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The influence of antioxidants in the thiyl radical induced lipid peroxidation and geometrical isomerization in micelles of linoleic acid

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ABSTRACT

The biomimetic model of micelles of linoleic acid containing 2-mercaptoethanol and the antioxidant was examined under gamma irradiation up to 400 Gy in aerobic or deoxygenated conditions where thiyl radicals are the main reactive species. Lipid peroxidation was retarded by ascorbic acid and α-tocopherol, whereas this process was strongly inhibited by resveratrol as effectively as the ascorbic acid/α-tocopherol mixture. Furthermore, antioxidants have a much stronger inhibitory effect on the peroxidation in the presence of 2-mercaptoethanol, and at the same time show protective properties of the double bond, decreasing the *cis–trans* isomerization. Under anaerobic conditions, *cis–trans* isomerization occurred and antioxidants efficiency increased along the series: resveratrol < α-tocopherol < ascorbic acid. This result is explained taking into account the double bond localization in the hydrophobic core of the micelle and the need of co-localization of the antioxidant in order to get an anti-isomerizing activity and protection of the natural lipid geometry.

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Free radicals; gamma-irradiation; geometrical isomerization; lipids; peroxidation; antioxidants

Introduction

Biomimetic lipid models are useful to explore lipid reaction outcomes and products in a simplified context that closely mimics the biological environment in living organism, thus allowing mechanistic basis of molecular processes to be assayed. There are two main classes of free radical reactions involving polyunsaturated fatty acids (PUFA). One of them is lipid peroxidation [1–3] and the second one is the process of geometrical isomerization of naturally occurring *cis* unsaturated lipids which can be catalyzed by thiyl radicals ($RS^{\bullet}$) [4–6]. It has been shown that one process can be parallel with the occurrence of the other and both lipid hydroperoxides and *trans* lipids are the resulting products of oxidative free radical conditions [7] (Scheme 1). Investigations of peroxidation and isomerization processes in liposome models were shown to occur by chemical agents, such as the antitumor drug bleomycin, known to form metal complexes, thus producing oxidative stress conditions [8]. Both processes induce profound changes of lipid structures, especially when phospholipids, present in organized compartments such as cell membranes, are involved. Indeed, the loss of PUFA from membrane phospholipids due to the oxidation processes is known to affect bilayer asset and its fluidity, as well as the release of lipid mediators from membranes and signaling cascades [9,10]. On the other hand, the transformation of the natural *cis* geometry into *trans* is a subtle change that influences membrane organization and biochemical processes, with harmful effects on health [11].

Preventive or repairing strategies for the lipid integrity and good model systems for testing their efficiency are therefore necessary. In recent studies of unilamellar liposomes, several aspects of the lipid isomerization of monounsaturated fatty acids (MUFA) in organized systems and the effectiveness of known antioxidants was were shown [12,13]. In linoleic acid (LH) micelles, the effects of aromatic amines and phenols to the mechanisms of Scheme 1 have been also recently addressed [14].

Here, we wish to present the micelle model made of LH (9*cis*,12*cis*-octadecadienoic acid, 9c,12c-18:2) as representative PUFA in membranes, to elucidate the

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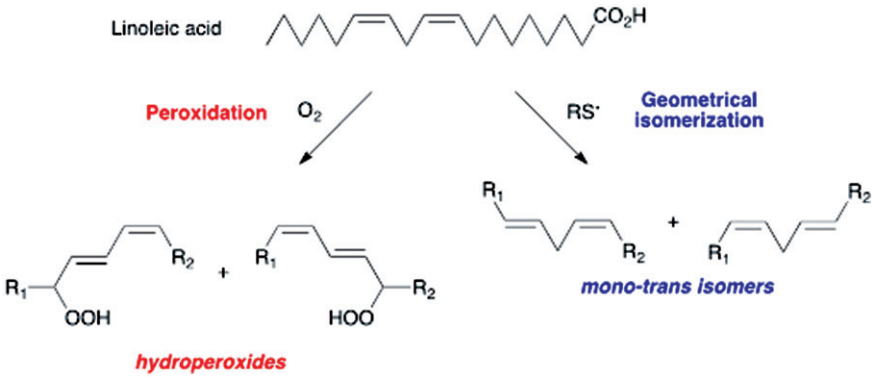


