Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 3541

www.rsc.org/obc

PAPER

Linoleic acid peroxidation *vs.* isomerization: a biomimetic model of free radical reactivity in the presence of thiols†‡

Branka Mihaljević,*[a] Ivana Tartaro,[a] Carla Ferreri*[b] and Chryssostomos Chatgilialoglu*[b]

Received 16th January 2011, Accepted 7th March 2011
DOI: 10.1039/c1ob05083d

Biomimetic models of free radical-induced transformation of polyunsaturated fatty acids, such as micelles and liposomes, have been used for the study of lipid peroxidation and lipid isomerization. Free radical reactivity of thiol compounds is the common link between the two processes, since lipid peroxidation is inhibited by thiols, due to their H-donation ability, whereas lipid isomerization is catalysed by S-centered radicals. In this paper the two processes are compared for the first time, in solution and under biomimetic conditions, demonstrating that hydroperoxides and *trans* lipids are formed to comparable extents as a result of oxidative free radical conditions. The biomimetic model of micelles of linoleic acid, prepared by addition of a non-ionic surfactant (TWEEN®-20) and 2-mercaptoethanol as the amphiphilic thiol, was irradiated by ionizing radiation up to 400 Gy under various conditions. In air-equilibrated solutions, the *cis–trans* isomerization process was observed with a catalytic cycle of 370 together with a substantial amount of hydroperoxides (LOOH). The effect of micelle size was also studied in order to envisage the effect of the supramolecular organization on the outcome of the two processes, and in particular, for the positional preference of the double bond isomerization.

1. Introduction

Lipids, like other biomolecules such as proteins and DNA, are targets of various reactive oxygen, nitrogen and sulfur species, nominated ROS, RNS and RSS, respectively.[1] In this context, the reactivity of polyunsaturated fatty acids (PUFA) toward free radicals is well known and there are two main classes of reactions. The far most known process regarding PUFA is the peroxidation process with implications that span from chemistry to biology and medicine.[2] Much recent study has evidenced the process of geometrical isomerization of unsaturated lipids catalysed by free radicals, in particular S-centered radicals,[3,4] highlighting the biological significance of the change of the *cis* double bond configuration, which is the structural characteristic of eukaryotic membrane fatty acids.

In Scheme 1 the first stages of the two processes are shown for linoleic acid. It is well documented that the first products formed in the radical-based oxidation are diene hydroperoxides having the conjugated *trans*,*cis* double bond geometry.[5–7] The initial step of peroxidation is the hydrogen abstraction from the bisallylic position by a variety of radicals followed by addition to oxygen. On the other hand, the first step of thiyl radical induced *cis–trans* isomerization produces geometrical mono-*trans* isomers.[3,8,9] In fact, the initial step is the addition of thiyl radical (as an example, only the attack at the 9-position is shown) followed by ejection of the same radical, which is an effective process even in the presence of oxygen. Interestingly, the two processes can superimpose by the known activity of thiols, which are repairing agents in case of lipid peroxidation, due to the H-donation towards peroxyl radicals.[10] In doing so, thiols are precursors of thiyl radicals that can act, in their turn, as isomerizing agents for the fatty acid double bonds. So far the two processes have not been studied in the same context, although the double-sword character of thiols towards biomolecules has been increasingly addressed in the last decade.[11] Kinetic investigations of the lipid isomerization process suggested that thiyl radicals can be very effective isomerizing agents also in the presence of oxygen due to their catalytic activity,[12] however no data are available to estimate the extent of fatty acid peroxidation in parallel with the isomerization in solution. Moreover, a biomimetic model of the occurrence of both processes is lacking, although this is an important piece of information at a molecular level since *trans* geometrical lipid isomers have been already reported as markers of free radical stress in biological samples.[13]

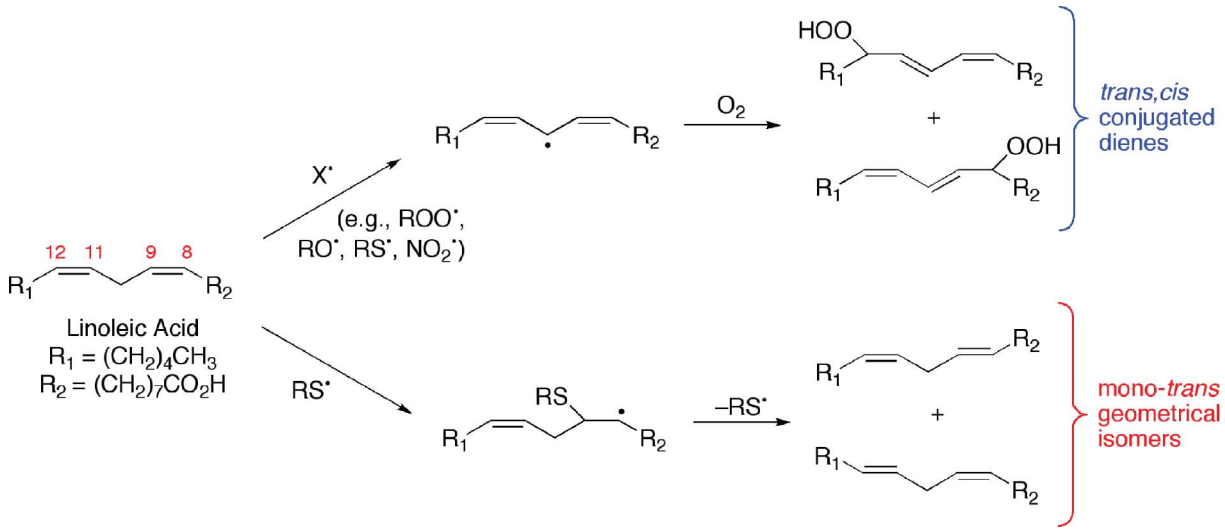
Biomimetic models allow this reactivity to be studied in a context closer to the biological environment and, in the case of lipids, micelles or liposomes are widely used for mimicking

[a]*Radiation Chemistry and Dosimetry Laboratory, Division of Materials Chemistry, Ruđer Bošković Institute, Bijenička 54, Zagreb, Croatia. E-mail: mihozeg@irb.hr*

[b]*ISOF, Consiglio Nazionale delle Ricerche, Via P. Gobetti 101, 40129 Bologna, Italy. E-mail: cferreri@isof.cnr.it, chrys@isof.cnr.it; Fax: +39 051 639 8349*

† Dedicated to the memory of Athel Beckwith, a pioneer in radical chemistry.

