Biophysical Chemistry 192 (2014) 20–26

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

# Reaction rates of α-tocopheroxyl radicals confined in micelles and in human plasma lipoproteins

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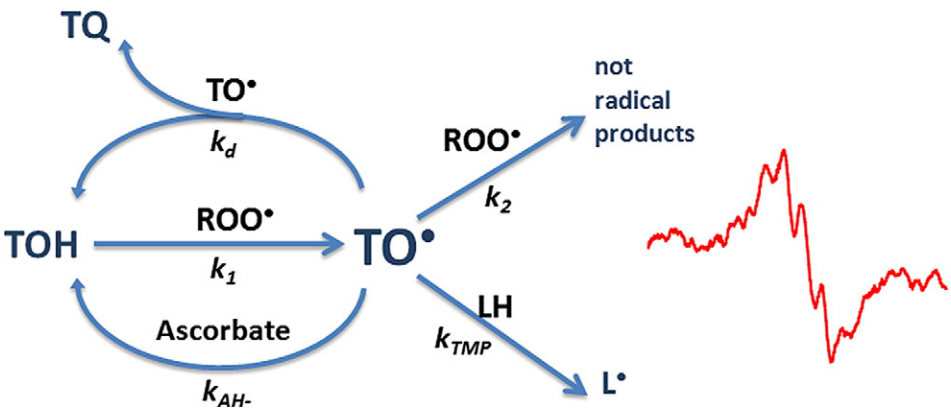
## HIGHLIGHTS

- Dismutation rate of tocopheroxyl radical is higher in micelle systems
- Dismutation kinetic constant of tocopheroxyl radical was calculated in human plasma
- Tocopheroxyl radical dismutation prevails under oxidative stress conditions

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 13 May 2014
Received in revised form 10 June 2014
Accepted 12 June 2014
Available online 21 June 2014

*Keywords:*
Vitamin E
Tocopherol
Dismutation rate constant
Ascorbate
Antioxidant
Tocopheryl quinone

## ABSTRACT

α-Tocopherol, the main component of vitamin E, traps highly reactive radicals which otherwise might react with lipids present in plasmatic lipoproteins or in cell membranes. The α-tocopheroxyl radicals generated by this process have also a pro-oxidant action which is contrasted by their reaction with ascorbate or by bimolecular self-reaction (dismutation). The kinetics of this bimolecular self-reaction were explored in solution such as ethanol, and in heterogeneous systems such as deoxycholic acid micelles and in human plasma. According to ESR measurements, the kinetic rate constant ($2k_d$) of the bimolecular self-reaction of α-tocopheroxyl radicals in micelles and in human plasma was calculated to be of the order of $10^5$ $M^{-1}$ $s^{-1}$ at 37 °C. This value was obtained considering that the reactive radicals are confined into the micellar pseudophase and is one to two orders of magnitude higher than the value we found in homogeneous phase. The physiological significance of this high value is discussed considering the competition between bimolecular self-reaction and the α-tocopheroxyl radical recycling by ascorbate.

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*Abbreviations:* α-TOH, α-tocopherol; ROO•, peroxyl radicals; ROOH, hydroperoxyl; α-TO•, α-tocopheroxyl radical; TMP, tocopherol mediated peroxidation; α-TQ, tocopheryl quinone; DCA, deoxycholic acid sodium salt; LG, rac1-lauroylglycerol; DPPH, diphenylpicrylhydrazyl; ABIP, 2,2′-azobis[2-(2-imidazolin-2-yl)propane] di-hydrochloride; HMW, high molecular weight; $R_0$, generation rate.

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http://dx.doi.org/10.1016/j.bpc.2014.06.002
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## 1. Introduction

The main component of vitamin E, α-tocopherol (α-TOH), has been considered the most important lipid-soluble radical-trapping antioxidant [1]. Unlike other antioxidants, this compound reacts selectively with hydroperoxyl radicals and protects the lipids present in plasmatic lipoproteins or in cell membranes. In the last 10 years new perspectives concerning vitamin E function are emerging, such as, for example, its potential role in gene regulation and cellular signaling [2]. In the light of this new research track, the oxidative fate of vitamin E and of its derivatives should gain even more importance. It has been largely demonstrated that vitamin E as antioxidant scavenges the peroxyl radicals ($ROO^{\bullet}$) and inhibits the free-radical chain reaction of lipid peroxidation so avoiding the formation of cytotoxic and genotoxic reaction products. This scavenging reaction generates α-tocopheroxyl radical (α-$TO^{\bullet}$) which, beyond to scavenge $ROO^{\bullet}$ radicals, may undergo several reactions [3]. In particular it can react with lipids and hydroperoxyl derivative producing, again, allilyc or $ROO^{\bullet}$ radicals (pro-oxidant action). This radical chain is referred to as the "tocopherol mediated peroxidation" (TMP) [4]. The pro-oxidant action of α-TOH is contrasted by the reaction of α-$TO^{\bullet}$ radical with ascorbate or by bimolecular self-reaction (dismutation). The rate of the bimolecular self-reaction appears a key factor to switch the α-TOH action from pro-oxidant to anti-oxidant and to give a physiological significance to the synergistic effect of ascorbate [5,6]. However the effective contribution of the α-$TO^{\bullet}$ bimolecular termination step (which occurs according to a second order kinetic constant $2k_d$) to α-TOH oxidative fate is still matter of debate since the $2k_d$ values reported until now in homogeneous systems vary in the range $0.061–6 \times 10^3\ M^{-1}\ s^{-1}$ [7–10]. Even though the self-reaction is recognized as a possible route of α-$TO^{\bullet}$ removal, its effective contribution is usually neglected [3]. As regards plasma lipoproteins, in which α-TOH is the major lipid-soluble antioxidant, a hypothetical $2k_d$ value of $1 \times 10^3\ M^{-1}\ s^{-1}$ has been suggested [4], while no values have been given in membranes or in α-TOH emulsions, where the rate of bimolecular decay of α-$TO^{\bullet}$ was found too fast to be measured [10,11]. The bimolecular decay of two α-$TO^{\bullet}$ radicals generates one molecule of α-TOH and one of tocopheryl quinone (α-TQ) via a multistep reaction [12]. α-TQ is a metabolite of α-TOH in vivo [13], and is a bio-reactive compound, which for example, could interfere with mitochondrial activity since it can behave as alternative substrate for some mitochondrial electron transfer enzymatic complex [12]. In the absence of water, the bimolecular self-reaction of α-$TO^{\bullet}$ produces also a highly reactive *o*-quinon methide intermediate [14,15], the structure of which is similar to that of 1,2-benzoquinone [7,9,16,17].

In this work we have studied the kinetics of the bimolecular decay of α-$TO^{\bullet}$, in ethanol solutions and in heterogeneous systems such as deoxycholate (DCA) micelles and human plasma by ESR. This technique, at difference from other spectroscopic techniques [18] allows the detection of α-$TO^{\bullet}$ produced by physiological concentrations of α-TOH. Kinetic rate constant values have been calculated in the bulk phase and in the lipid phase taking into account the effects of the confinement of the reacting species.