Scheme 1. Radical-based peroxidation and geometrical isomerization processes of linoleic acid (9*cis*,12*cis*-octadecadienoic acid, 9c,12c-18:2).

influence of different naturally occurring antioxidants on both peroxidation and geometrical isomerization processes. Interesting insights of the lipid protection emerged in aerobic and anaerobic conditions, highlighting the role of molecular diversity of antioxidants in "oil in water" heterogeneous systems.

Materials and methods

Materials

LH (9*cis*,12*cis*-octadecadienoic acid, 9c,12c-18:2, >99% pure), non-ionic surfactant polyoxyethylene sorbitan monolaureate (Tween®-20), 2-mercaptoethanol (RSH), and resveratrol (ResOH) (>99%, pure) were purchased from Sigma-Aldrich. DL-α-Tocopherol (α-TOH) (97%, pure) and L−(+) ascorbic acid (AscH) were purchased from "Alfa Aesar". Sodium dihydrogen phosphate (≥98%) obtained from Carlo Erba, ferrous sulfate ($FeSO_4 \times 7\,H_2O$), and potassium thiocyanate (KSCN) from Merck. These products were used as received and all other used chemicals were of analytical reagent grade purity. Water was triply distilled, first from potassium permanganate and sodium dichromate, then without additives. On the basis of UV absorption, all solvents used were of analytical grade purity.

LH micelles

Model system containing mixed surfactant micelles and buffer was prepared by slow solubilization of LH in non-ionic surfactant micelles previously formed by mixing Tween®-20 and NaH_2PO_4, pH 6.5. The composition of investigated model systems was typically 5.0×10^{-4} M LH, 2.8×10^{-4} M Tween®-20, and 5.0×10^{-3} M NaH_2PO_4 (pH ~5). Different antioxidants of define concentrations were added during preparation of model systems. The addition of the amphiphilic thiol RSH (2.8×10^{-3} M) is added to previously prepared micelles just before irradiation.

Continuous irradiation and product studies

Gamma radiolysis was performed at room temperature using panoramic ^{60}Co source at different doses and dose rates. LH micelles were irradiated in air-equilibrium or after saturation with N_2O. After irradiation, LH components were extracted with a solvent mixture of CH_2Cl_2/MeOH (2:1, v/v) de-aerated by nitrogen, and an aliquot of the sample was taken out from the lower layer for the quantitative determination of hydroperoxide of LH (LOOH) by spectrophotometric method. The concentration of LOOH was determined by the spectrophotometric ferric thiocyanate method following the procedure described by us earlier, using UV/VIS spectrophotometer Varian Cary 4000 [15]. The rest of the lipid extract was used for GC analysis of geometrical isomers using known conditions for the separation of *cis* and *trans* isomers [7,16]. In order to transform LH and its geometrical isomers in the corresponding methyl ester, the reaction solutions were treated with an ethereal solution of diazomethane [17]. We used Varian 450 gas chromatograph equipped with a flame ionization detector and an Rtx-2330 (90% biscyanopropyl/10% phenylcyanopropyl polysiloxane capillary column; 105 m × 0.25 mm). Temperature started from 180 °C held for 35 min, followed by increase in 10 °C/min up to 250 °C and held for 5 min. Methyl esters were identified by comparison with the retention times of authentic samples, which were commercially available.