‡ Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/c1ob05083d



Scheme 1 Mechanisms for the radical-based peroxidation and isomerization processes of linoleic acid.

their chemical behavior.[14,15] Free fatty acids reaching their critical concentration can organize themselves in micelles, simply exposing the polar heads to water and keeping the hydrophobic tails in the core.[16] The addition of surfactants, such as the non-ionic surfactant TWEEN®-20, to linoleic acid micelles affords an oil-in-water emulsion widely studied for susceptibilities to oxidation,[16] for the activity of the surfactant itself or for the partitioning of antioxidants added in the medium.[17,18] It is also worth adding that the information gathered on thiol compounds in a micelle system is also interesting *per se*, since there are a few examples of thiol reactivity when added to micellar systems, *i.e.*, in the case of nucleophilic esterolysis mimicking the acyl transfer processes,[19] and in the case of a synergic effect of antioxidant activity with ascorbic acid and cathecol against styrene oxidation in SDS micelles.[20]

First of all, the system composed of linoleic acid in the presence of TWEEN®-20 and 2-mercaptoethanol, as the amphiphilic thiol, was studied in homogeneous solution (water–alcohol) for gathering information on the two processes of isomerization and peroxidation, alone and in competition, under free radical conditions generated by the use of gamma irradiation. Then, under the same irradiation conditions the biomimetic model was used, made of linoleic acid micelles in the presence of TWEEN®-20 and the thiol. Comparing the efficiency of the peroxidation process with and without thiol, and combining with the isomerization process in the presence or absence of oxygen, a complete scenario of the main reactivity features was obtained and discussed. The biomimetic model of free radical reactivity, where the micelles composed by the fatty acid and the surfactant are added with the thiol, showed some interesting features deriving from the supramolecular disposition of the lipid components and the different distribution and diffusion of reactive species. More importantly, the micelle system offered for the first time a biomimetic environment to demonstrate that diene hydroperoxides and mono-*trans* fatty acids can be formed to a comparable extent under oxidative free radical conditions.

2. Results and discussion

2.1 Irradiation of linoleic acid in homogeneous solutions with and without thiols

Linoleic acid (LH) is soluble in EtOH–H_2O (1/1, v/v). γ-Radiolysis of the ethanol–water system led to the transient species shown in eqn (1) and (2), where R$^\bullet$ represents the alcohol radicals produced (*i.e.*, $^\bullet CH_2CH_2OH$, $CH_3{}^\bullet CHOH$, $CH_3CH_2O^\bullet$).[21] Solvated electrons (e_{sol}^-) in N_2O-saturated solutions are transformed into HO$^\bullet$ radical [eqn (3)]. Hydrogen abstraction from ethanol by HO$^\bullet$ radical and H$^\bullet$ atoms increases the production of alkyl radicals [eqn (4)]. Alkyl radicals react with 2-mercaptoethanol (2-ME) to give the corresponding thiyl radicals [eqn (5)].[22]

$$CH_3CH_2OH \rightsquigarrow e_{sol}^-, R^\bullet \quad (1)$$

$$H_2O \rightsquigarrow e_{sol}^-, HO^\bullet, H^\bullet \quad (2)$$

$$e_{sol}^- + N_2O + H_2O \rightarrow N_2 + HO^- + OH^\bullet \quad (3)$$

$$HO^\bullet/H^\bullet + CH_3CH_2OH \rightarrow H_2O/H_2 + R^\bullet \quad (4)$$

$$R^\bullet + HOCH_2CH_2SH \rightarrow RH + HOCH_2CH_2S^\bullet \quad (5)$$

The blank experiment of γ-irradiation of phosphate buffered air-equilibrated or N_2O-saturated ethanol–water (1/1, v/v) solutions (pH 5) containing 5×10^{-4} M of LH was carried out. After 100 Gy of irradiation, lipid components were extracted with a solvent mixture of CH_2Cl_2–MeOH (2 : 1, v/v), deaerated by nitrogen and analyzed for quantitative determination by the spectrophotometric ferric thiocyanate method, as described earlier.[23] It is worth noting that this detection method is one of the most reliable procedures used for direct diene hydroperoxide measurements, using the 500 nm absorbance of a ferric-thiocyanate complex, which is an ideal wavelength for minimizing the interferences of many substances in complex systems (*e.g.*, with thiol compounds). Moreover, this method has a very low detection limit, and this allows the quantitation of the corresponding hydroperoxides to be

performed accurately. It is also worth noting that, for the aim of this work, further transformations of the formed hydroperoxides were not considered. The blank experiment showed that after 100 Gy the LOOH formation was of 1.52×10^{-5} and 1.40×10^{-5} M in air-equilibrated or N_2O-saturated solutions, respectively. In parallel the analysis of *trans* fatty acid formation at 100 Gy was carried out by GC, after treatment of the lipid extracts with an ethereal solution of CH_2N_2 in order to transform quantitatively the free fatty acid into the corresponding methyl ester (LAME). In the absence of thiol no trace of *trans* fatty acid was detected.

Next, TWEEN®-20 (a non-ionic detergent soluble in ethanol–water) was added to the linoleic acid solution, in the presence and absence of thiol, followed by γ-irradiation. In comparison with the blank experiment reported above, the amount of lipid peroxidation was found to be similar under the various conditions (Table 1, third column) using the same dose of irradiation. On the other hand, when 2-ME was added to the system, a decrease of LOOH was evident both in anaerobic and aerobic conditions, indicating the inhibition of the peroxidation process (fourth column). After esterification, GC-analysis of the solutions irradiated at 100 Gy evidenced the formation of *trans* isomers of linoleic acid that are in higher amount in N_2O saturated solution than in air-equilibrated solution. It is worth underlining that the two mono-*trans* 9c,12t and 9t,12c isomers were formed in equal amounts in all experiments.

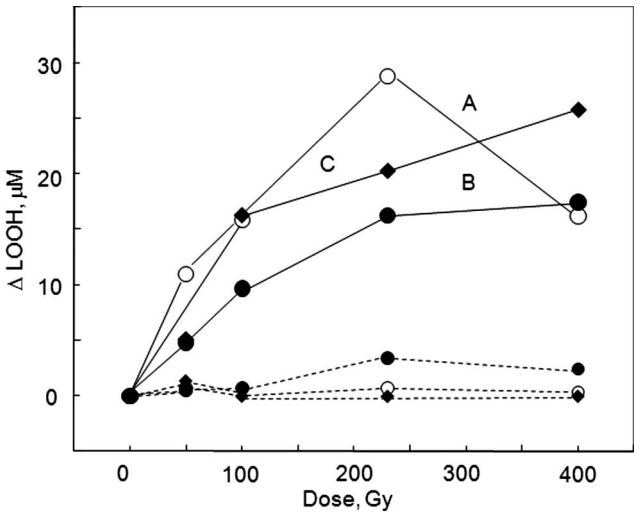


Fig. 1 The formation of LOOH in micelles as a function of irradiation dose at 274.8 Gy min^{-1} dose rate; aerobic (full lines) or anaerobic conditions (dashed lines), (○) system A (added 2-ME), (●) system B (incorporated 2-ME), (◆) control C; LH micelles formed by 5×10^{-4} M LH, 2.8×10^{-4} M TWEEN®-20, 5×10^{-3} M PB and 2.8×10^{-3} M 2-ME at pH 5. Reported values represent the mean of three independent measurements ($p < 0.05$); errors are ± 5%.

