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Effect of oxygen free radicals on ubiquinone in aqueous solution and phospholipid vesicles

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The purpose of this study was to evaluate the direct effect of oxygen free radicals produced by ultrasonic irradiation on ubiquinone and to compare the efficiency with which the antioxidant can compete with these radicals when it is both in aqueous solution and within the lipid bilayer. The main product obtained after insonation of aqueous solutions of ubiquinone-0 was ubiquinol, moreover some degradation occurred. The direct electron donor responsible for most of the ubiquinol generated by ultrasonic irradiation appeared to be superoxide radical. Addition reactions of hydroxyl radicals with aromatic ring structure led probably to degradation products of ubiquinone, which were not identified. Experiments were also performed to evaluate the efficiency with which ubiquinone-3 could react with oxygen radicals when it was within the lipid bilayer. The effect of presence or absence of a net surface charge was studied selecting a suitable bilayer including dimyristylphosphatidic acid or stearylamine in uncharged dimyristylphosphatidylcholine vesicles. In these systems hydroxyl radicals did not represent a potential danger for the antioxidant, the reaction between superoxide and ubiquinone-3 instead was significant only in positively charged membranes and gave rise to ubiquinol. It is suggested that ubiquinone acts as an antioxidant by stopping the propagation reaction.

Introduction

It has been known that ultrasonic waves initiate redox reactions in aqueous solutions which are often similar to the reactions produced by ionizing radiations [1].

Water is decomposed to yield H_2 and H_2O_2, both under oxygen and other gases [2,3]. It was suggested that hydrogen peroxide is formed under argon by the recombination of HO· radicals. The submission of water to the action of ultrasonic radiations under oxygen produces additional pathways for the formation of H_2O_2 [4]. While our understanding of radiolysis of water is excellent [5], little is known about the yields of radicals in sonolysis. As the achievement of a good level of predictability and reproducibility for ultrasonic reactions has hystorically been a problem to standardize conditions the rate of hydrogen peroxide formation at a determined pH and temperature is currently followed [6–8].

Sonication, that allows to incorporate comparable amounts of ubiquinone homologues not depending upon the length of the isoprenoid side chain [9] in monolamellar vesicles, has been the method utilized to prepare membrane models used in the study of the antioxidant effect of quinones [10,11].

Abbreviations: Q_n, ubiquinone with n isoprenoid units; Q_0, ubiquinone-0; Q_3, ubiquinone-3; DMPC, L-α-dimyristylphosphatidylcholine; DMPA, L-α-dimyristylphosphatidic acid.

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We showed that antioxidant properties of Q homologues from Q_2 to Q_{10} are the same [10] and that they were already exhibited during the preparation of vesicles by sonication [11]. The present study was undertaken to extend our earlier work and to evaluate the direct effect of radicals formed in sonolysis on ubiquinone.

The following points have been investigated: (a) whether ubiquinone can scavenge radicals produced in the sonolysis of water, (b) whether chemical reactions that occur in liquids subjected to ultrasonic irradiation are able to degrade this quinone, (c) whether the ultrasonic effect on ubiquinone is different when quinone is in aqueous solution or in phospholipid membranes.

Materials and Methods

Ubiquinone homologues were a kind gift of Eisai Co., Tokio, Japan. Synthetic L-α-dimyristylphosphatidic acid, L-α-dimyristylphosphatidylcholine, stearylamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and superoxide dismutase were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Inactivated superoxide dismutase was prepared by boiling, which reduced the specific activity from 3080 to 45 units/mg protein. Superoxide dismutase activity was measured according to Mc Cord and Fridovich [12]. Other reagents used were of analytical grade. Silica gel thin-layer chromatography precoated plates with fluorescent indicator were from Farmitalia Carlo Erba (Milan, Italy).

The homologue chosen for the majority of this study was ubiquinone-0 because most experiments were performed in buffered aqueous media and Q_0 is the unique quinone completely water-soluble.