## 2. Materials and methods

### 2.1. Materials

Carboxy proxyl; deoxycholic acid sodium salt (DCA); rac1-lauroylglycerol (LG); diphenylpicrylhydrazyl (DPPH); and α-TOH, sodium mono- and diphosphate, dichloromethane, ethanol, methanol, ascorbate oxidase from *Cucurbita* sp., uricase from *Bacillus fastidiosus*, and catalase from *Aspergillus niger* were purchased from Sigma-Aldrich (Buchs, Switzerland). The 2,2′-azobis[2-(2-imidazolin-2-yl)propane] di-hydrochloride (ABIP) was a kind gift of Wako Chemicals (Germany). All the aqueous solutions were prepared with distilled water. Buffer solutions were equilibrated in batch with Chelex-100 (Bio-Rad, Richmond, CA, USA) to minimize the concentration of heavy metal ions.

### 2.2. Preparation of reagent solutions

A stock solution of 25 mM α-TOH was prepared in ethanol 99.8% and stored in dark at room temperature and used within the day. The concentration of α-TOH was monitored by a Varian Cary 50 Scan UV-visible spectrophotometer. DPPH (10 mM) was prepared in methanol 99.9% and stored at 4 °C. ABIP (0.5 M) was solubilized in water and stored at −20 °C.

### 2.3. ESR analysis

X band (9.8 GHz) ESR spectra were acquired by a Bruker ER200D spectrometer, equipped with a TE standard cavity, and interfaced with a personal computer for data acquisition and processing. The modulation frequency was 100 kHz. The samples to be analyzed were transferred by a short glass transfer line into a quartz flat cell inserted in the ESR cavity and kept at 37.0 ± 0.2 °C. A carboxy proxyl solution was used as calibration standard for concentration measurements [19]. To calculate the α-$TO^{\bullet}$ radical concentration, the whole spectrum area was compared with that of the carboxy proxyl at known concentration.

### 2.4. Homogeneous solutions

DPPH (0.15 mM final concentration) was added to a solution of α-TOH (0.3 mM, final concentration) in ethanol, containing 2% v/v of water, equilibrated at 37 °C. The reaction system was immediately transferred into the quartz flat cell inserted in the ESR cavity and the spectrum acquisition was started within 1 min from the DPPH addition. Because the reaction is very fast, and since the line shape of α-$TO^{\bullet}$ signal does not change during the kinetic experiments, only the central peak was recorded to reduce the acquisition time (scan rate 0.66 G $s^{-1}$, time constant 2 s). The peak to peak height (hpp) of the central peak was used instead of the whole spectrum area (A) and the radical concentration was calculated from the ratio hpp/A of a α-$TO^{\bullet}$ radical spectrum normalized to the whole spectrum of carboxy proxyl at known concentration.

In the experiments where a stationary concentration of α-$TO^{\bullet}$ radicals was needed, ABIP was used to generate a constant flux of peroxyl radicals. In these experiments, α-TOH was diluted in ethanol at 37 °C and its final concentration was in the range 0.003–23 mM. ABIP 8 mM final concentration, previously dissolved in water, was added to the α-TOH solutions. After this addition, the water concentration in the alcoholic solution was 10% (v/v). The reaction solution was transferred into the quartz flat cell. When steady state conditions were reached for the α-$TO^{\bullet}$ radical, its concentration was calculated from the whole area of its ESR spectrum. This spectrum was acquired with higher resolution than that used for the kinetic experiment (scan rate 0.25 G $s^{-1}$, time constant 5 s).

### 2.5. Micelle system

The micelle system was prepared drying 100 μL of 10 mM LG in dichloromethane and by dissolving the obtained film in 2 mL of 20 mM phosphate buffer containing 5 mM DCA and 110 mM NaCl, pH 7.4. α-TOH (2–150 μM final concentration) was added and the solution was vortexed for 1 min and equilibrated with atmospheric oxygen by stirring at 37 °C [20]. After the thermal equilibrium was achieved, ABIP was added in the range of 0.5–16 mM and the resulting solution was immediately transferred into the quartz flat cell. In the case of kinetics carried out with the lowest α-TOH amounts, the α-$TO^{\bullet}$ radical concentration was calculated as the mean value of some consecutive scans, to increase the signal to noise ratio.

### 2.6. Human plasma

Human blood samples were withdrawn from volunteers after overnight fasting into heparinized glass tubes. The samples were immediately centrifuged at 1000 ×g for 10 min for plasma separation. Plasma was purified from ascorbic and uric acid by enzymatic treatment or by molecular exclusion chromatography.

To estimate the time required for the complete oxidation of ascorbate and uric acid, standard solutions reproducing the usual plasma concentrations were used. Standard solutions of 50 μM ascorbic acid in PBS, pH 7.4, and of 300 μM uric acid were incubated with ascorbate oxidase ($2 \times 10^{-3}$ U/mL) and with uricase (1.3 U/mL) and catalase (6 U/mL), respectively. The oxidation of ascorbate and uric acid was monitored by the disappearance of absorbance at 268 and 291 nm, respectively. To be sure that the complete oxidation of ascorbic and of uric acid took place, plasma was incubated with the same enzyme levels for a period twice as much the time required for the standard solutions.

The high molecular weight (HMW) fraction was quickly obtained from the fresh samples of human plasma by molecular exclusion chromatography. A PD-10 column Sephadex™ G-25 Medium (Amersham Pharmacia Biotech) was used to separate high (Mr > 5000) from low (Mr < 1000) molecular weight substances. After the column equilibration with the buffer (20 mM phosphate buffer, containing 0.5 M NaCl, pH 7.4), 2.5 ml of plasma was added. A preliminary elution test was carried out to set up the procedure: fractions of 0.5 ml were successively collected and the protein concentration was measured at 280 nm, while ascorbic and uric acid levels were monitored by UV-vis spectrophotometry, before and after addition of ascorbate oxidase, uricase and catalase in each fraction. It appeared that the HMW fraction was eluted completely within the first 4.5 mL while ascorbic and uric acid were eluted starting from the 5 mL fraction. Therefore, only the first 4 mL fraction of eluted plasma was collected and HMW concentration was measured at 280 nm.

The HMW solution and the plasma depleted of ascorbic and uric acid were equilibrated with atmospheric oxygen by stirring at 37 °C. After the thermal equilibrium was achieved, 4 mM ABIP (final concentration) was added and the solution was immediately transferred into the quartz flat cell.