2.2 Irradiation of linoleic acid in micelles: evaluation of peroxidation process with and without thiol

LH (5.0×10^{-4} M) was added to micelles of non-ionic surfactant, previously formed by mixing TWEEN®-20 (2.8×10^{-4} M) and NaH_2PO_4, pH ~ 5. The addition of the amphiphilic thiol 2-ME was carried out in two different ways: *System A* where 2-ME is added to previously prepared micelles of LH and TWEEN®-20 just before irradiation, and *System B* where 2-ME is incorporated with linoleic acid during the micelle formation, that is, together with linoleic acid before mixing with TWEEN®-20. γ-Radiolysis of micelles was performed at room temperature. The samples were irradiated by 50, 100, 230 and 400 Gy under air-equilibration or N_2O-saturation. The effect of two different dose rates, *i.e.*, 1.31 and 274.8 Gy min^{-1} was also studied. After irradiation, lipid components were extracted with CH_2Cl_2–MeOH (2/1, v/v) and analyzed, either spectrophotometrically for LOOH determination or by GC, after esterification for the geometrical isomer identification as previously explained. Fig. 1 shows the dose profiles of lipid hydroperoxide (LOOH) formation as a function of the dose, at a dose rate of 274.8 Gy min^{-1}. The analogous experiment at 1.31 Gy min^{-1} dose rate is reported in the ESI (Figure S1‡). In both systems A and B LOOH was formed under aerobic conditions, whereas formation was negligible in N_2O-saturated solutions.

In Fig. 1 it is clearly shown that the thiol incorporation in the micelle system represents an important element for the reactivity. Micelles where 2-ME was incorporated during the preparation are more resistant to oxidation than the micelles with 2-ME added just before irradiation. Since the micelle size does not significantly change, the different behavior of the two systems under the same oxidative conditions could be explained by the different distribution of the thiol in the water phase and in the micelles depending on the preparation procedure. In other words, thiol is a more effective antioxidant when it reaches the micelle core, therefore its position is closer to the fatty acid during the propagation of lipid peroxidation, whereas in the system A the thiol distribution was not at the same extent. The efficiency of the antioxidant behavior is reported to be connected to the distribution of the antioxidant.[19]

Table 1 γ-Irradiation with 100 Gy of air-equilibrated or N_2O-saturated ethanol–water (1/1, v/v) solutions (pH 5) containing 5×10^{-4} M of LH and TWEEN®-20 and in the presence or absence of 2.8×10^{-3} M of 2-mercaptoethanol (2-ME)[a,b]

		[LOOH]/10^{-5} M		% Isomers 9c,12c/9c,12t/9t,12c/9t,12t
[TWEEN-20]/M		No thiol	2-ME	2-ME
2.8×10^{-4}	Air	1.50 ± 0.02	1.18 ± 0.09	84.6/7.2/6.9/1.3
	N_2O	1.30 ± 0.08	0.43 ± 0.00	19.4/23.8/22.9/33.9
2.8×10^{-3}	Air	1.58 ± 0.02	1.16 ± 0.07	88.8/5.0/4.8/1.4
	N_2O	1.19 ± 0.03	0.55 ± 0.02	25.7/24.0/23.3/27.0

[a] Dose rate = 274.8 Gy min^{-1}. [b] [PB] = 5×10^{-3} M.

In order to understand the effect of the dose rate on the lipid oxidation, system B (2-ME incorporated in micelles) was chosen for further investigation. Fig. 2 shows the dose profiles of LOOH formation at dose rates of 0.15, 1.31, 6.79 and 274.8 Gy min^{-1} under air-equilibration and N_2O-saturation conditions. It is evident that for the same dose the higher amounts of LOOH are associated with lower dose rate. For example at 230 Gy the concentration of LOOH varies from 20 to 150 μM. It is worth recalling that in the absence of oxygen it was ≪1 μM.

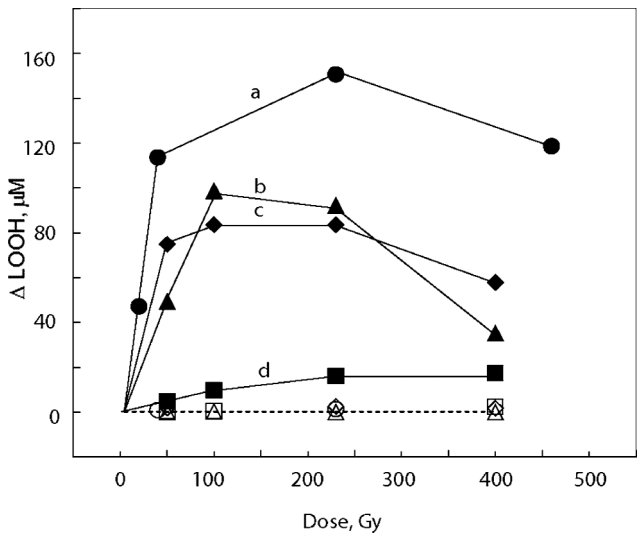


Fig. 2 The influence of dose and dose rates on the formation of LOOH in system B (incorporated 2-ME) under air-equilibration (solid symbols) and N_2O-saturated conditions (open symbols); dose rates: (a) 0.15 Gy min^{-1}, (b) 1.31 Gy min^{-1}, (c) 6.8 Gy min^{-1}, (d) 274.8 Gy min^{-1}; LH micelle: 5×10^{-4} M LH, 2.8×10^{-4} M TWEEN®-20, 5×10^{-3} M PB and 2.8×10^{-3} M 2-ME at pH 5. Reported values represent the mean of three independent measurements ($p < 0.05$); errors are ± 5%.

Scheme 2 shows the steps of the γ-irradiation initiated autoxidation of linoleic acid. Radiolysis of neutral water leads to e_{aq}^-, HO$^\bullet$ and H$^\bullet$ as shown in eqn (6). The values in parentheses represent the radiation chemical yields (G) in units of μmol J^{-1}. From known kinetic data,[22] the HO$^\bullet$ radicals (and part of H$^\bullet$ atom) should be partitioned between the reactions with thiol and TWEEN®-20/LH to give thiyl and alkyl radicals R$^\bullet$, respectively [eqn (7) and (8)].[24,25] The H$^\bullet$ atom should be quenched by oxygen too (eqn 9).[26] On the other hand, e_{aq}^- are partitioned between oxygen and thiol [eqn (9) and (10)]. Assuming that the concentration of O_2 in air-equilibrated solutions is ≤2.66×10^{-4} M (*i.e.*, equal or less than an air-saturated aqueous solution), having 2.8×10^{-3} M 2-ME and taking into consideration the related rate constants with e_{aq}^- and H$^\bullet$,[22] the main products will be peroxyl radicals [eqn (11)] and superoxide radical anion [eqn (9)] or its protonated form.[26] While superoxide radical anion does not react with LH,[27] HOO$^\bullet$, ROO$^\bullet$ and RS$^\bullet$ radicals are expected to react with LH generating the bisallylic radical L$^\bullet$ [eqn (12)].[27–30] In the first propagation step molecular oxygen adds to L$^\bullet$, whereas in the second propagation step LOO$^\bullet$ abstracts hydrogen atom from the bisallylic position at rate k_p to generate L$^\bullet$ in what is the rate-determining step of the propagation sequence [eqn (13) and (14)].[28] The termination steps involve couplings of peroxyl radicals [eqn (15)].