Results and discussion

Formation of LOOH in irradiated LH micelles under aerobic conditions

LH micelles were irradiated by 50, 100, 200, 300, and 400 Gy doses under air-equilibration. After irradiation, lipid components were extracted with CH_2Cl_2–MeOH (2/1, v/v) and analyzed spectrophotometrically for lipid hydroperoxide (LOOH) determination. Figure 1 shows

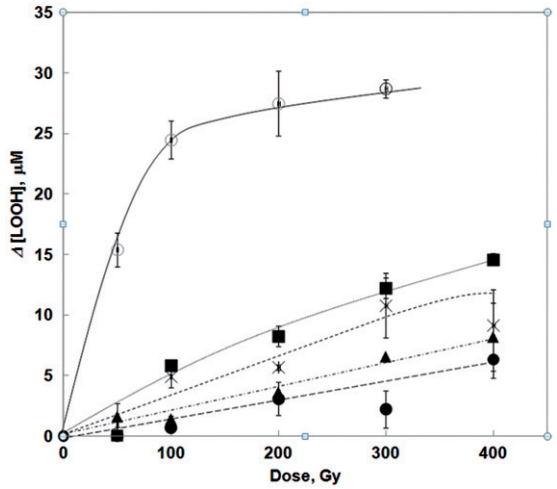


Figure 1. The formation of LOOH in LH micelles as a function of irradiation dose (dose rate 274.8 Gy/min) under air-equilibration: (○) LH micelles without antioxidants; (*) LH micelles in the presence of 60 μM AscH; (▲) LH micelles in the presence of 80 μM ResOH; (■) LH micelles in the presence of 50 μM α-TOH; (●) LH micelles in the presence of mixture of 50 μM α-TOH and 60 μM AscH; LH micelles formed by 5.0×10^{-4} M LH, 2.8×10^{-4} M Tween®-20, 5.0×10^{-3} M NaH_2PO_4, and 2.8×10^{-3} M RSH at pH 5. Reported values represent the mean of three independent measurements.

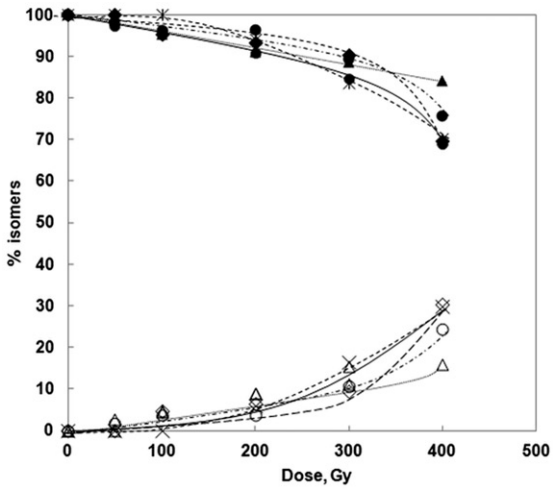


Figure 2. The geometrical isomer distribution of linoleic acid methyl ester (symbols (▲, ●, *, ◆) represent 9*cis*,12*cis*-18:2 and symbols (◊, ×, ○, Δ) represent the sum of *trans* isomers) in the presence of natural occurring antioxidants under air-equilibration (dose rate: 274.8 Gy/min): (*, ×) LH micelles in the presence of 60 μM AscH; (●, ○) LH micelles in the presence of 80 μM ResOH; (▲,Δ) LH micelles in the presence of 50 μM α-TOH; (◆, ◊) LH micelles in the presence of mixture of 50 μM α-TOH and 60 μM AscH; LH micelles formed by 5.0×10^{-4} M LH, 2.8×10^{-4} M Tween®-20, 5.0×10^{-3} M NaH_2PO_4, and 2.8×10^{-3} M RSH at pH 5. Reported values represent the mean of three independent measurements.