### 2.7. Kinetic scheme

By considering a system where ROO$^\bullet$ are generated in the presence of α-TOH, the reactions involving the scavenging of ROO$^\bullet$ radicals by α-TOH can be summarized as follow:

$$\text{ROO}^\bullet + \alpha\text{-TOH} \xrightarrow{k_1} \text{ROOH} + \alpha\text{-TO}^\bullet \quad (1)$$

$$\text{ROO}^\bullet + \alpha\text{-TO}^\bullet \xrightarrow{k_2} \text{not radical products} \quad (2)$$

$$\alpha\text{-TO}^\bullet + \alpha\text{-TO}^\bullet \xrightarrow{k_d} \alpha\text{-TOH} + \alpha\text{-TQ} \quad (3)$$

In the presence of a constant source of ROO$^\bullet$ radicals, when the steady-state conditions for the radical species involved (ROO$^\bullet$ and α-TO$^\bullet$) are reached, the corresponding steady-state equations are as follows:

$$d[\text{ROO}^\bullet]/dt = R_0 - k_1 [\text{ROO}^\bullet][\alpha\text{-TOH}] - k_2 [\text{ROO}^\bullet][\alpha\text{-TO}^\bullet] \approx 0 \quad (4)$$

$$d[\alpha\text{-TO}^\bullet]/dt = k_1 [\text{ROO}^\bullet][\alpha\text{-TOH}] - k_2 [\text{ROO}^\bullet][\alpha\text{-TO}^\bullet] - 2k_d [\alpha\text{-TO}^\bullet]^2 \approx 0 \quad (5)$$

where $R_0$ is the rate of generation of ROO$^\bullet$ radicals [20].

From the above equations the following expression can be easily obtained

$$2k_d = \left(R_0/[\alpha\text{-TO}^\bullet]^2\right)\ (K[\alpha\text{-TOH}] - [\alpha\text{-TO}^\bullet])/(K[\alpha\text{-TOH}] + [\alpha\text{-TO}^\bullet]) \quad (6)$$

where $K = k_1/k_2$.

From Eq. (6) we obtain

$$K = k_1/k_2 = \left(R_i[\alpha\text{-TO}^\bullet] + 2k_d[\alpha\text{-TO}^\bullet]^3\right)/\left(R_i[\alpha\text{-TOH}] - 2k_d[\alpha\text{-TOH}]\ [\alpha\text{-TO}^\bullet]^2\right) \quad (7)$$

When α-TO$^\bullet$ concentration approaches zero Eq. (7) becomes

$$K = k_1/k_2 = [\alpha\text{-TO}^\bullet]/[\alpha\text{-TOH}] \quad (8)$$

while increasing α-TOH concentration Eq. (6) becomes

$$2k_d = R_0/[\alpha\text{-TO}^\bullet]^2 \quad (9)$$

which means that all the ROO$^\bullet$ radicals are captured by α-TOH (saturation condition).

## 3. Results and discussion

### 3.1. Formation and decay of tocopheroxyl radical in solutions

The addition of DPPH to a solution of α-TOH in ethanol, at [α-TOH]/[DPPH] > 1, brought to the almost instantaneous decay of DPPH as it is shown by the disappearance of the characteristic UV-Vis signal of the DPPH radical with the concomitant formation of α-TO$^\bullet$ radicals. The α-TO$^\bullet$ ESR spectrum is shown in Fig. 1, inset. The α-TO$^\bullet$ radicals decay according to reaction 3, that is by a second order process, is shown in Fig. 1.

For this process we calculated a kinetic rate constant $2k_d = (7.6 \pm 0.5) \times 10^3\ M^{-1}\ s^{-1}$, at 37 °C. Analogous experiments, carried out in ethanol solution in the presence of 5 mM DCA, gave similar results ($2k_d = (7.4 \pm 0.4) \times 10^3\ M^{-1}\ s^{-1}$, at 37 °C, see Table 1) indicating that DCA molecules are not involved in the decay of α-TO$^\bullet$ radicals. These $2k_d$ values are higher than the $2k_d$ value measured in ethanol by Rousseau-Richard et al. [9], obtained using DPPH as α-TO$^\bullet$ radical generator ($2k_d = 1.4 \times 10^3\ M^{-1}\ s^{-1}$ at 20 °C). This difference can be attributed to the dissimilarity between the experimental temperatures.

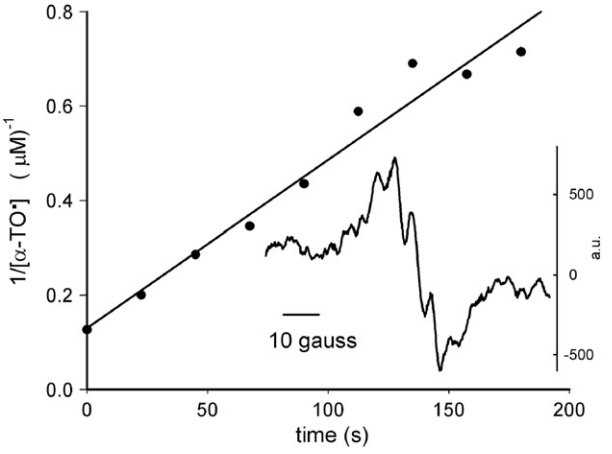


**Fig. 1.** Decay of α-TO$^\bullet$ radicals in ethanol solution. The reaction was performed in an ethanol solution containing 2% of $H_2O$, 150 μM DPPH and 300 μM α-TOH, at 37 °C. Inset: ESR spectrum of α-TO$^\bullet$ radical. Instrumental ESR settings: modulation amplitude 2 Gpp, scan rate 0.66 G $s^{-1}$, scan time 12 s, time constant 2 s and microwave power 20 mW.

**Table 1**
Rate constant of α-TO• dismutation in various systems at 37 °C.

| System | $2k_d$ $M^{-1}$ $s^{-1}$ |
|---|---|
| Ethanol | DPPH $(7.6 \pm 0.5) \times 10^3$ |
| Ethanol, 10% $H_2O$ | ABIP $(1.21 \pm 0.08) \times 10^4$[a] |
| Ethanol, 10% $H_2O$ | ABIP $(1.72 \pm 0.2) \times 10^4$[b] |
| DCA micelles | ABIP $(1.03 \pm 0.02) \times 10^5$[c,g] |
| DCA micelles | ABIP $(1.43 \pm 0.33) \times 10^5$[d,g] |
| DCA micelles | ABIP $(1.58 \pm 0.11) \times 10^5$[e,g] |
| Plasma | ABIP $2.0 \times 10^5$[f,g] |

[a] ABIP 8 mM, α-TOH in the range 0.003–23 mM.
[b] 12 mM α-TOH, ABIP in the range 0.5–16 mM.
[c] ABIP 2 mM, α-TOH in the range 2–150 μM.
[d] 0.1 mM α-TOH, ABIP 8 mM, DCA in the range 5–100 mM.
[e] 0.1 mM α-TOH, ABIP in the range 0.5–12.8 mM.
[f] ABIP 4 mM.
[g] These values were calculated considering the volume of micelles or of lipoproteins.