Initiation Steps

$$H_2O \rightsquigarrow e_{aq}^-(0.27), HO^\bullet(0.28), H^\bullet(0.062) \quad (6)$$

$$HO^\bullet/H^\bullet + HOCH_2CH_2SH \rightarrow H_2O/H_2 + HOCH_2CH_2S^\bullet \quad (7)$$

$$HO^\bullet/H^\bullet + \text{TWEEN-20/LH} \rightarrow H_2O/H_2 + R^\bullet \quad (8)$$

$$H^\bullet/e_{aq}^- + O_2 \rightarrow O_2^{\bullet-} \quad (9)$$

$$e_{aq}^- + HOCH_2CH_2SH \rightarrow HOCH_2CH_2^\bullet + HS^- \quad (10)$$

$$R^\bullet + O_2 \rightarrow ROO^\bullet \quad (11)$$

$$ROO^\bullet/HOCH_2CH_2S^\bullet + LH \rightarrow ROOH/HOCH_2CH_2SH + L^\bullet \quad (12)$$

Propagation Steps

$$L^\bullet + O_2 \rightarrow LOO^\bullet \quad (13)$$

$$LOO^\bullet + LH \xrightarrow{k_p} LOOH + L^\bullet \quad (14)$$

Termination Steps

$$LOO^\bullet + LOO^\bullet \xrightarrow{2k_t} \text{non-radical products} \quad (15)$$

Scheme 2 γ-Radiolysis of water initiated peroxidation chain process of linoleic acid (LH).

The rate of radiation-induced formation of the product LOOH is represented by the following expression:

$$d[LOOH]/dt = G(LOOH)\cdot\rho\cdot P = k_p[LH][LOO^\bullet] \quad (16)$$

where G is the radiation-chemical yield, ρ the density, and P the dose rate. The application of the steady-state approximation to the intermediates LOO$^\bullet$ and L$^\bullet$, taking into consideration eqn (16) and assuming high concentration of oxygen,[31] affords the final expression:

$$G(LOOH) = \frac{k_p}{\sqrt{2k_t}}[LH]\sqrt{G(L^\bullet)/\rho P} = G_0(LOOH)\frac{1}{\sqrt{P}} \quad (17)$$

The logarithmic form of eqn (17) gives the relationship between G(LOOH) and the dose rate, *i.e.*, inverse square root of the dose rate, from which the radiation chemical yield at unit dose rate G_0(LOOH) can be calculated:

$$\log G(LOOH) = \log G_0(LOOH) - a \log P \quad (18)$$

When G(LOOH) values were expressed according to eqn (18), a linear dependence of log G(LOOH) *vs.* log P was found, and from the intercept and slope of the straight line, it can be calculated: G_0(LOOH) = 1.7 μmol J^{-1} and a = 0.51 (see ESI‡). This dose rate dependence of peroxidation found in our model system indicated the validity of the inverse square-root relationship with dose rate [eqn (18)], which means that a relationship giving the yield of formation of peroxidation products (LOOH) derived for homogeneous medium can be applied in our model micellar system. Generally, lower dose rates were found to be more efficient in producing lipid hydroperoxidation in model membranes,[32] although no inverse square-root correlation with dose rate was originally tested in those systems. Assuming that the radiation chemical yield of radicals that abstract hydrogen generating the bisallylic radical is *ca.* 0.09 μmol J^{-1} [eqn (12)], which corresponds to *ca.* 15% of the initial radical species [eqn (6)], from G_0(LOOH) = 1.7 μmol J^{-1} we calculated a chain propagation length of *ca.* 19.

2.3 Irradiation of linoleic acid in micelles with thiols: evaluation of isomerization *vs.* peroxidation processes

As previously explained, thiols act as inhibitors of the lipid peroxidation by hydrogen donation with the formation of thiyl radicals which in their turn are isomerizing agents, Therefore, the same micelle experiments reported in Fig. 1 were analyzed for determining the geometrical isomer distribution in anaerobic and aerobic conditions.

First of all under anaerobic conditions, where the peroxidation process did not occur, Fig. 3 reports the formation of the geometrical isomers as a function of the dose under anaerobic conditions; the results are shown for System A, where 2-ME is added before irradiation (upper), and System B, where 2-ME is incorporated in the micelles (lower). We recall that in both anaerobic systems no LOOH was observed (*cf.* Fig. 1). In both plots, the disappearance of 9c,12c–C18:2 (●) is replaced by the formation of mono-*trans* isomers (▲) and the di-*trans* isomer 9t,12t–C18:2 (■). The two mono-*trans* isomers, *i.e.*, 9t,12c–C18:2 and 9c,12t–C18:2, were found in almost equal amounts and are reported together in the plot. Interestingly, the two micelle preparation procedures (A *vs.* B) showed a different efficiency of the isomerization process. Taking the 9c,12c–C18:2 decrease as a measure for the isomerization efficiency, the process of isomerization was found to be 2 times more effective in system A then in system B under anaerobic conditions. Micelles with incorporated 2-ME (system B) are more resistant to isomerization, therefore the distribution of molecular species within the organized system play an important role in their reactivity to generate thiyl radicals and, consequently, for the isomerization outcome. The influence of different dose rates (274.8 *vs.* 1.31 Gy min^{-1}) on the isomerization efficiency in N_2O-saturated suspensions (using system B) is also reported in the ESI (Figure S3‡), indicating a more effective isomerization process at lower dose rates, for the same dose of irradiation.