Ultrasonic irradiation was carried out using a Labsonic 2000 (B. Braun Melsungen AG, F.R.G.) with a titanium probe, 127 mm length, 9.5 mm diameter, at a power flow of 26 $W \cdot cm^{-2}$, as measured by calorimetric methods, in all the experiments. In a typical run, 8 ml of a 18–70 μM ubiquinone-0 solution in 0.05 M Hepes buffer (pH 7.2), in the presence or absence of radical scavengers were pipetted in a Pyrex tube (volume 40 ml). The irradiation vessel, thermostated at 0–4 °C, was fitted at constant position and the probe tip was steeped into the buffer solution at a constant position. The solutions were sonicated for different time spans up to 20 min under a nitrogen atmosphere. Sonication was carried out intermittently for 30 s, followed by a 30 s resting period. The solutions were centrifuged to remove any probe particles and then the absorption spectrum of Q_0 was recorded in a Perkin-Elmer 559-UV/VIS spectrophotometer.

Thin-layer chromatography of the quinone products after sonolysis was performed according to Petterson [13]. The solvent systems used were chloroform or ethanol/*n*-butanol/2 M ammonium hydroxide (3 : 5 : 3, v/v). The spots were removed by scraping the silica gel from the plates and the ultraviolet spectrum of each compound, previously eluted using absolute ethanol, was then determined.

Q_3 containing vesicles were prepared by a previously used procedure [10,11]. Mixture of stock solutions of lipid in chloroform, stored at −20 °C, containing 34 μmol DMPC, 0.61 μmol Q_3 and 4 μmol DMPA or stearylamine were dried under nitrogen and dispersed in 8 ml of 0.05 M Hepes buffer (pH 7.2), with a vortex mixer. The same amount of Q_3 was also incorporated in uncharged phospholipid membranes (38 μmol DMPC). The suspensions were then subjected to ultrasonic irradiation as above. During this procedure, temperature was maintained slightly above the transition point (24 °C).

Absorption spectra of Q_3 incorporated into vesicles after different sonication times were recorded. Potassium deoxycholate (pH 8.2), was added to vesicles to give a final concentration of 1% (w/v) before measurements of absorbance. The sonochemical formation of hydrogen peroxide was determined fluorimetrically according to Loschen et al. [14].

Results

A relatively simple procedure, common to most experiments, was adopted to follow the course of reactions; ubiquinone-0 dissolved in 0.05 M Hepes buffer (pH 7.2) or ubiquinone-3 dissolved in synthetic phospholipids were exposed to ultrasonic irradiation and the course of their modification measured spectrophotometrically.

Aqueous Q_0 solutions showed a linear decrease with time in the ultraviolet absorbance maximum at 268 nm during insonation at constant ultrasonic intensity.

Fig. 1 shows that the decrease rate for two different initial concentrations was fairly constant over at least a 20 min insonation period. This duration time corresponds to that normally used in the preparation of vesicles utilized in our studies of the antioxidant properties of quinones [9–11].

The characteristic changes observed in Q_0 absorbance spectrum are shown in Fig. 2. The unirradiated material had a spectrum typical of Q_0 with a maximum at 268 nm in water [15]. As irradiation proceeded, the 268 nm peak decreased and shifted progressively to a longer wavelength. The reduction in intensity of the ultraviolet absorbance band was initially attributed only to degradation of the benzoquinone ring. However when insonated Q_0 solutions were left under air and sequential ultraviolet spectra were recorded, there was a very slow return towards the initial conditions of the unirradiated quinone (Fig. 3); this did not occur when insonated Q_0 solutions were maintained under nitrogen (see Fig. 3). These findings suggested that the reactive species produced during ultrasonic irradiation could react with Q and partially reduce the quinone to quinol. In fact, when the $NaBH_4$ solution (5 $mg \cdot ml^{-1}$) was added at the end of the ultrasonic irradiation,

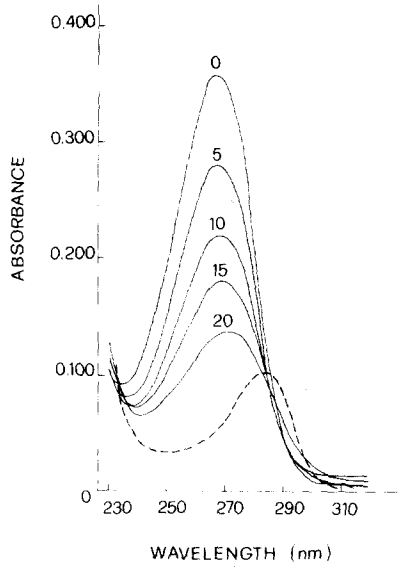

Fig. 2. Sequential spectra of 21 μM solution of ubiquinone-0 in 0.05 M Hepes (pH 7.2) exposed to insonation. — — —, spectrum of the same Q_0 solution upon addition of $NaBH_4$ solution at the end of insonation time. Numbers indicate insonation time in minutes.