the dose profiles of LOOH formation in absence or presence of various antioxidants. Vitamin E (α-TOH) is the major lipophilic, radical-scavenging antioxidant *in vivo* [18]. Using concentration of 50 μM in this work, its efficiency was confirmed. Another phenolic antioxidant like resveratrol (ResOH) showed an analogous behavior, for example using 80 μM of ResOH the formation of LOOH is about half of that one using 50 μM α-TOH [19]. The effect of 60 μM AscH to limit LOOH formation was also remarkable in our model (see below the section on AscH). In the presence of α-TOH/AscH mixture, oxidation of LH was inhibited better than with α-TOH or AscH alone. The synergistic or additive action of α-TOH and AscH to inhibit propagation of lipid peroxidation is known also in liposomes [18,20] and in biological systems, essentially connected either to a recycling of tocopherol radical by ascorbate anion or to the typical distribution between lipophilic and hydrophilic compartments of the two antioxidants [18,20]. Such effect was confirmed in this model of LH micelles where the two antioxidants can act by chemical recycling and by trapping of radicals distributed in the system.

Geometrical isomerization in irradiated LH micelles under aerobic conditions

The same samples described above for the measurements of LOOH were used for the follow-up of the geometrical isomerization of LH. Lipid components were extracted as methyl esters after treatment of the reaction mixture for esterification, as previously described [7]. GC analysis is the gold standard for the geometrical isomer identification and quantification. Figure 2 shows the dose profiles of LH disappearance and *trans* isomers appearance in the presence of antioxidants, that is, AscH (60 μM), α-TOH (50 μM), and ResOH (80 μM) as well as mixture of 50 μM α-TOH and 60 μM AscH. In all cases, the *trans* isomers increased with dose, indicating that the *cis–trans* isomerization parallels the peroxidation process even in the presence of various antioxidants.

Geometrical isomerization in irradiated LH micelles under anaerobic conditions

LH micelles were saturated by N_2O prior to irradiation (anaerobic conditions). The samples were irradiated by 50, 100, 200, 300, and 400 Gy doses and treated as previously explained to obtain the isomerization profiles. The disappearance of LH is replaced by its *trans* isomers as shown in Figure 3 in function of the dose and in absence or presence of various antioxidants, that is, AscH (60 μM), α-TOH (50 μM), and ResOH (80 μM). AscH

in the presence of RSH is the most effective inhibitor of radiation-induced *trans*-isomer formation. This result confirms the efficiency of this hydrophilic antioxidant to counteract lipid isomerization, as already known for MUFA in liposome models [12,13]. Slightly weaker effect was observed by the addition of an AscH/α-TOH mixture, while in the case of ResOH inhibition of *trans*-isomer formation did not significantly differ from that process in the control micelles.

Thiyl radical generation and cis–trans isomerization mechanism

Radiolysis of neutral water leads to reactive species, including solvated electrons (e^-_{aq}), hydrogen atoms ($H^\bullet$), and hydroxyl radicals ($HO^\bullet$) as shown in Reaction 1. The values in parentheses represent the radiation chemical yields (G) in units of μmol J^{-1} [21]. In a N_2O-saturated solution (~0.02 M of N_2O), e^-_{aq} are transformed into $HO^\bullet$ (Reaction 2, $k_2 = 9.1 \times 10^9\ M^{-1}\ s^{-1}$), affording a $G(HO^\bullet) = 0.55$ μmol J^{-1}, that is, $HO^\bullet$ radicals and $H^\bullet$ atoms account for 90% and 10%, respectively, of the reactive species. In the presence of thiols such as the amphiphilic 2-mercaptoethanol (RSH), hydrogen abstraction by $HO^\bullet$, and $H^\bullet$ directly produce thiyl radicals (Reaction 3, $k_3 = 6.8 \times 10^9\ M^{-1}\ s^{-1}$ for $HO^\bullet$ and $k_3 = 1.7 \times 10^9\ M^{-1}\ s^{-1}$ for $H^\bullet$) [21].

