental model system emerged as a ''gold standard'' for evaluation of antioxidative potency. Castelli et al. (1997) proposed the use of AAPH-induced peroxidation of linoleic acid in DPPC-LUVs to evaluate the potency of free radical scavengers. Their choice of LUVs was based on the following rationale:

(a) LUVs maintain their structural integrity when exposed to ROS generated outside of the bilayer (i.e. in aqueous media).
(b) LUVs are more susceptible to oxidation than multilamellar vesicles, and
(c) The use of LUVs (unlike MLVs) avoids the problems of being dependent on the availability of an undefined fraction of the lipid bilayers of the multilamellar assemblies to the inducer of peroxidation.

Accordingly, these authors advocated using LUVs made of ''unoxidizable lipids as host lipids, doped with minimal quantities of peroxidizable lipids''. In their studies, they used an 8/1 molar ratio (DPPC/linoleic acid), which they defined as being ''an optimal compromise'' that allows both minimal changes in the physical state of the bilayers and significant and well-detectable accumulation of peroxidation products after an exposure to an initiator of free radical peroxidation.

Although, the use of liposomes to assay the antioxidative potency of antioxidants has several advantages, the biological significance of such assays is questionable, because the effects of an antioxidant on the peroxidation of liposomal lipids depend on the structure of the liposomes and may be different for biological membrane. Furthermore, the effect of an antioxidant is not always a monotonic function of its concentration.

In spite of these limitations, some issues can be addressed only in the relatively simple liposomal model systems. The following examples demonstrate the usefulness of model system studies in advancing our knowledge in a way that could not have been achieved in the much more complex biological systems.

(1) Using a liposomal system, Shi et al. (2004) demonstrated that mixtures of vitamin E and lycopene, present in tomatoes, exhibit a markedly synergistic antioxidative activity. Such synergism may also occur in the much more complex biological membranes in which we may not be able to observe it.

(2) Using liposomes, Woodall et al. (1997) investigated the relationship between the structure of different carotenoids and their ability to protect liposomal lipids against peroxidation induced either by AAPH or by AMVN. This analysis also demonstrated the importance of the position and orientation of different carotenoids in the bilayer. Similar results were also reported earlier by Lim et al. (1992). In relation to the significance of their findings, Woodall et al. (1997) speculated ''this information may be relevant in studies examining the role of carotenoids in the prevention of age-related macular degeneration''. This hypothesis is yet to be tested.

(3) Gabrielska et al. (2004) showed that quercetin protects liposomal lipids against oxidation induced by UV-irradiation and attributed this protection to complexation of residues of transition metals by quercetin. These authors also proposed that the high antioxidative activity of a mixture of quercetin and tocopherol is due to different locations of these antioxidants in the liposomal bilayers.

(4) The HMG-CoA reductase inhibitor fluvastatin protects liposomal lipids against peroxidation induced either by iron or by free radical generators, both water-soluble (AAPH) and lipid soluble (AMVN) (Yamamoto et al. 2001). By contrast, pravastatin is also a potent antioxidant but only against transition metal-induced peroxidation. The obvious conclusion is that in vivo fluvastatin acts as an effective antioxidant ''non-enzymatically'' (i.e. not via its effect on any enzyme). Such a conclusion could have been reached only in a simplified model system, such as liposomes.

(5) Using PLPC monolayers, Morandat et al. (2003) have shown that exposure of the monolayer to UV-irradiation resulted in a decrease of the molecular area and of the surface elasticity modulus of monolayer, and that inclusion of plasmalogens in the monolayer inhibited these effects. Similarly, hyaluronan and its derivatives protected liposomes made of skin lipids against UV-induced peroxidation (Trommer et al. 2003). Given the constant exposure of skin to UV irradiation, the authors proposed to use hyaluronan in sunscreens. Such information could not have been straightforwardly obtained in the much more complex biological systems.

(6) In an attempt to gain understanding of the mechanism(s) involved in the protective activities of curcumin and of ginger-derived phenolic 1,3-diketones against Fe^{2+}/ascorbate-induced peroxidation in model systems, Patro et al. (2002) evaluated the relative contribution of various structural features of lipid-soluble and water-soluble antioxidants to the protective potency of the studied antioxidants (including the number of phenolic hydroxyl groups and the hydrophobicity of the 1,3-diketons). Such information, again, could not have been gained in biological membrane.