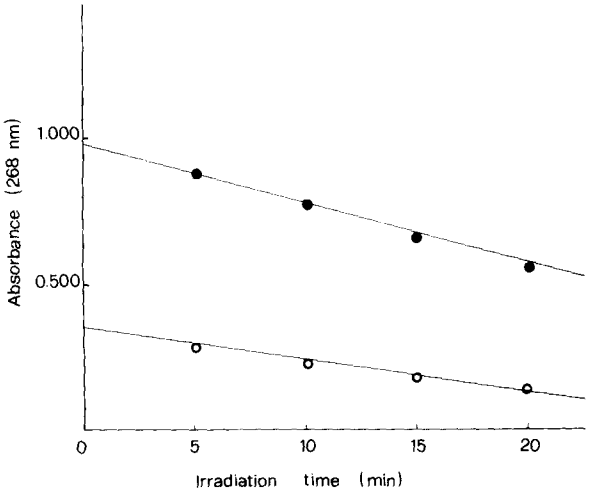

Fig. 1. Time course of the absorbance decrease at 268 nm of ubiquinone-0 solutions insonated under conditions described in Materials and Methods. ○——○, 24 μM Q_0; ●——● 64 μM Q_0.

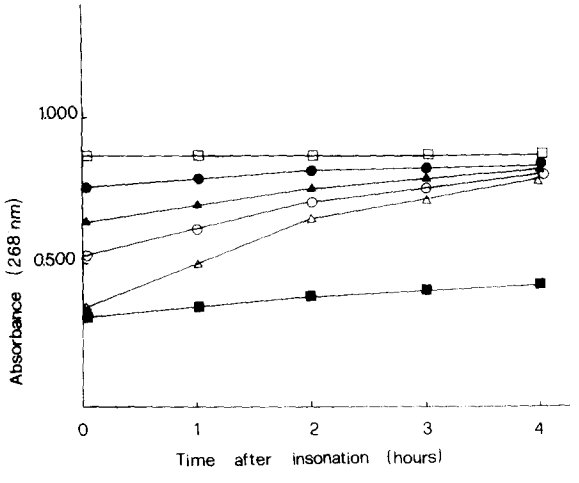

Fig. 3. Time course of the absorbance increase at 268 nm of 64 μM solutions of ubiquinone-0 after different time of insonation. □——□, not exposed to insonation; insonation time: 5 min (●——●), 10 min (▲——▲), 15 min (○——○), 20 min (△——△), 20 min and maintained under nitrogen (■——■).

the main absorbance band was bleached and a smaller band was formed with a maximum at 284 nm and there was an evident and well defined isosbestic joint at 285 nm (see Fig. 2). Furthermore, upon addition of a KOH solution the ubiquinone solution displayed again a spectrum with a maximum at 268 nm.

These spectral features are typical of the reduced and oxidized forms of ubiquinone, respectively. The last figure shows also that, four hours after the sonication, the absorbance values of insonated solutions never reached those of unirradiated solutions, suggesting that in our experiments radical reactions may have lowered the quinone yield. Probably either products with identical spectra, but different molar absorption coefficients or products with the ring cleaved may be formed.

A further attempt to investigate the products obtained after ultrasonic irradiation of Q_0 was made utilizing chromatographic analysis. The thin-layer chromatography, performed according to Petterson [13], was used to investigate whether the sonolyzed solution eventually contained isomeric *p*-benzoquinones in different stages of hydroxylation and the reduced and oxidized forms of the quinone. The ethanolic extract of the sample recorded prior to be chromatographed, showed a shoulder in the spectral feature of the oxidized form (Fig. 4), suggesting again the contemporaneous presence of ubiquinol and ubiquinone.

The presence of quinone and quinol in the irradiated sample was identified by cochromatography with not irradiated samples as controls and by their spectra. Hydroxylated forms of the quinone were not evidenced. This was probably due to the degradation of these molecules which led to compounds that could not be resolved by the solvent systems used.

No other attempt was made to identify these species.