The reaction steps are based on rate constants of 1.2×10^{10} and 9.1×10^9 M^{-1} s^{-1} for the reaction of e_{aq}^- with 2-ME (2.8 mM) and N_2O (20 mM),[22] that is, 85% of hydrated electrons are trapped by N_2O to increase the formation of HO• radicals [eqn (3)]. HO• radicals and H• atoms should be partitioned between thiol and TWEEN®-20/LH to give thiyl and alkyl radicals [eqn (7) and (8)]. In the absence of oxygen, alkyl radicals are essentially trapped by thiol to give extra thiyl radical, which can isomerize double bonds of unsaturated fatty acids.[3,8]

Scheme 3 shows the reaction mechanism, involving reversible addition of $HOCH_2CH_2S$• radical (X•) to the double bond to form a radical-adduct. The reconstitution of the double bond is obtained by β-elimination of X•, and the result favours the *trans* geometry, the most thermodynamically-stable configuration. It should be noted that X• acts as a catalyst for the *cis–trans* isomerization.[3,12]

Under air-equilibrated conditions, the *cis–trans* isomerization process could be finally observed in parallel with the occurrence of the peroxidation process. Fig. 4 shows the dose dependence of the various geometrical isomers for system A, where 2-ME was added before irradiation (upper), and system B, where 2-ME was incorporated (lower). It is worth recalling that under these conditions the LOOH formation was observed (*cf.* Fig. 1, upper part). The disappearance of 9c,12c–C18:2 (●) matches well with the formation of mono-*trans* isomers 9t,12c–C18:2 + 9c,12t–C18:2 (▲). No detectable amount of 9t,12t–C18:2 (■) was observed. As under anaerobic conditions, the process of isomerization was found to be 2 times more effective in system A than in system B. The influence of dose rates (274.8 *vs.* 1.31 Gy min^{-1}) on the geometrical isomer distribution in the air-equilibrated system B is also reported in the ESI (Figure S4‡), indicating a more effective

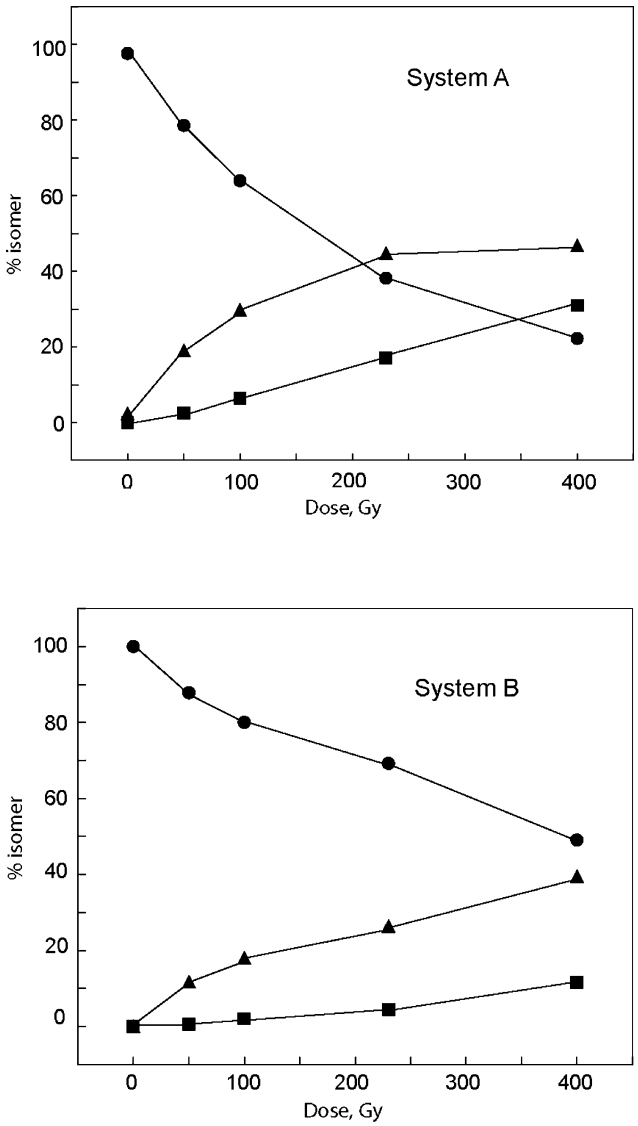
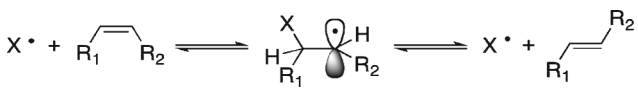


Fig. 3 The geometrical isomer distribution of linoleic acid isomers as a function of the irradiation dose (274.8 Gy min^{-1}) in system A and system B under anaerobic conditions (N_2O-saturated); the disappearance of 9c,12c–C18:2 (●), the formation of 9t,12c–C18:2 + 9c,12t–C18:2 (▲), and the formation of 9t,12t–C18:2 (■) are reported (the two mono-*trans* isomers are found in equal amounts); LH micelle: 5×10^{-4} M LH, 2.8×10^{-4} M TWEEN®-20, 5×10^{-3} M PB and 2.8×10^{-3} M 2-ME at pH 5. Reported values represent the mean of three independent measurements ($p < 0.05$); errors are ± 2%.



Scheme 3 Reaction mechanism for the *cis–trans* isomerization catalysed by $HOCH_2CH_2S$• radical (X•).

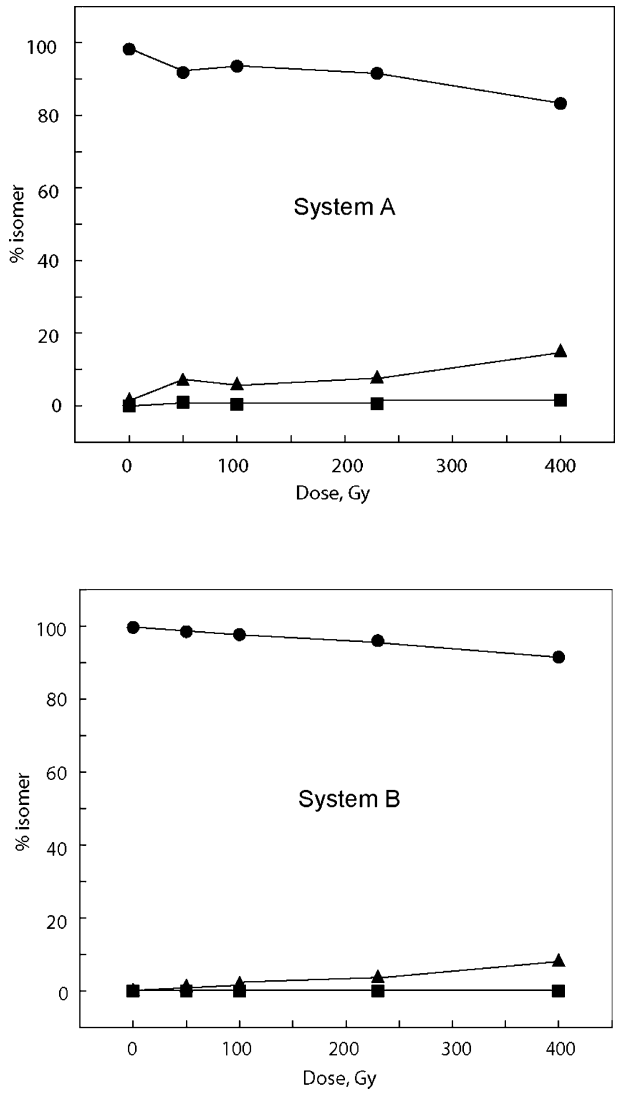


Fig. 4 The geometrical isomer distribution of LAME as a function of the irradiation dose (274.8 Gy min^{-1}) in system A and system B under aerobic conditions (air-equilibrated); the disappearance of 9c,12c–C18:2 (●), the formation of 9t,12c–C18:2 + 9c,12t–C18:2 (▲), and the formation of 9t,12t–C18:2 (■) are drawn (the two mono-*trans* isomers were found in equal amounts); LH micelle: 5×10^{-4} M LH, 2.8×10^{-4} M Tween®-20, 5×10^{-3} M PB and 2.8×10^{-3} M 2-ME at pH 5. Reported values represent the mean of three independent measurements ($p < 0.05$); errors are ± 2%.

isomerization process at lower dose rates for the same dose of irradiation.