α-TO• radicals were also generated in ethanol containing 10% of $H_2O$, by reaction of α-TOH with ROO• radicals produced at constant rate $R_0$, by ABIP ($R_0 = 38.7$ nM $s^{-1}$). The steady-state concentration of α-TO• radicals obtained under these conditions was monitored by ESR, and the dependence of this concentration on that of α-TOH has been reported in Fig. 2. From this figure it appears that, at α-TOH concentrations below 1 mM, a linear relationship between the concentrations of α-TO• and α-TOH holds. At α-TOH concentrations above 2 mM this dependence deflects from linearity, and a saturation effect appears. From the linear part of the plot of Fig. 2 the ratio $K = k_1/k_2$, calculated according to Eq. (8), was $(0.42 \pm 0.03) \times 10^{-3}$. This datum is close to the $K$ value of $1.7 \times 10^{-3}$ which can be calculated from the data of Bowry and Ingold [4], obtained in tert-butanol. Furthermore, from the asymptote of Fig. 2 and according to Eq. (9), a $2k_d$ value of $(1.21 \pm 0.08) \times 10^4$ $M^{-1}$ $s^{-1}$ was calculated at 37 °C.

We also studied the dependence of α-TO• concentration on $R_0$, being $R_0$ (M $s^{-1}$) $= (4.84 \pm 0.30) \times 10^{-6}$ [ABIP] in the range $(0.5–16) \times 10^{-3}$ M ABIP. We carried out these experiments with a bulk concentration of α-TOH high enough (12 mM) to make reaction 2 negligible with respect to reaction 1. Under these conditions Eq. (9) holds and, from the linear dependence of α-TO• concentration on the square root of $R_0$ ($R_0$ was in the range 5–80 nM $s^{-1}$), a $2k_d$ value of $(1.72 \pm 0.2) \times 10^4$ $M^{-1}$ $s^{-1}$ was calculated, in agreement with the $2k_d$ value obtained from the data of Fig. 2.

The $2k_d$ values, found from the dependence of α-TO• on ABIP and on α-TOH concentrations, are slightly higher than that we have calculated from the decay of α-TO• radical after addition of DPPH in ethanol (Table 1). This difference may be due to the higher concentration of water that increases the polarity of the reaction medium in the experiments carried out with ABIP. In fact solvent polarity appears one of the causes of the different values measured for $2k_d$ [9].

### 3.2. Formation and decay of tocopheroxyl radical in a micelle system

Experiments were performed in aqueous solution containing DCA micelles and various concentrations of α-TOH. ABIP in mM range was added to this system to generate a constant flow of peroxyl radicals. We investigated the dependence of α-TO• radicals on α-TOH concentrations, at constant DCA (5 mM). In Fig. 3 we show the relationship between the bulk concentration of [α-TO•] and [α-TOH]. From this figure, it appears that the α-TO• radical concentration increases linearly with the α-TOH concentration in the low micro-molar range. A value of $K = k_1/k_2 = (1.02 \pm 0.07) \times 10^{-3}$ was calculated from this straight line, according to Eq. (8). Furthermore the [α-TO•] tends to a plateau value of about 15 nM at [α-TOH] ≥ 50–60 μM. The plateau concentration in the nano-molar range is at least ~$10^2$ times lower than that we have measured in ethanol, and clearly indicates that the rate of disappearance of α-TO• radicals is much higher than that we found in ethanol solutions. Accordingly, from Eq. (9) and from the asymptotic value of [α-TO•] of Fig. 3, an apparent $2k_d$ value of $4.04 \times 10^7$ $M^{-1}$ $s^{-1}$ was calculated. This value is about 3000–4000 times higher than the $2k_d$ value we have measured in ethanol (Table 1) and any previously reported value for the bimolecular self-reaction of α-TO• [7]. Bowry and Ingold suggested that a $XH_2$ species (derived from a spiro-dimer of quinone methide reduction) is present as impurity in α-TOH and contributes to the α-TO• disappearance, leading to the overestimation of $2k_d$ [7]. This does not seem to be our case since, from the experiments performed in ethanol using DPPH as α-TO• generator, we found a $2k_d$ value very close to that reported in literature [9].

Reactions in surfactant solutions are usually treated using pseudophase models in which the reactants distribute between the bulk aqueous phase and the micellar pseudophase. The sum of the rates of concurrent reactions in the two phases constitutes the overall rate [21,22]. However in our case the hydrophobic tail of α-TOH concentrates this compound in the lipophilic domain of DCA micelles, and inhibits its inter-micellar transfer [23]. Therefore DCA micelles can be

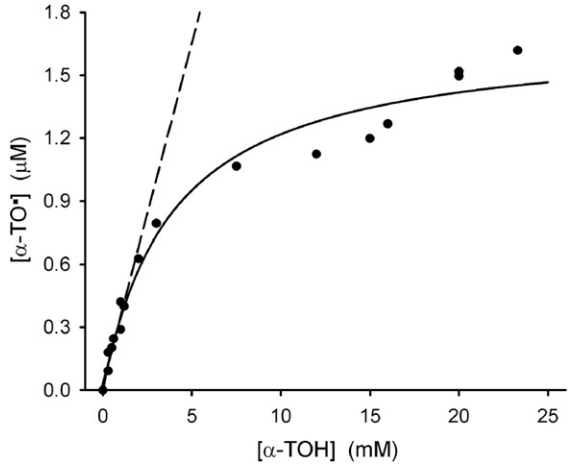


**Fig. 2.** Dependence of α-TO• concentration on that of α-TOH in ethanol solution in the presence of a system generating ROO• radicals at constant rate. The reaction was performed in ethanol solutions containing 10% of $H_2O$, 8 mM ABIP and α-TOH, at 37 °C. Instrumental ESR settings: modulation amplitude 4 Gpp, scan rate 0.25 G $s^{-1}$, time constant 5 s and microwave power 20 mW.

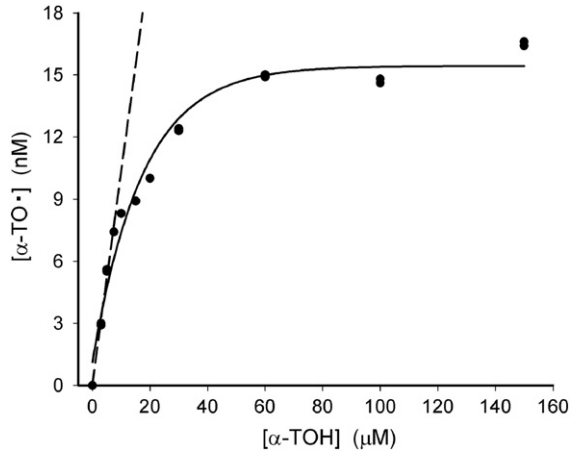


**Fig. 3.** Dependence of α-TO• concentration on that of α-TOH in an aqueous solution containing DCA micelles in the presence of a system generating ROO• radicals at constant rate. The reaction was performed in 20 mM phosphate buffer solutions, pH 7.4, containing 110 mM NaCl, 5 mM DCA, 0.5 mM LG, 2 mM ABIP and α-TOH, at 37 °C. Instrumental ESR settings as in Fig. 2.