In subsequent experiments we tested the nature of free radicals initiating the reduction and/or the degradation of Q_0. The reactive species produced in water and aqueous solution subjected to ultrasonic irradiation are hydroxyl radicals, hydrated electrons, hydrogen atoms (1), thus water is decomposed to yield H_2 plus H_2O_2. In the presence of oxygen, e^-_{aq} and H· are efficiently converted by

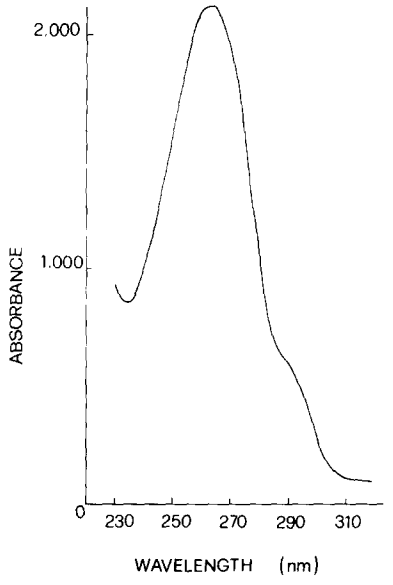

Fig. 4. Spectrum of ethanolic extract of ubiquinone-0 insonated for 20 min. After acidification of the buffered Q_0 solution with concentrated hydrochloric acid quinone was extracted with diethyl ether. The solvent was evaporated and the extract dissolved in ethanol.

oxygen into O_2^- [4,16] and additional pathways for the formation of H_2O_2 are produced. Under our experimental conditions the yield of hydrogen peroxide formed was 5.5 nmol · ml^{-1} · min^{-1}.

A direct proof of the role of superoxide radicals in reducing Q was obtained from studies on the inhibition of the absorbance decrease at 268 nm by superoxide dismutase. A 4 $\mu g \cdot ml^{-1}$ concentration of enzyme was able to considerably suppress the reduction process, as shown in Figs. 5A and 6. At the end of the insonation time the absorbance decrease at 268 nm of Q_0 was approximately 3-fold lower in the presence of the enzyme than in its absence (Fig. 6). Moreover, while in the absence of superoxide dismutase there was a relevant absorbance decrease with a red shift (Fig. 2) in its presence the red shift did not occur (Fig. 5A). Inactivated superoxide dismutase exhibited a slight inhibitory effect (Fig. 6) that could be probably explained by the remaining activity.

A check for possible involvement of hydroxyl radicals in the degradation of Q was made by carrying out some of the irradiations in the presence of 0.05 M formate. This scavenger was only

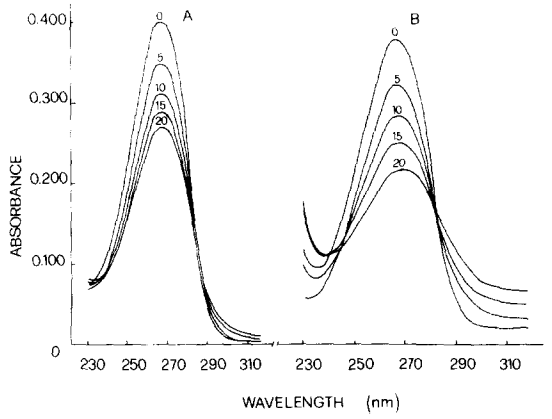

Fig. 5. (A) Sequential spectra of 25 μM solution of ubiquinone-0 in 0.05 M Hepes (pH 7.2) exposed to insonation in the presence of superoxide dismutase. (B) Sequential spectra of 18 μM solution of ubiquinone-0 in 0.05 M Hepes (pH 7.2) exposed to insonation in the presence of 0.05 M sodium formate. Numbers indicate insonation time in minutes.

able to provide about 42% protection against the absorbance decrease at 268 nm after a sonication time of 20 min (Fig. 6). In this case an absorbance decrease with a red shift occurred (Fig. 5B). About the same results were obtained also in the presence of 0.02 M formate (not shown). The experiments reported in Figs. 5B and 6 point to the fact that part of HO$^{\cdot}$ radicals were not accessible to formate.