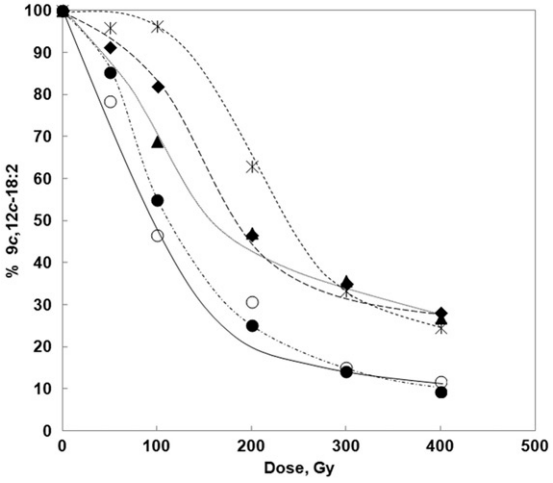


Figure 3. The disappearance of LH (9c,12c-18:2 isomer) versus dose in the presence of different natural occurring antioxidants under anaerobic conditions (dose rate: 274.8 Gy/min): (○) LH micelles without antioxidants; (*) LH micelles in the presence of 60 μM AscH; (●) LH micelles in the presence of 80 μM ResOH; (▲) LH micelles in the presence of 50 μM α-TOH; (◆) LH micelles in the presence of mixture of 50 μM α-TOH and 60 μM AscH; LH micelles formed by 5.0×10^{-4} M LH, 2.8×10^{-4} M Tween®-20, 5.0×10^{-3} M NaH_2PO_4, and 2.8×10^{-3} M RSH at pH 5.

$$H_2O \quad e^-_{aq}(0.27),\ HO^\bullet(0.28), H^\bullet(0.06) \qquad (1)$$

$$e^-_{aq} + N_2O + H_2O \rightarrow HO^\bullet + N_2 + HO^- \qquad (2)$$

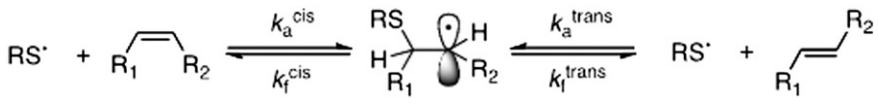
$$HO^\bullet/H^\bullet + RSH \rightarrow RS^\bullet + H_2O/H_2 \qquad (3)$$

Scheme 2 shows the reaction mechanism of *cis–trans* isomerization that consists of $RS^\bullet$ radical reversible addition to the double bond. Indeed, the reconstitution of the double bond is obtained by β-elimination of $RS^\bullet$ and the result is in favor of *trans* geometry, the most thermodynamically favorable disposition. The rate constants for $RS^\bullet$ addition to the *cis* and *trans* isomers (oleic acid and elaidic acid, respectively) were found to be rather similar ($k_a^{cis} = 1.6 \times 10^5\ M^{-1}\ s^{-1}$ and $k_a^{trans} = 2.9 \times 10^5\ M^{-1}\ s^{-1}$), whereas the thiyl radical elimination from the intermediate adduct radicals were substantially different ($k_f^{cis} = 1.7 \times 10^7\ s^{-1}$ and $k_f^{trans} = 1.6 \times 10^8\ s^{-1}$) [22,23]. It is worth noting that the $RS^\bullet$ radical acts as a catalyst for *cis–trans* isomerization. Considering LH, the isomerization mechanism occurs as a step-by-step process, that is, each isolated double bond behaves independently [4,16].

The disappearance of the starting material (mol kg^{-1}) divided by the absorbed dose (1 Gy = 1 J kg^{-1}) gives the radiation chemical yield or G[-(9c,12c-18:2)]. By treating the data of Figure 3 in this way and by plotting the G[-(9c,12c-18:2)] versus dose (see Figure S1 in Supporting Information), we obtained further information on the catalytic process. For example, the extrapolation to zero dose for LH micelles without antioxidants gives $G = 192$ μmol J^{-1}. Assuming that the $G(RS^\bullet)$ is 0.52 μmol J^{-1} (ca. 85% of the initial radical species), we calculated the catalytic cycle to be 370 at the initial phase. For the analogous isomerization of LH micelles in the presence of AscH, a catalytic cycle of 96 was calculated, whereas the effect of ResOH was negligible since the catalytic cycle is close to the one without antioxidants (Figure S1).