considered as a homogeneous phase where the reactions involving α-TOH and its radical are confined, and the corrected $2k_d$ values can be obtained by supposing the volume of DCA micelles as the reaction volume. According to this hypothesis and assuming a molar volume of 0.515 L/mol for DCA [24], the local α-TOH and α-TO$^\bullet$ concentrations in 5 mM DCA micelles are about 400 times higher than those in the total volume. The much higher effective concentration of the confined α-TO$^\bullet$ radicals determines a higher decay rate of these radicals. This increase of decay rate explains the low concentration of α-TO$^\bullet$ (in nano-molar range) we measured in DCA micelles with respect to homogeneous systems. On the basis of the radical confinement, the asymptotic concentration value of α-TO$^\bullet$ in Fig. 3 has to be multiplied by a factor of 400. We considered that this asymptotic value corresponds to the condition of the complete capture by α-TOH of the ROO$^\bullet$ radicals generated by ABIP. We also assumed that this capture reaction occurs at the micelle outer layer, where α-TOH exposes its hydroxyl reactive group which transfers into the lipophilic phase the radical character beard by hydrophilic ROO$^\bullet$ [25]. Under these conditions Eq. (9) can be applied and we obtained $2k_d = (1.03 \pm 0.02) \times 10^5\ M^{-1}\ s^{-1}$.

To confirm the hypothesis that the α-TO$^\bullet$ dismutation process occurs in the DCA micelles and that the apparent value of the kinetic constant should be corrected taking into account the effective micelle volume, we changed the bulk concentration of DCA (from 5 to 100 mM) while the concentration of α-TOH in the total volume was kept constant (100 μM) to achieve the asymptotic α-TO$^\bullet$ concentration. In Table 2, column 3, we reported the concentration of α-TO$^\bullet$ confined in DCA micelles (obtained on the basis of their bulk concentration measured by ESR and corrected for the micelle volume), while in the last column we reported the corrected $2k_d$ values calculated according to Eq. (9). Although the DCA bulk concentration varied by a factor of 20, the calculated $2k_d$ values are invariant, being $2k_d = (1.43 \pm 0.33) \times 10^5\ M^{-1}\ s^{-1}$, confirming the correctness of our approach.

In DCA micelles in the presence of a bulk concentration of 100 μM α-TOH, a linear dependence of the concentration of α-TO$^\bullet$ radicals on the square root of the rate of ROO$^\bullet$ generation ($R_0$) was found. In these experiments, $R_0$ ranged between 2.5 and 62 nM $s^{-1}$. From the slope of this linear plot a $2k_d$ value of $(1.58 \pm 0.11) \times 10^5\ M^{-1}\ s^{-1}$ was calculated, according to Eq. (9), in agreement with the values reported in Table 1.

The values $2k_d$ we have measured in DCA micelles are about one order of magnitude higher than that we have found in ethanol under similar experimental conditions (Table 1). Since inter-phase mass transfer effects must be excluded [23], the observed difference between $2k_d$ values should be ascribed to the aqueous environment. In fact in water the radical of the soluble analog trolox dismutes with a rate constant of $10^5$–$10^7\ M^{-1}\ s^{-1}$, depending on pH [26].

### 3.3. Formation and decay of α-tocopheroxyl radical in human plasma

Experiments were carried out using the high molecular weight fraction of freshly withdrawn human plasma. This fraction contains the plasmatic lipoproteins where the vitamin E is concentrated, and is free from ascorbate and uric acid and from other unknown low molecular weight molecules with possible antioxidant action. ABIP (4 mM) (final concentration) was added to the HMW fraction kept at 37 °C, to obtain

**Table 2**
Rate constant of α-TO$^\bullet$ dismutation in different DCA concentration.

| $[DCA]_{bulk}$ mM | DCA volume mL | $[\alpha\text{-TO}^\bullet]_{bulk}$ nM | $[\alpha\text{-TO}^\bullet]_{DCA}$ μM | $(R_0)_{DCA}$ μM $s^{-1}$ | $2k_d$ $M^{-1}\ s^{-1}$ |
|---|---|---|---|---|---|
| 100 | 50 | 98.6 | 1.97 | 0.77 | $1.99 \cdot 10^5$ |
| 50 | 25 | 82.6 | 3.3 | 1.55 | $1.42 \cdot 10^5$ |
| 25 | 12.5 | 60.6 | 4.84 | 3.1 | $1.32 \cdot 10^5$ |
| 12.5 | 6.25 | 46.9 | 7.54 | 6.2 | $1.09 \cdot 10^5$ |
| 5 | 2.5 | 27 | 10.8 | 15.51 | $1.33 \cdot 10^5$ |

The experiments were carried out with $[\alpha\text{-TOH}]_{bulk} = 100$ μM and $[ABIP]_{bulk} = 8$ mM.

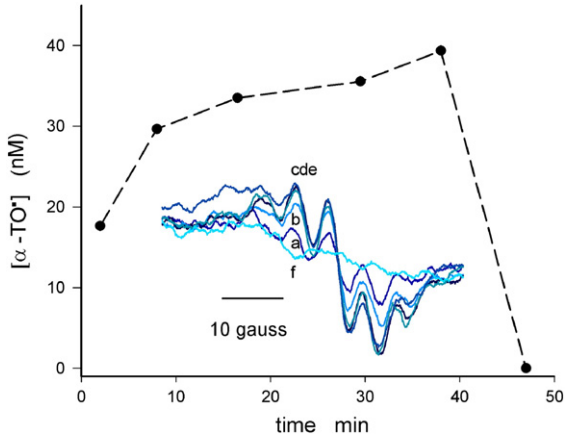


**Fig. 4.** Time dependence of α-TO$^\bullet$ radical in high molecular weights (HMW) of human plasma in the presence of a system generating ROO$^\bullet$ radicals at constant rate. The experiment was performed in HMW in the presence of 4 mM ABIP, at 37 °C. Inset: ESR spectrum of α-TO$^\bullet$ radical at 2 min (a), 8 min (b), 16.5 min (c), 29.5 min (d), 38 min (e), and 47 min (f). ESR settings: modulation amplitude 4 Gpp, scan rate 0.875 G $s^{-1}$, time constant 5 s and microwave power 20 mW.

a rate of peroxyl radical generation of about 20 nM $s^{-1}$. The ESR spectrum of α-TO$^\bullet$ radical showed a transient increase, which was over within few minutes from ABIP addition to HMW, see Fig. 4. This increase was not observed in DCA micelles. Since all the free low-molecular weight antioxidants were removed by exclusion chromatography, the transient increase could be ascribed to the antioxidants present in HMW, which compete with vitamin E for peroxyl radicals, till their complete consumption. These antioxidants may be ubiquinol-10 and bilirubin bound to albumin which are consumed before α-TOH [4]. After the increase phase, α-TO$^\bullet$ maintains a steady-state value of about 30 nM for about 30–40 min. In this period of time vitamin E, the concentration of which is in the range 20–30 μM, is consumed [27]. Analogous experiments were carried out using freshly withdrawn human plasma incubated for few minutes with ascorbate oxidase and uricase to eliminate ascorbate and uric acid. These experiments gave similar results, and also in this case the α-TO$^\bullet$ steady state concentration was about 30 nM. This behavior strongly indicates that the generation of α-TO$^\bullet$ radicals, at the constant rate $R_0$, is balanced by the fast decay of these radicals as observed in the α-TOH-DCA micelles. Various routes of α-TO$^\bullet$ removal could account for the α-TO$^\bullet$ constancy at a concentration of about 30 nM that we observed in the presence of HMW or of human plasma deprived of ascorbate and uric acid.