(7) In our studies on the effects of tocopherol, urate, ascorbic acid and mixtures of these antioxidants, we observed that all these biologically relevant reducing agents act either as antioxidants or as prooxidants, depending on the composition of the system. The actual effects of each of these compounds depended on several factors, including the inducer of peroxidation and its concentration, the composition of the liposomes and the way of introduction of these antioxidants to the mixture of inducer and liposomes. For each of the studied antioxidants, we also studied the effect of the presence and concentrations of other antioxidants. These studies (Gal et al. 2003; Bittner et al. 2002; Schnitzer et al. 2007; Samocha et al., unpublished results) yielded the following information:

   (a) Vitamin E co-sonicated with lipids protects PLPC against copper-induced peroxidation, whereas vitamin E added in ethanol to preformed liposomes of similar compositions promoted peroxidation (Gal et al. 2003). Given the limited but significant solubility of vitamin E in aqueous media, we have attributed the observed prooxidative effect of vitamin E to tocopheryl radicals formed in the aqueous solution upon reduction of Cu^{2+} to Cu^{+}.

   (b) Increasing the concentration of copper (i.e. increasing the ''oxidative stress'') results in promotion of antioxidative potency of ascorbic acid against copper-induced oxidation of liposomal PLPC (Bittner et al. 2002).

   (c) Both urate and ascorbate are potent inhibitors of AAPH-induced peroxidation of PLPC liposomes. By contrast, both these antioxidants promote copper-induced peroxidation of PLPC liposomes but inhibit peroxidation when the liposomes contain either cholesterol (16 mol%) or tocopherol (Samocha et al., unpublished results; Schnitzer et al. 2007), probably via different mechanisms.

   (d) In negatively charged liposomes made of PLPC and PA, the effect of tocopherol was qualitatively similar to that observed in the absence of PA. By contrast, PS-containing (negatively charged) liposomes are protected by nanomolar concentrations of tocopherol. Similar extraordinary effects were observed for 12 out of 37 studied phenolic antioxidants (Gal et al.,

unpublished results). Based on analysis of the structure-activity relationship of these compounds, we propose that the mechanism responsible for the observed unusual protective effect involves replenishment of the ''extraordinary active'' antioxidant within a PS-copper-antioxidant ternary complex and that such process requires formation of an intermediate, relatively stable semiquinone-like radicals.

(8) In an attempt to gain understanding of the mechanism of copper-induced lipid peroxidation of non-fractionated serum lipids (on which we based our optimized assay of the susceptibility of serum lipids to peroxidation; Schnitzer et al. 1998), we have investigated the effect of serum albumin on copper-induced peroxidation of liposomal PLPC (Samocha et al. 2004). The results of this study, conducted with the relatively simple liposomal system, taught us that a 2:1 copper–albumin complex is the inducer of peroxidation in our assay. This complex is a known inducer of peroxidation (Marx and Chevion 1986), which is less potent than ''free'' copper but much more potent than either a 1:1 copper–albumin complex or a copper–citrate complex. Hence, in systems containing free copper, the commonly recognized antioxidative effect of albumin, attributed to its capacity to bind transition metal ions, was also observed in the liposomal system, but at higher copper concentrations, the redox-active 2:1 copper/albumin complex is formed. In our optimized assay (Schnitzer et al. 1998), the concentration of ''free'' copper is negligible because the serum contains an excess of citrate. Citrate binds copper with a lower binding constant than albumin, but the concentration of citrate used in our assay is sufficiently high to reduce the concentration of ''free'' copper to levels that are insufficient to induce peroxidation of serum lipids. Under these conditions, oxidation is induced by the copper–albumin 2:1 complex.

In conclusion, evaluation of antioxidative capability of various antioxidants and of the dependence of antioxidative potency on various factors yielded much knowledge and some understanding of the mechanisms responsible for these effects. As discussed above, peroxidation in liposomal systems is not very simple, but biological systems are much more complex. The intriguing issue, which is far from being clear is, whether peroxidation in model-membrane studies relevant to peroxidation in biological systems. Essentially, all the relevant publications dealt with this issue, some in greater detail than the others. In their study of the dependence of antioxidative effect of tocopherol on liposome composition, Jacob and Mason (2005) noted that the effect of tocopherol depends on its antioxidant properties rather than on alteration of the lipid bilayer. This view accords with the previous investigations of Morandat et al. (2003) and Wiseman et al. (1990). These (and several other) studies indicated that a clear correlation exists between the potency of various antioxidants to inhibit lipid oxidation in liposomes and their protective effects against oxidation in biological systems.