It was gratifying to see that in the presence of 2.8 mM of the amphiphilic thiol in the biomimetic model under air equilibrated conditions containing 500 μM linoleic acid, the two processes of peroxidation and *cis–trans* isomerization are both effective (Fig. 1, 2d and 4). In this system it was estimated that after 400 Gy 20 μM of LOOH (by the methodology described above[23]) and 50 μM of mono-*trans* isomers were formed. The micelle model offers for the first time the possibility to parallel two free radical processes involving lipids and thiols. Further work is in progress on a detailed product analysis in order to complete the scenario of these transformations from a quantitative point of view. These results point out the need to better define the occurrence of these parallel processes in the biological environment.

It is worth mentioning that a quantitative follow-up of linoleic acid geometrical isomers in Fig. 3A and 4A (*i.e.*, in the absence of oxygen or in air-equilibrated solutions) was performed by GC analysis, using methyl stearate as internal standard, showing that by increasing reaction time other products should be formed accounting for a full mass balance. In fact, at up to 100 Gy in aerobic and anaerobic experiments the sum of all geometrical isomers accounted for ≥95% of the starting LH, whereas at 400 Gy the yields dropped down to 60% and 71% in the absence of oxygen or in air-equilibrated solutions, respectively. Fig. 5 shows the plot of *G*(9c,12c–C18:2) *versus* dose for both cases. The extrapolation to zero dose gives $G = 220$ μmol J^{-1} in the absence of oxygen and $G = 190$ μmol J^{-1} in air-equilibrated solutions. Assuming that the $G(RS^{\bullet})$ is 0.52 μmol J^{-1}, corresponding to *ca.* 85% of the initial radical species [eqn (6)], we calculated the catalytic cycle to be 420 and 370, respectively, at the initial phase.

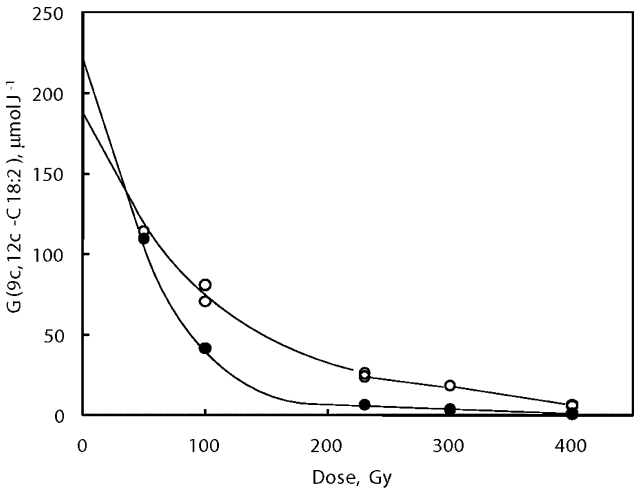


Fig. 5 The chemical radiation yields (*G*) of disappearance of 9c,12c–C18:2 *versus* dose in system A (●) under anaerobic conditions (N_2O-saturated) and (○) under aerobic conditions (air-equilibrated); LH micelle in system A: 5×10^{-4} M LH, 2.8×10^{-4} M TWEEN®-20, 5×10^{-3} M PB and 2.8×10^{-3} M 2-ME at pH 5 at dose rate 274.8 Gy min^{-1}. The data are a combination of two independent experiments.

The decrease of *G* in air-equilibrated solutions may have its origin from different factors and we suggest *inter alia* that the peroxidation products (*trans*,*cis* conjugated dienes, see Scheme 1) are inhibitors of the *cis–trans* isomerization process, since it is well known that thiyl radicals add to conjugated dienes with rate constants two-orders of magnitude higher than for addition to isolated double bonds.[33,34] Another aspect worthy of further comment is the *G* value under anaerobic conditions. If one considers that (i) $G = 230$ μmol J^{-1} was obtained for the isomerization of methyl oleate in *t*-BuOH and $G = 120$ μmol J^{-1} in POPC-LUVET in the absence of oxygen,[35] and (ii) $G = 20$ μmol J^{-1} was obtained for the analogous isomerization of 0.15 M methyl linoleate in *t*-BuOH,[8] we suggest that the *cis–trans* isomerization of LH in micelles is effectively like the isomerization of methyl oleate in solution, and the described competitive hydrogen abstraction from the bisallylic position is substantially limited. We suggest that the supramolecular organization of micelles controls the diffusion

Table 2 The dependence of micelle size on the concentrations of TWEEN®-20 and LH and the effect of the size on the formation of LOOH and geometrical isomers after 100 Gy irradiation in air-equilibrated or N_2O-saturated conditions[a]

				AIR		N_2O
Entry	d_0, nm	TWEEN®-20, M	LH, M	ΔLOOH, μM[b]	% Isomers 9c,12c/9c,12t/9t,12c/9t,12t	% Isomers 9c,12c/9c,12t/9t,12c/9t,12t
1	92.2 ± 2.9	2.8×10^{-4}	4.8×10^{-3}	35.5 ± 5.0	99.1/0.5/0.4/0	97.5/1.2/1.2/0.1
2	22.5 ± 0.8	2.8×10^{-4}	5.0×10^{-4}	9.7 ± 0.4	97.7/1.1/1.2/0	80.0/8.3/9.7/2.0
3	9.1 ± 0.7	2.8×10^{-3}	5.0×10^{-4}	13.0 ± 0.8	94.2/2.5/2.9/0.4	45.5/18.9/23.5/12.1

[a] System B (incorporated 2-ME in micelles); dose rate = 274.8 Gy min^{-1}; 5×10^{-3} M PB and 2.8×10^{-3} M 2-ME at pH 5.

of thiyl radicals (see also next section), and disfavors the optimum transition state for the hydrogen abstraction.