### 3.4. 1th route: α-TO$^\bullet$ disappears by the TMP process

A possible route is the reaction of α-TO$^\bullet$ with unsaturated lipids (LH) of plasma lipoproteins that is the starting step of the TMP process. In this case, under the steady-state conditions of Fig. 4 we can write:

$$d[\alpha\text{-TO}^\bullet]/dt = R_0 - k_{TMP}[LH]\ [\alpha\text{-TO}^\bullet] \approx 0$$

According to this equation and on the bases of the data of Bowry et al. which reported a TMP value of the propagation step $k_{TMP}$ [LH] ≈ 0.03 $s^{-1}$ in the case of LDL [4], we estimated that 0.65 μM α-TO$^\bullet$ should be present under our experimental conditions. The much lower experimental α-TO$^\bullet$ concentration strongly indicates that the TMP process is negligible. This is in accord also with the findings of Bowry and Stocker [28]. In fact these authors reported that in the presence of ROO$^\bullet$ fluxes similar to those we used in our experiments the amount of generated hydroperoxides LOOH in LDL is much smaller than the amount of α-TOH disappeared. We confirmed that TMP process is negligible under the conditions of Fig. 4 performing the following experiment. Briefly, a fresh amount of HMW (20%) was added to a

solution of 4 mM ABIP as source of ROO$^\bullet$ at 37 °C. The rate of $O_2$ consumption due to the generation of ROO$^\bullet$ radicals from ABIP did not change after the addition of fresh HMW (data not shown). This indicates that vitamin E present in HMW and the products of its reaction with ROO$^\bullet$ do not fire up a peroxidation process.

Alternatively from the 1st route, in the absence of the TMP process, a second and a third reaction routes for α-TO$^\bullet$ decay appear possible: the reaction between α-TO$^\bullet$ and ROO$^\bullet$ radicals or the bimolecular self-reaction of α-TO$^\bullet$ radicals.

### 3.5. 2nd route: α-TO$^\bullet$ disappears by reaction with ROO$^\bullet$ radicals

In this case we can neglect the term $2k_d[\alpha\text{-TO}^\bullet]^2$ in Eq. (5) and therefore Eq. (8) holds. This equation establishes a linear relationship between α-TO$^\bullet$ and α-TOH concentration. However the data of Fig. 4 are in contrast with this hypothesis, since during the α-TO$^\bullet$ steady-state, α-TOH is progressively consumed [4].

### 3.6. 3rd route: α-TO$^\bullet$ disappears by bimolecular self-reaction

In this case the term $2k_d[\alpha\text{-TO}^\bullet]^2$ predominates in Eq. (5) and therefore Eq. (9) can be applied. Under our experimental conditions the lifetime $\tau$ of α-TO$^\bullet$ is about 1.5 s ($\tau = [\alpha\text{-TO}^\bullet]_{\text{steady-state}}/R_0$) and $2k_d = 2 \times 10^7\ M^{-1}\ s^{-1}$ can be calculated considering the α-TOH concentration in bulk.

The intriguing question is how two α-TO$^\bullet$ radicals can meet together. The α-TO$^\bullet$ radicals (the concentration of which is about 30 nM) are distributed among the lipoproteins particles, the concentration of which in human plasma is in the low μM range. Then, in average about 1/100 of the lipoprotein particles contain one α-TO$^\bullet$ radical under our steady-state conditions. Therefore, the encounter between two radicals confined in two different lipoproteins should be highly improbable.

The diffusion of α-TO$^\bullet$ radical inside a lipoprotein particle is very fast (the time necessary to diffuse from one side to the opposite site of a LDL particle is of the order of few ms) [27]. As a consequence, the presence of two α-TO$^\bullet$ radicals in the same lipoprotein particle should bring to their rapid disappearance. Conversely, the diffusion between particles is very slow due to the strong anchoring of tocopherols by their hydrophobic tail to the lipid phase [23]. The phospholipid-transfer protein present in plasma, which is responsible for the homeostasis of vitamin E, can contribute to the transfer of α-TO$^\bullet$ radicals between different lipoprotein particles [29]. However, being this process protein-mediated, we expect to be slow [30]. A possible reaction pathway should be the intermicellar transfer of a hydrogen atom rather than the α-TO$^\bullet$. The hydrogen transfer between two lipoprotein particles can be also mediated by the lipid hydroperoxides (LOOH) which are presumed to reside on the membrane surface or by the metal ions weakly bound to phospholipid head [26,31]. So that the probability that two α-TO$^\bullet$ are in the same particle is increased. The mediated mechanism occurs according to the scheme:

$$(\alpha\text{-TO}^\bullet)_A + (\text{LOOH})_B \rightarrow (\alpha\text{-TOH})_A + (\text{LOO}^\bullet)_B$$
$$(\text{LOO}^\bullet)_B + (\alpha\text{-TOH})_B \rightarrow (\text{LOOH})_B + (\alpha\text{-TO}^\bullet)_B$$

and:

$$(\alpha\text{-TO}^\bullet)_A + (\text{Fe(II)})_B + H^+ \rightarrow (\alpha\text{-TOH})_A + (\text{Fe(III)})_B$$
$$(\alpha\text{-TOH})_B + (\text{Fe(III)})_B \rightarrow (\alpha\text{-TO}^\bullet)_B + (\text{Fe(II)})_B + H^+$$

where the suffix A and B refer to different lipoprotein particles. A fast micelle diffusion followed by electron transfer has already been reported [32]. These mechanisms require that the α-TO$^\bullet$ radicals are confined in plasmatic lipoproteins and therefore, if they are sufficiently fast, the apparent kinetic rate constant of the bimolecular self-reaction should be recalculated taking the lipoprotein volume as the reaction volume. Since the approximate volume occupied by lipoproteins is about 1% of the volume of plasma [33], on the basis of Eq. (9) we obtain $2k_d = 2 \times 10^5\ M^{-1}\ s^{-1}$. This value is close to that we measured in DCA micelles.