The effect of ascorbic acid or resveratrol concentration on the LOOH formation and geometrical isomers distribution

Next, we considered the role of AscH in our model system in some details at fixed irradiation dose of 100 Gy.



Scheme 2. Reaction mechanism for the *cis–trans* isomerization catalyzed by thiyl radicals.

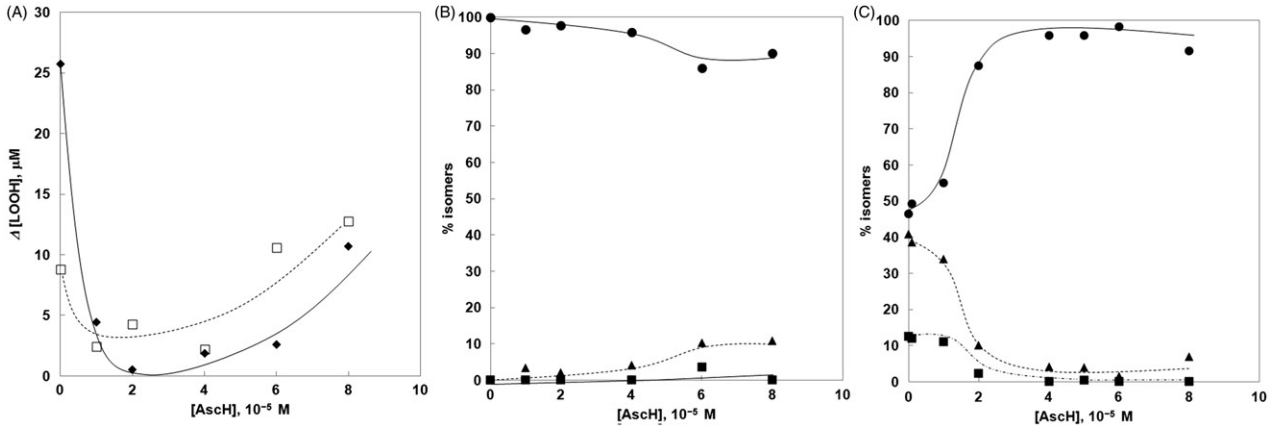


Figure 4. (A) Effect of the AscH concentrations on LOOH formation after 100 Gy of irradiation (dose rate: 274.8 Gy/min) under air-equilibration: (□) LH micelles and AscH (0–8) $\times 10^{-5}$ M; (◆) LH micelles, AscH (0–8) $\times 10^{-5}$ M and RSH 2.8 mM; (B) Effect of the AscH concentrations on the distribution of linoleic acid methyl ester geometrical isomers after 100 Gy of irradiation of LH micelles, AscH (0–8) $\times 10^{-5}$ M and RSH 2.8 mM under air-equilibration: (●) 9c,12c-18:2, (▲) 9t,12c-18:2 and 9c,12t-18:2, (■) 9t,12t-18:2; (C) like in B under anaerobic conditions; LH micelles formed by 5.0 $\times 10^{-4}$ M LH, 2.8 $\times 10^{-4}$ M Tween®-20, 5.0 $\times 10^{-3}$ M NaH_2PO_4, and 2.8 $\times 10^{-3}$ M RSH at pH 5. Reported values represent the mean of three independent measurements (errors ±5%).