2.4 The effect of micelle size

Next we considered the effect of micelle size, by changing TWEEN®-20 or LH concentrations, on the peroxidation and isomerization processes in the system B (incorporated 2-ME). In particular, the apparent hydrodynamic diameter (d_0) of micelles was determined by means of dynamic light scattering. In Table 2, entry 2 corresponds to the experiments described in the previous section. The diameter was found to be (22.5 ± 0.8) nm. By increasing the amount of LH or TWEEN®-20 the micelle size can increase or decrease, respectively. Entry 1 shows that d_0 increased to (92.2 ± 2.9) nm by increasing the LH concentration by one order of magnitude, while TWEEN®-20 is kept constant. On the other hand, for the same concentration of LH an increase of TWEEN®-20 by 10-fold caused d_0 to decrease from 22.5 to 9.1 (entry 3).

The micelles were irradiated with 100 Gy both in under air-equilibrated and N_2O-saturated conditions. Under anaerobic conditions it was observed that *cis–trans* isomerization increased as the micelle size became smaller and, interestingly, the two mono-*trans* isomers (9c,12t and 9t,12c) were formed in different amounts (see also Figure S5‡). This effect indicates that a defined supramolecular arrangement of the fatty acid tail (hydrocarbon) is present in small aggregates, which renders the double bond in position 9 not equivalent to that in position 12 for its reaction with thiyl radicals.[36] Obviously, LOOH is absent under N_2O-saturated conditions, whereas it formed at μM level under aerobic conditions. The extent of formation of LOOH showed relatively small differences as a function of micelle size, whereas the positional effect in case of peroxidation will be considered in further studies. As expected, the *cis–trans* isomerization is less pronounced under aerobic conditions, but still significant. At the same concentrations of thiol and LH previously described (Fig. 1 and 2d) but at a lower micelle size (entry 3), after 100 Gy of irradiation the formation of 13 μM of LOOH and a 6% conversion of linoleic acid into mono-*trans* isomers (*ca.* 30 μM) were estimated. In the 9 nm micelle size the two mono-*trans* isomers (9c,12t and 9t,12c) were formed in different amounts, indicating that in organized systems the positions of the two double bonds of the linoleic fatty acid chain are not equivalent in terms of their exposure to the attacking radicals.

3. Conclusions

Competition is a relevant feature of free radical transformations especially in the biological environment, where the fate of a molecule can be partitioned among several molecular mechanisms and paths, influenced by the "local" factors and conditions.[37] In particular, with thiol being a very important chemical functionality reaching a not negligible concentration (up to 10 mM) in the biological environment, thiyl radical formation is a crucial event in living systems, that can be connected with the concept of "repair" of free radical damage and with the reactivity of thiyl radicals with polyunsaturated compounds occurring in parallel. For the first time, peroxidation and isomerization processes supported by thiol compounds were evaluated in a biomimetic model of linoleic acid micelles, showing that one process can be parallel with the occurrence of the other, and a 1 : 2 ratio can be preliminary estimated under our conditions. These results give further support to the role of mono-*trans* fatty acid isomers of PUFA residues as relevant markers of thiyl radical reactivity in complex systems. Indeed, mono-*trans* isomers were already reported in biological specimens correlated with free radical stress conditions.[13,38] These findings also suggest further model studies of lipid transformations be carried out in an integrated manner, in order to provide a comprehensive view of free radical stress in the presence of thiols to be extrapolated to living systems.

Experimental

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 the investigated 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). Gamma radiolysis of the solutions with typical composition and in the presence of 2-ME, which was added prior to irradiation (System A) and incorporated within the micelle during preparation (System B) was performed. Radiolysis was performed at room temperature using panoramic ^{60}Co source at different dose and dose rates.[39] Micelles were irradiated in air-equilibrium or after saturation with N_2O. After irradiation, lipid components were extracted with a solvent mixture of CH_2Cl_2–MeOH (2 : 1, v/v) deaerated by nitrogen, and an aliquot of the sample was taken out from the lower layer for the quantitative determination of LOOH. All further analysis was carried out in three independently prepared solutions. The concentration of LOOH was determined by the spectrophotometric ferric thiocyanate method following a published detailed procedure,[23] using UV/VIS spectrophotometer Varian Cary 4000. 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.[8] In order to transform linoleic acid and its geometrical isomers into the corresponding methyl esters the reaction solutions were treated with an ethereal solution of diazomethane.[40] A Varian 450 gas chromatograph equipped with a flame ionization detector and a Rtx-2330 (90% biscyanopropyl/10% phenylcyanopropyl-lpolysiloxane capillary column; 105 m × 0.25 mm) was used with the following oven program: temperature started from 180 °C, held for 35 min, followed by increase of 10 °C min^{-1} up to 250 °C and held for 5 min. Methyl esters were identified by comparison with the retention times of authentic samples, which are commercially available. The apparent hydrodynamic diameter of mixed micelles was determined by means of a dynamic light scattering (DLS) technique using Zetasizer Nano ZS (Malvern, UK) equipped with a 532 nm "green" laser.[41]

Acknowledgements

The support by the COST Action CM0603 "Free Radicals in Chemical Biology" is kindly acknowledged. The authors wish to thank Sikirić Dutour M. and Jurašin D. for their help with DLS technique. We are grateful to F. Luschtinetz for her helpful intercomparison in the micelle hydrodynamic radius measurements obtained with fluorescence correlation spectroscopy.