To confirm these values we estimated the $k_d$ value from the data reported in literature for similar experiments. From the kinetic experiments of Bisby and Parker [34] performed in phosphatidylcholine bilayer, we estimated a $2k_d$ value of about $3 \times 10^7\ M^{-1}\ s^{-1}$ in bulk and of $2 \times 10^4\ M^{-1}\ s^{-1}$ if we assume the lipid bilayer as the actual reaction volume. The values of $k_d$ calculated in DCA micelles, phospholipids bilayer and lipoproteins confirm the soundness of the experimental results and of the assumption we made.

According to the measured kinetic constant, the bimolecular self-reaction in human plasma becomes competitive with the recycling of α-TOH by ascorbate under oxidative stress conditions. In fact, under physiological conditions the concentration of ascorbate in human plasma is in the range 20–60 μM [35], and the radical flux ($R_0$) in vivo is lower than 1 nM $s^{-1}$ [36]. On these bases a value of $[\alpha\text{-TO}^\bullet]_{\text{bulk}}$ of the order of $10^{-9}$ M or below is expected. These concentrations make the dismutation self-reaction negligible with respect to the recycling by ascorbate which occurs with a kinetic constant $k_{rc} = 3 \times 10^5\ M^{-1}\ s^{-1}$ in phoshatidylcholine liposomal membranes [37,38]. Conversely, under conditions of acute oxidative stress, when high fluxes of radicals may be generated (70 nM $s^{-1}$ [39]), the simultaneous presence of the ascorbyl and of the tocopheroxyl radical ESR signals was observed in liposome suspensions [38] and in human plasma [39].

The occurrence, in some cases, of the predominance of the α-TO$^\bullet$ bimolecular self-reaction is supported by the finding that in atherosclerotic plaques, resulting from oxidative conditions, α-TQ can reach 11% of the total α-TOH [40] while the concentration of α-TQ in fresh serum samples of healthy volunteers is about 0.33% [41]. Besides the possible reactions of the reactive α-TQ with some components of human plasma, and the role of α-TQ in regulation and cellular signaling, the formation of α-TQ leads to a loss of vitamin E. Therefore the high values of $2k_d$ we found for dismutation of α-TO$^\bullet$ radicals support the importance of the presence of physiological concentrations of ascorbate which preserves completely the antioxidant potential of vitamin E.

## 4. Conclusions

The oxidative fate of vitamin E in biological systems strongly depends on the reaction route of its oxidation product α-TO$^\bullet$. We investigated the behavior of α-TO$^\bullet$ in lipoprotein-mimicking systems (DCA micelles) and in the high molecular weight fraction of plasma by ESR. This technique permitted the monitoring of the α-TO$^\bullet$ in the nanomolar range, that is at concentrations undetectable by optical spectroscopy, since the molar extinction coefficient of this radical is $\leq 6 \times 10^3\ M^{-1}\ cm^{-1}$ [8]. We could demonstrate that in human plasma the decay of the α-TO$^\bullet$ by self-reaction prevails in the presence of high rates of radical generation. The transfer of a hydrogen atom between lipoproteins increases the probability that two α-TO$^\bullet$ are simultaneously in the same lipoprotein. This most likely accounts for the mechanism of disappearance of α-TO$^\bullet$ through dismutation in human plasma deprived of low molecular weight antioxidants. We enlightened the significance of considering the micelle volume to calculate the kinetic constants for the α-TO$^\bullet$ self-reaction in heterogeneous system. Furthermore, we calculated and compared the kinetic rate constants in homogeneous and heterogeneous phases (ethanol solutions, micelles and human plasma). We found that the kinetic constants in heterogeneous systems are at least one order of magnitude higher than that in homogeneous solutions. On the basis of the obtained kinetic constant values, we discussed the physiological significance of the tocopheroxyl radical decay through the bimolecular self-reaction under oxidative stress conditions, though the recycling by ascorbate predominates under physiological free radical fluxes.