However, it is important to note that the protective effects of different antioxidants against peroxidation of different oxidizable lipids that reside in liposomes of different compositions and induced by different inducers may differ considerably. One should therefore be careful in using the data obtained for liposomal systems to seek understanding of the mode of action of these antioxidants in biological systems.

Effect of the peroxidation of membrane lipids on the biophysical properties of the membrane

An important issue in membrane biophysics is the possible causal relationship between chemical and biophysical consequences of lipid peroxidation. Specifically, peroxidation of polyunsaturated fatty acid residues of membrane phospholipids results in changes of the membrane composition, which also alter the physical properties of the membrane. Such changes depend on the products of peroxidation. The formation of conjugated dienic hydroperoxides at the first step of peroxidation, introduces a relatively polar hydroperoxyl group into the hydrophobic milieu of the bilayers (Buettner 1993). This, in turn, may alter the localization of the lipids in the bilayer. Moreover, subsequent degradation of the hydroperoxides results in cleavage of the hydrocarbon chains. The resultant fatty acid fragments may partition between the bilayer and the aqueous solution. Thus, the consequences of lipid peroxidation may depend on the chemical character of the oxidizable lipids as well as on the inducer of peroxidation and other experimental details.

In biological membranes, the peroxidation of lipids is usually accompanied by oxidation of membrane proteins (Yukawa et al. 1983; Mattson 1998; Jacob and Mason 2005). Furthermore, peroxidized lipids may also alter membrane proteins (e.g. by forming Schiff bases with aldehydes or/and by activating membrane-bound enzymes). In addition, lipid peroxidation products may alter the arrangement of the proteins in the bilayers and by that interfere with the physiological role of the proteins (Yukawa et al. 1983; Dinis et al. 1993; Goel et al. 1993; Mattson 1998; Sevanian and Ursini 2000). All these changes affect the function of the membrane in a very complex fashion. The relatively simple liposomal model membranes are still

quite complex, but, unlike biological membranes, they enable evaluation of the effects of varying the lipid composition on the consequences of lipid peroxidation.

The specific effects of peroxidation include changes of the packing of the membrane lipids, which govern all measurable physical properties of the membrane, including the width and/or the molecular volume of the bilayer, as well as the bilayer microviscosity, fluidity and rate of lateral diffusion. Furthermore, the products of peroxidation may alter the lateral homogeneity (domain formation) within the bilayers and the membrane's permeability to water and to water-soluble low molecular weight compounds (i.e. the ''leakiness'' of the membrane). All these possible consequences of peroxidation have been investigated in liposomes of varying compositions, oxidized by various inducers.

Many studies have been conducted with the aim of gaining understanding of kinetic, mechanistic and structural effects of peroxidation of liposomal lipids on the physical properties of the liposomes. Yet, the results published thus far are insufficient to reach generalizations because they are not always consistent with each other. Moreover, in many cases, the results of different studies are apparently contradictory. As an example, several investigators reported that peroxidation results in ''fluidization'' of the bilayers whereas, others reported that peroxidation results in bilayer rigidization (Marathe and Mishra 2002; Mishra 2002; De Guidi et al. 2005).

In spite of these inconsistencies, the examples described below may be used to form a basis for working hypotheses that may hopefully help gaining understanding of these issues not only in model membranes but in biological membranes as well:

(1) In an attempt to elucidate the effect of lipid peroxidation on the organization of phospholipids and cholesterol in biological membranes, Jacob and Mason (2005) exposed liposomes made of DLPC and cholesterol to conditions of spontaneous peroxidation (''under physiological conditions'') and compared the organization of lipids within the liposomal bilayer, before and after peroxidation, using small angle X-ray scattering (SAXS). The major finding of this study was that moderate levels of hydroperoxides cause a concentration-dependent formation of discrete immiscible cholesterol-rich domains. In non-oxidized liposomes, such isothermal lateral phase separation occurs only when the cholesterol content of the bilayer exceeds 50 mol%, whereas after moderate levels of hydroperoxides are formed upon oxidation, such a phase separation occurs at merely 28 mol% cholesterol. This phase separation correlated directly with the level of hydroperoxides. Interestingly, the cholesterol-poor domains formed after partial peroxidation, were 1 nm thinner than the cholesterol-rich domains. Relating to these results, the authors speculate that the peroxidation-induced alteration of biological membranes is similar to that observed in their model membrane. Although this speculation is reasonable, it still has to be verified experimentally.