References

1 B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, 4th ed., University Press, Oxford, 2007.
2 E. Niki, *Free Radical Biol. Med.*, 2009, **47**, 469.
3 (*a*) C. Chatgilialoglu and C. Ferreri, *Acc. Chem. Res.*, 2005, **38**, 441; (*b*) C. Ferreri and C. Chatgilialoglu, *ChemBioChem*, 2005, **6**, 1722.
4 (*a*) C. Chatgilialoglu, C. Ferreri, I. N. Lykakis and P. Wardman, *Bioorg. Med. Chem.*, 2006, **14**, 6144; (*b*) C. Chatgilialoglu and C. Ferreri, in *Cis-Trans Isomerization in Biochemistry*, C. Dugave, (ed.), Wiley-VCH, Weinheim, 2006, pp 95–112.
5 W. Liu, H. Yin, Y. O. Akazawa, Y. Yoshida, E. Niki and N. A. Porter, *Chem. Res. Toxicol.*, 2010, **23**, 986.
6 K. A. Tallman, C. L. Rector and N. A. Porter, *J. Am. Chem. Soc.*, 2009, **131**, 5635.
7 K. A. Tallman, B. Roschek Jr. and N. A. Porter, *J. Am. Chem. Soc.*, 2004, **126**, 9240.
8 C. Ferreri, C. Costantino, L. Perrotta, L. Landi, Q. G. Mulazzani and C. Chatgilialoglu, *J. Am. Chem. Soc.*, 2001, **123**, 4459.
9 C. Ferreri, A. Samadi, F. Sassatelli, L. Landi and C. Chatgilialoglu, *J. Am. Chem. Soc.*, 2004, **126**, 1063.
10 (*a*) A. W. T. Konings, J. Damen and W. B. Trieling, *Int. J. Radiat. Biol.*, 1979, **35**, 343; (*b*) L. R. C. Barclay, K. A. Dakin and J. A. Y. Khor, *Res. Chem. Intermed.*, 1995, **21**, 467; (*c*) K. Hildenbrand and D. Schulte-Frohlinde, *Int. J. Radiat. Biol.*, 1997, **71**, 377.
11 (*a*) C. Schöneich, *Chem. Res. Toxicol.*, 2008, **21**, 1175; (*b*) C. von Sonntag, *Free-Radical-Induced DNA Damage and Its Repair*, Springer-Verlag, Berlin, 2006.
12 (*a*) C. Chatgilialoglu, A. Altieri and H. Fischer, *J. Am. Chem. Soc.*, 2002, **124**, 12816; (*b*) C. Chatgilialoglu, A. Samadi, M. Guerra and H. Fischer, *ChemPhysChem*, 2005, **6**, 286.
13 (*a*) C. Ferreri, M. R. FaraoneMennella, C. Formisano, L. Landi and C. Chatgilialoglu, *Free Radical Biol. Med.*, 2002, **33**, 1516; (*b*) C. Ferreri, S. Kratzsch, O. Brede, B. Marciniak and C. Chatgilialoglu, *Free Radical Biol. Med.*, 2005, **38**, 1180; (*c*) L. Zambonin, C. Ferreri, L. Cabrini, C. Prata, C. Chatgilialoglu and L. Landi, *Free Radical Biol. Med.*, 2006, **40**, 1549.
14 Y. Chevalier and T. Zemb, *Rep. Prog. Phys.*, 1990, **53**, 279.
15 *Liposomes a practical approach*, R. R. C. New, (ed.), IRL Press, Oxford, 1990.
16 (*a*) P. Sherman, *Emulsion Science*, Academic Press, London, 1968; (*b*) E. Jungermann, *Cationic Surfactants*, Marcel Dekker Inc., NewYork, 1969; (*c*) D. Myers, *Surfactant Science and Technology*, VCH Publisher, New York, 1988.
17 L. R. C. Barclay, S. J. Locke and J. M. MacNeil, *Can. J. Chem.*, 1983, **61**, 1288.
18 D. J. McClements and E. A. Decker, *J. Food Sci.*, 2000, **65**, 1270; Y.-J. Cho, D. J. McClements and E. A. Decker, *J. Agric. Food Chem.*, 2002, **50**, 5704; S.-W. Huang, A. Hopia, K. Schwarz, E. N. Frankel and J. B. German, *J. Agric. Food Chem.*, 1996, **44**, 444; L. R. C. Barclay, S. J. Locke and J. M. MacNeil, *Can. J. Chem.*, 1985, **63**, 366.
19 R. Fornasier and U. Tonellato, *Bioorg. Chem.*, 1982, **11**, 428.
20 F. Xi and L. R. C. Barclay, *Can. J. Chem.*, 1998, **76**, 171.
21 K.-D. Asmus, H. Möckel and A. Henglein, *J. Phys. Chem.*, 1973, **77**, 1218.
22 (*a*) G. V. Buxton, C. L. Greenstock, P. W. Helman and A. B. Ross, *J. Phys. Chem. Ref. Data*, 1988, **17**, 513; (*b*) A. B. Ross, W. G. Mallard, W. P. Helman, G. V. Buxton, R. E. Huie, P. Neta, *NDRL-NIST Solution Kinetic Database – Ver. 3*, Notre Dame Radiation Laboratory, Notre Dame, IN and NIST Standard Reference Data, Gaithersburg, MD, 1998.
23 B. Mihaljević, B. Katušin-Ražem and D. Ražem, *Free Radical Biol. Med.*, 1996, **21**, 53.
24 The reactions of HO$^{\bullet}$/H$^{\bullet}$ with TWEEN®-20 generate alkyl radicals R$^{\bullet}$ by hydrogen abstraction, whereas the reaction of HO$^{\bullet}$/H$^{\bullet}$ with LH occurs also by addition to give R$^{\bullet}$.[25a] However, it can be supposed that the abstraction of hydrogen atom giving R$^{\bullet}$ could occur preferentially in micellar medium[25b].
25 (*a*) M. Al-Sheikhly, J. Silverman, M. Simic and B. Michael, *J. Phys. Chem. B*, 2004, **108**, 17618; (*b*) L. K. Patterson and K. Hasegawa, *Ber. Bunsenges. Phys. Chem.*, 1978, **82**, 951.
26 The pK_a of HOO$^{\bullet}$ is 4.8, see: B. H. J. Bielski, D. E. Cabelli and R. L. Arudi, *J. Phys. Chem. Ref. Data*, 1985, **14**, 1041.
27 J. M. Gebicki and B. H. Bielski, *J. Am. Chem. Soc.*, 1981, **103**, 7020.
28 N. A. Porter, *Acc. Chem. Res.*, 1986, **19**, 262.
29 C. Schöneich, U. Dillinger, F. von Bruchhausen and K.-D. Asmus, *Arch. Biochem. Biophys.*, 1992, **292**, 456.
30 L. R. C. Barclay, *Can. J. Chem.*, 1993, **71**, 1.
31 B. Katušin-Ražem and D. Ražem, *J. Phys. Chem. A*, 2000, **104**, 1482.
32 (*a*) J. A. Raleigh, W. Kremers and B. Gaboury, *Int. J. Radiat. Biol.*, 1977, **31**, 203; (*b*) A. Petkau, *Health Phys.*, 1972, **22**, 239; (*c*) M. Al-Sheikhly and M. G. Simic, *J. Phys. Chem.*, 1989, **93**, 3103.
33 C. Chatgilialoglu, L. Zambonin, A. Altieri, C. Ferreri, Q. G. Mulazzani and L. Landi, *Free Radical Biol. Med.*, 2002, **33**, 1681.
34 M. D'Aquino, C. Dunster and R. L. Willson, *Biochem. Biophys. Res. Commun.*, 1989, **161**, 1199.
35 C. Chatgilialoglu, C. Ferreri, M. Ballestri, Q. G. Mulazzani and L. Landi, *J. Am. Chem. Soc.*, 2000, **122**, 4593.
36 The fatty acid moieties are able to give supramolecular aggregates in water that influence the regioselectivity of double bond epoxidation[13].
37 C. C. Winterbourn, *Nat. Chem. Biol.*, 2008, **4**, 278.
38 C. M. Zghibeh, G. V. Raj, C. D. Poff, J. R. Falck and M. Balazy, *Anal. Biochem.*, 2004, **332**, 137.
39 J. W. T. Spinks and R. J. Woods, *An Introduction to Radiation Chemistry*, 3rd ed., Wiley, New York, 1990; p. 100.
40 J. Glaastrup, *J. Chromatogr., A*, 1998, **827**, 133.
41 F. Luschtinetz and C. Dosche, *J. Coll. Interface Sci.*, 2009, **338**, 312.