## References


[1] G.W. Burton, A. Joyce, K.U. Ingold, First proof that vitamin E is the major lipid-soluble, chain-breaking antioxidant in human blood plasma, Lancet 320 (1982) 327.
[2] R. Brigelius-Flohe, F. Galli, Vitamin E: a vitamin still awaiting the detection of its biological function, Mol. Nutr. Food Res. 54 (2010) 583–587.
[3] E. Niki, Role of vitamin E as a lipid-soluble peroxyl radical scavenger: in vitro and in vivo evidence, Free Radic. Biol. Med. 66 (2014) 3–12.
[4] V.W. Bowry, K.U. Ingold, The unexpected role of vitamin E (α-Tocopherol) in the peroxidation of human Low-Density Lipoprotein, Acc. Chem. Res. 32 (1999) 27–34.
[5] Kagan, E.A. Serbinova, T. Forte, G. Scita, L. Packer, Recycling of vitamin E in human low density lipoproteins, J. Lipid Res. 33 (1992) 385–397.
[6] E. Niki, N. Noguchi, Dynamics of antioxidant action of Vitamin E, Acc. Chem. Res. 37 (2004) 45–51.
[7] V.W. Bowry, K.U. Ingold, Extraordinary kinetic behavior of the α-Tocopheroxyl (Vitamin E) radical, J. Org. Chem. 60 (1995) 5456–5467.
[8] K. Mukai, A. Ouchi, A. Mitarai, K. Ohara, C. Matsuoka, Formation and decay dynamics of vitamin E radical in the antioxidant reaction of vitamin E, Bull. Chem. Soc. Jpn. 82 (2009) 494–503.
[9] R. Rousseau-Richard, C. Richard, R. Martin, Kinetics of biomolecular decay of α-tocopheroxyl free radicals studied by ESR, FEBS Lett. 233 (1988) 307–310.
[10] A. Watanabe, N. Noguchi, A. Fujisawa, T. Kodama, K. Tamura, O. Cynshi, E. Niki, Stability and reactivity of aryloxyl radicals derived from a novel antioxidant BO-653 and related compounds. Effects of substituent and side chain in solution and membranes, J. Am. Chem. Soc. 122 (2000) 5438–5442.
[11] K. Ohara, A. Shimizu, Y. Wada, S. Nagaoka, Photochemical formation and decay of tocopheroxyl radical in vitamin E emulsion: a laser-photolysis study, J. Photochem. Photobiol. A 210 (2010) 173–180.
[12] L. Gille, K. Staniek, T. Rosenau, J.C. Duvigneau, A.V. Kozlov, Tocopheryl quinones and mitochondria, Mol. Nutr. Food Res. 54 (2010) 601–615.
[13] H. Shi, N. Noguchi, E. Niki, Comparative study on dynamics of antioxidative action of alpha-tocopheryl hydroquinone, ubiquinol, and alpha-tocopherol, against lipid peroxidation, Free Radic. Biol. Med. 27 (1999) 334–346.
[14] K. Muller, H.G. Korth, H. de Groot, M. Kirsch, Reaction of Vitamin E compounds with N-nitrosated tryptophan derivatives and its analytical use nonaqueous disproponation, Chem. Eur. J. 13 (2007) 7532–7542.
[15] I. Kohar, M. Baca, C. Suarna, R. Stocker, P.T. Southwell-Keely, Is α-tocopherol a reservoir for α-tocopheryl hydroquinone? Free Radic. Biol. Med. 19 (1995) 197–207.
[16] T. Doba, G.W. Burton, K.U. Ingold, M. Matsuo, α-Tocopheroxyl decay: lack of effect of oxygen, J. Chem. Soc. Chem. Commun. 7 (1984) 461–462.
[17] M. Lucarini, G.F. Pedulli, M. Cipollone, Bond-dissociation enthalpy of alpha-tocopherol and other phenolic antioxidants, J. Org. Chem. 59 (1994) 5063–5070.
[18] J.E. Packer, T.F. Slater, R.L. Wilson, Direct observation of a free radical interaction between vitamin E and vitamin C, Nature 278 (1979) 737–738.
[19] M. Rossetto, P. Vanzani, M. Lunelli, M. Scarpa, F. Mattivi, A. Rigo, Peroxyl radical trapping activity of anthocyanins and generation of free radical intermediates, Free Radic. Res. 41 (2007) 854–859.
[20] L. Zennaro, M. Rossetto, P. Vanzani, V. De Marco, M. Scarpa, L. Battistin, A. Rigo, A method to evaluate capacity and efficiency of water soluble antioxidants as peroxyl radical scavengers, Arch. Biochem. Biophys. 462 (2007) 38–46.
[21] D.M. Davies, S.J. Foggo, Kinetic treatment of the reaction of m-chloroperbenzoic acid and iodide in mixed anionic/non-ionic micelles, J. Chem. Soc. Perkin Trans. 2 2 (1998) 248–251.
[22] F.M. Menger, C.E. Portnoy, Chemistry of reactions proceeding inside molecular aggregates, J. Am. Chem. Soc. 89 (1967) 4698–4703.
[23] L. Castle, M.J. Perkins, Inhibition kinetics of chain-breaking phenolic antioxidants in SDS micelles. Evidence that intermicellar diffusion rates may be rate-limiting for hydrophobic inhibitors such as.alpha.-tocopherol, J. Am. Chem. Soc. 108 (1986) 6381–6382.
[24] S. Glasstone, Textbook of physical chemistry, D. Van Nostrand Company, University of Cornell, 1946.
[25] J. Salgado, J. Villalain, J.C. Gomez-Fernadez, Magic angle spinning $^{13}$C-NMR spin-lattice relaxation study of the location and effects of α-tocopherol, ubiquinone-10 and ubiquinol-10 in unsonicated model membranes, Eur. Biophys. J. 22 (1993) 151–155.
[26] D.E. Cabelli, B.H.J. Bielski, Studies of the reactivity of trolox with Mn3 +/Fe3 + complexes by pulse radiolysis, Free Radic. Biol. Med. 2 (1986) 71–75.
[27] M. Alessi, T. Paul, J.C. Scaiano, K.U. Ingold, The contrasting kinetics of peroxidation of Vitamin E-containing phospholipid unilamellar vesicles and human Low-Density Lipoprotein, J. Am. Chem. Soc. 124 (2002) 6957–6965.
[28] V.W. Bowry, R. Stocker, Tocopherol-mediated peroxidation. The prooxidant effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein, J. Am. Chem. Soc. 115 (1993) 6029–6044.
[29] S. Lemaire-Ewing, C. Desrumaux, D. Néel, L. Lagrost, Vitamin E transport, membrane incorporation and cell metabolism: is α-tocopherol in lipid rafts an oar in the lifeboat? Mol. Nutr. Food Res. 54 (2010) 631–640.
[30] N. Kono, U. Ohto, T. Hiramatsu, M. Urabe, Y. Uchida, Y. Satow, H. Arai, Impaired α-TTP-PIPs interaction underlies familial vitamin E deficiency, Science 340 (2013) 1106–1110.
[31] S. Nagaoka, K. Kouhei Sawada, Y. Fukumoto, U. Umpei Nagashima, S. Katsumata, K. Mukai, Mechanism of prooxidant reaction of vitamin E. Kinetic, spectroscopic, and ab initio study of proton-transfer reaction, J. Phys. Chem. 96 (1992) 6663–6668.
[32] N. Dhenadhayalan, C. Selvaraju, Role of photoionization on the dynamics and mechanism of photoinduced electron transfer reaction of coumarin 307 in micelles, J. Phys. Chem. B 116 (2012) 4908–4920.
[33] S. Mora, J.D. Otvos, R.S. Rosenson, A. Pradhan, J.E. Buring, P.M. Ridker, Lipoprotein particle size and concentration by nuclear magnetic resonance and incident Type 2 diabetes in women, Diabetes 59 (2010) 1153–1160.
[34] R.H. Bisby, A.W. Parker, Reactions of the α-tocopheroxyl radical in micellar solutions studied by nanosecond laser flash photolysis, FEBS Lett. 290 (1991) 205–508.
[35] M. Levine, C. Conry-Cantilena, Y. Wang, R.W. Welch, P.W. Washko, K.R. Dhariwal, J.B. Park, A. Lazarev, J.F. Graumlich, J. King, R.L. Cantilena, Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 3704–3709.
[36] K.U. Ingold, V.W. Bowry, R. Stocker, C. Walling, Autoxidation of lipids and antioxidation by α-tocopherol and ubiquinol in homogeneous solution and in aqueous dispersions of lipids: unrecognized consequences of lipid particle size as exemplified by oxidation of human low density lipoprotein, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 45–49.
[37] R.H. Bisby, A.W. Parker, Reaction of ascorbate with the α-tocopheroxyl radical in micellar and bilayer membrane systems, Arch. Biochem. Biophys. 317 (1995) 170–178.
[38] M. Scarpa, A. Rigo, M. Maiorino, F. Ursini, C. Gregolin, Formation of α-tocopherol radical and recycling of α-tocopherol by ascorbate during peroxidation of phosphatidylcholine liposomes: an electron paramagnetic resonance study, BBA Gen. Subj. 810 (1984) 215–219.
[39] M.K. Sharma, G.R. Buettner, Interaction of vitamin C and vitamin E during free radical stress in plasma: an ESR study, Free Radic. Biol. Med. 14 (1993) 649–653.
[40] A.C. Terentis, R.T. Shane, J.A. Burr, D.C. Liebler, R. Stocker, Vitamin E oxidation in human atherosclerotic lesions, Circ. Res. 90 (2002) 333–339.
[41] D. Pollok, H.U. Melchert, Determination of alpha-tocopherolquinone in human serum samples by liquid chromatography with fluorescence detection and on-line post-column derivatization, J. Chromatogr. A 1056 (2004) 257–262.