(2) In his review on irradiation-induced oxidative damages to membranes, Mishra (2002) refers to the previous studies of his group regarding the damage caused to egg-PC liposomes upon gamma irradiation. Using fluorescence and ESR probes, this author studied the effects of irradiation on the fluidity and permeability of liposomal membranes. The most intriguing result of this study was that irradiation by low doses (up to 10 Gy) caused ''fluidization'' of the liposomal bilayers, whereas irradiation at higher doses caused rigidization of the bilayers. Notably, the permeability of the liposomal membranes to entrapped solutes increased monotonically upon increasing the dose of irradiation. As expected, the ''permeabilization'' correlated with the radiation-induced generation of ROS, indicating that the observed changes in permeability indeed result from peroxidation of the liposomal lipids. Another important observation of this study was that inclusion of cholesterol in the membranes protects them against irradiation-induced permeabilization. In this respect, cholesterol has protective effects qualitatively similar to those of tocopherol and ascorbate (probably via different mechanisms).

(3) Using SAXS, Mason et al. (1997) studied the consequences of Fe^{2+}/ascorbate-induced peroxidation of liposomal DLPC. The main results of these studies were that peroxidation resulted in a marked reduction of the width of the hydrocarbon core, a similar decrease of the overall width of the membrane, and a decrease of the molecular volume of the bilayers, accompanied by interdigitation of the terminal methyl segments of the fatty acid chains. Similar effects were observed in liposomes made of the lipids extracted from cardiac membranes. Based on the latter similarity, the authors proposed that the observed peroxidation-induced perturbations of the membrane structure (and function) may be ''associated with aging and cardiovascular diseases''. This proposal is yet to be experimentally tested.

(4) Using cholesterol-containing liposomes, several studies have shown that peroxidation promotes lateral phase separation in cholesterol-phospholipid liposomes, as described above. Furthermore, in a recent investigation, Ayuyan and Cohen (2005) found that

lipid peroxidation promotes the production of large ''rafts'' in giant unilamellar liposomes made of sphingomyeline, cholesterol and unsaturated phospholipids. Such an effect may contribute to oxidative stress-related disorders. Yet, the existing data is insufficient to validate this speculation.

In relating to the effects of peroxidation on the membrane properties, it is important to note that alteration of the membrane properties affects the peroxidation. Hence, peroxidation-induced changes in the composition (and physical properties) of the bilayers are likely to affect the propagation of peroxidation. As an example, on the basis of their studies on the peroxidation of liposomes of different phospholipid compositions, De Guidi et al. (2005) concluded that the sensitivity of oxidizable lipids to phototoxic damage is an increasing function of membrane fluidity. In turn, Udilova et al. (2003) have shown that incorporation of hydroperoxides into lipid bilayers decreases the membrane fluidity and promotes Fe^{2+}/ascorbate-induced peroxidation. Whether or not there is a casual relationship between the oxidation-induced fluidization and the rate of continuing peroxidation, as opposed to being merely due to the product-induced autoacceleration discussed above, remains to be discovered.

Understanding of the peroxidation-induced damages to liposomal membranes is important not just because it is an interesting basic issue. Studies addressing this issue also raise reasonable biologically relevant possibilities. In fact, the studies described above on the consequences of peroxidation of liposomal lipids on the properties of the liposomal membranes addressed the relevance of their results, obtained in model-membrane studies, to biological membranes. For example, in their discussion of the peroxidation-induced reduction of both the width and the molecular volume of the hydrocarbon core of liposomal bilayers, Mason et al. (1997) proposed that similar perturbations in the structure of biological membranes might affect ''the overall structure of biological membranes and the function of the membrane proteins''. This, they claim, may ''contribute to aging and cardiovascular disease'' (Mason et al. 1997). Similar results were reported and similar speculations were made in a more recent investigation of this group (Jacob and Mason 2005). Specifically, in the latter investigation, the authors claim that their observations on the formation of peroxidation-induced, cholesterol-rich domains ''may contribute to changes in membrane function during aging and oxidative stress-related disorders''. Peroxidation-induced permeabilization of liposomal bilayers was also claimed to ''trigger processes leading to apoptotic cell death after radiation exposure'' (Mishra 2002).

Although reasonable, all these speculations require further investigations. If proven to be relevant, the dependence of peroxidation in liposomal membranes on their compositions and on the details of the induction of peroxidation can be very valuable for gaining mechanistic understanding on how the peroxidation occurs in biological membranes. This will hopefully help designing ways to reduce oxidative damage to biological membranes.

Acknowledgments We thank the Israel Science Foundation (research grant 362/02) and the Lady Davis Chair of Biochemistry for financial support.

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