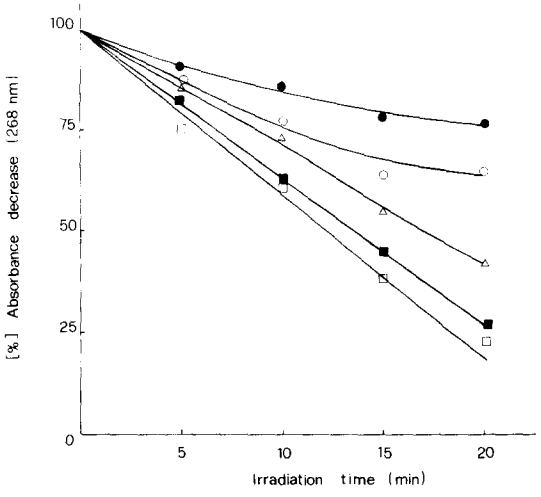

Fig. 6. The effect of superoxide dismutase, inactivated superoxide dismutase, formate, superoxide dismutase plus formate on the time course of the absorbance decrease of ubiquinone-0 in 0.05 M Hepes (pH 7.2). ●———●, Q_0 insonated in the presence of superoxide dismutase (4 $\mu g \cdot ml^{-1}$) and 0.05 M sodium formate, ○———○, Q_0 insonated in the presence of superoxide dismutase (4 $\mu g \cdot ml^{-1}$); ■———■, Q_0 insonated in the presence of inactivated superoxide dismutase (4 $\mu g \cdot ml^{-1}$); △———△, Q_0 insonated in the presence of 0.05 M sodium formate; □———□, Q_0 insonated under standard conditions described in Materials and Methods.

The contemporary addition of superoxide dismutase and formate to the Q solution subjected to sonication produced an absorbance decrease of only about 20% after an irradiation time of 20 min.

We then proceeded to investigate the reaction of oxygen radicals with Q in a membrane environment corresponding closely to its normal location in tissue. The effect of the presence or absence of a net surface charge was studied selecting a suitable bilayer including 10% of dimyristyl-phosphatidic acid or stearylamine in uncharged dimyristyl-phosphatidylcholine membranes.

Fig. 7 shows that only a positive surface charge allowed relatively easy access to oxygen radicals that could decrease Q_3 absorbance at 277 nm, while negatively charged or uncharged vesicles kept the radicals away from the quinone ring. When the spectra of Q_3 incorporated into DMPC-stearylamine vesicles were recorded after different sonication time, the 277 nm peak decreased and shifted progressively to a longer wavelength. In this case active superoxide dismutase

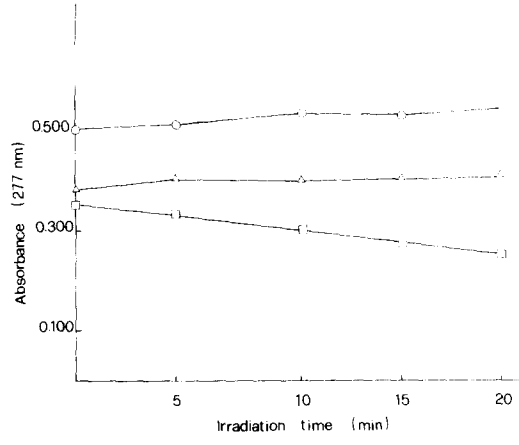

Fig. 7. Effect of the surface charge on the time course of the absorbance change of ubiquinone-3 in DMPC vesicles (○———○), in DMPC-DMPA vesicles (△———△) and in DMPC-stearylamine vesicles (□———□) insonated under conditions described in Materials and Methods.

was able to completely inhibit Q reduction (not shown).

Discussion

The main product of Q obtained after ultrasonic irradiation was ubiquinol, as confirmed by spectral features and by thin-layer chromatography analysis. The presence of the quinone reduced form, was not so evident in the spectra recorded after different irradiation times (cf. Fig. 2). On the other hand, when ubiquinone and ubiquinol were mixed in the same cuvette, the absorbance maximum of the mixture occurred between 268 and 284 nm, depending on the relative concentrations of the two forms. The poor resolution between the ubiquinone and the ubiquinol peaks did not allow to detect the isosbestic point (not shown).

During sonolysis oxygenated aqueous solutions buffered at pH 7.2 contain H_2O_2, $HO^{\cdot}$ and $O_2^{\cdot-}$ radicals. After 20 min sonication hydrogen peroxide concentration was estimated about 0.1 mM in the vessel. Such an amount added to a Q_0 solution was able to decrease the absorbance maximum of only about 5% without changing the spectral features of the spectrum (not shown). Thus the reduction in intensity of the ultraviolet absorbance band could not be attributed to H_2O_2, but to oxygen radicals.