In particular, Figure 4(A) shows the increase in AscH concentration range from zero to 8×10^{-5} M in the absence (□) or presence (◆) of 2.8 mM RSH under air-equilibration. At zero concentration of AscH, the LOOH yield is higher with thiol than in the absence of thiol, indicating that thiyl radicals activate peroxidation. By increasing the concentration of AscH to 2×10^{-5} M, AscH alone has a small inhibiting effect, whereas in the presence of both AscH and thiol complete inhibition of peroxidation occurs, indicating an effective synergism between AscH and RSH. With further increase in AscH concentration, a pro-oxidative effect was observed although the synergic effect of RSH remains. Figure 4(B) shows the geometrical isomers distribution for the same experiments when the thiol is present. Therefore, the *cis–trans* isomerization process operates in parallel with lipid peroxidation. Figure 4(C) shows the analogous experiments under anaerobic conditions, where the process of lipid peroxidation is suppressed. At zero concentration of AscH, 50% of LH is transformed in mono-*trans* (40%) and di-*trans* (10%) isomers. By increasing the concentration of AscH, the isomerization process with *trans*-isomer formation decreased and at 4×10^{-5} M is almost completely inhibited.

It is worth mentioning that rate constants for the reactions of $RS^{\bullet}$ (from cysteamine, cysteine, and glutathione) and ascorbate were reported to be $\sim 10^9$ M^{-1} s^{-1} by pulse radiolysis determination [24]. It is also known that the $RS^{\bullet}$ radicals are moderate oxidants (e.g. $E(HOCH_2CH_2S^{\bullet}, H^+/HOCH_2CH_2SH) = 1.3$ V) and oxidize ascorbate to ascorbyl radicals which terminate due to disproportionation ($k = 10^6$ M^{-1} s^{-1}). Based on these results, it can be assumed that in the model system of LH, a rapid reaction between AscH and $RS^{\bullet}$ radicals in the hydrophilic part of the micelles takes place so that quenching of the majority of $RS^{\bullet}$ occurs, preventing the sulfur-center radical migration in the hydrophobic part of micelles to reach the double bond, thereby inhibiting the isomerization process.

Table 1. The influence of AscH and ResOH on the formation of geometrical isomers of LH micelles after 100 Gy of irradiation at 274.8 Gy/min under anaerobic conditions[a].

Sample	% 9c,12c	% 9c,12t + 9t,12c	% 9t,12t
LH	38.9	43.5	17.5
LH +100 μM ResOH	47.8	39.9	12.3
LH +60 μM AscH	98.4	1.6	0
LH +100 μM ResOH +60 μM AscH	88.2	10.9	1.7

[a]LH micelles formed by 5.0 $\times 10^{-4}$ M LH, 2.8 $\times 10^{-4}$ M Tween®-20, 5.0 $\times 10^{-3}$ M NaH_2PO_4, and 2.8 $\times 10^{-3}$ M 2-mercaptoethanol at pH 5. Reported values represent the mean of three independent measurements.

Similar experiments with ResOH are performed and reported in Figure S2 (Supporting Information). The results of the geometrical LH isomer distribution in LH micelles in the presence of RSH obtained in anaerobic conditions at irradiation dose of 100 Gy are summarized in Table 1. It is evident that ResOH inhibits isomerization, but it is not so effective as compared with AscH. Using the mixture of the two antioxidants, the isomerization process shows intermediate reactivity.

Conclusion

The "oil in water" system of micelles was used as biomimetic model of the lipid isomerization and peroxidation reactions occurring under irradiation conditions

and inhibited by natural compounds known as antioxidants. Efficiency of the inhibition was correlated to the compartment where radical initiation occurs and to the double bond location in organized systems, that is in the lipophilic interior.

Under air-equilibrated conditions, the addition of different natural occurring antioxidants retarded the process of lipid peroxidation along the series: α-TOH < AscH < ResOH < AscH/α-TOH mixture. At the same time and conditions, isomerization was observed at low radical concentration in the presence of different antioxidants as compared to control LH system. Under anaerobic conditions, isomerization is a process more effective than in aerobic conditions. The presence of antioxidants in LH model systems decreased *trans*-isomer level after gamma irradiation at low dose (up to 200 Gy) along the series: ResOH < α-TOH < AscH/α-TOH mixture < AscH.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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