It has been known that quinones can react with superoxide to give rise first to semiquinones [17,18], which via dismutation reaction, can lead to the formation of quinols and quinones again.

In the presence of superoxide dismutase the superoxide radical was converted to H_2O_2. Since the effect of H_2O_2 on the Q spectrum was minimal the reduction in intensity of the ultraviolet absorbance band in the presence of superoxide dismutase was attributed mainly to the formation of products due to addition reaction of $HO^{\cdot}$ with aromatic ring structure.

The presence of formate in the insonation vessel was expected to lead to the conversion of all the $HO^{\cdot}$ radicals to superoxide as a final product, as it occurs when these radicals are produced by radiolysis of aqueous solutions [5,19]. In this case the absorbance decrease with the red shift (Fig. 5B) would have depended upon the formation of the quinol. Thus the contemporary addition of superoxide dismutase and formate was expected to provide a total protection against the absorbance decrease. However, this did not occur (cf. Fig. 6). In fact in the case of ultrasonic irradiations the radicals are produced in oscillating tiny gas bubbles, and what is known from chemical studies on radiation in homogeneous solutions does not determine the efficiency of a scavenger for sonolytically produced hydroxyl radicals [20].

The experiments reported in Fig. 6 could point to the fact that a part of $HO^{\cdot}$ radicals are not accessible to formate. The quinone molecules, instead, being less polar, can be expected to enter into the gas bubbles and to be preferentially degraded.

The side-chain of ubiquinones places strategically Q molecules in membranes in order to fulfill the protective role against lipoperoxidation and, in mitochondria, to perform the most important role in electron transfer and energy conservation. Since the antioxidant effect is probably to be ascribed only to the substituted quinone ring and not also to the side-chain, as previously suggested [21], these data with a water-soluble Q homologue may partly clarify the reactions of this molecule with oxygen free radicals. However, as the quinone usual location is within the lipid bilayer, experiments were performed to evaluate the efficiency with which this antioxidant can compete for oxygen radicals when it is not in solution.

Our experiments reported in Fig. 7 point out that reactions of oxygen radicals with Q_3 incorporated into vesicles may be of minor significance since membranes present a barrier to their penetration. Sonolytically produced hydroxyl radicals do not threaten the antioxidant incorporated into lipid bilayer since they react as soon as formed with whatever organic molecule is in their vicinity. This reaction depends on the relative concentrations of target molecules: in the membrane system tested there were about 100 molecules of phospholipids for every Q_3 and the rate constants of reaction of $HO^{\cdot}$ with all organic components are likely to be similar [22,23]. The reaction between $O_2^{\cdot-}$ and Q_3 is significant only in the DMPC-stearylamine vesicles. In this system the positive field was evidently able to cause $O_2^{\cdot-}$ to be an effective reductant. In negative charged or uncharged membranes, the field effect was insuffi-

cient to overcome the removal of O_2^- by the dismutation reaction.

The first qualitative conclusion of this study is that, when model membranes are prepared by ultrasonic irradiation, radical production and a possible and consequent degradation of unsaturated phospholipids have to be considered.

As far as Q degradation by oxygen free radicals, in living as well as in model systems, we suggest that only HO· radicals formed in the vicinity of, or inside the membrane represent a potential danger for the antioxidant. The O_2^- radicals, instead, can act as one-electron reducing agent of ubiquinone to give rise to ubisemiquinone [17,18]. The quinol arising by spontaneous dismutation exhibits a higher antioxidant effect than the oxidized form [10,24].

Our results suggest that the mechanism by which Q could act as an antioxidant during the preparation of vesicles by sonication is not to be ascribed to its reaction with the radicals responsible for the initiation of lipoperoxidation. In our opinion Q acts as a chain-breaking antioxidant scavenging lipid radicals which propagate the free radical chain oxidation. The same mechanism can be suggested to explain the antioxidant role of ubiquinone in biological systems. Q molecules embedded in phospholipid bilayer probably do not interact with initiating free radicals produced in the aqueous environment. On the contrary, ubiquinone, stacking among phospholipid molecules and keeping the quinone ring in non polar phase [25], can prevent autocatalytic free radical reaction by inhibiting the propagation reaction.

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