Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Photochemistry and Photobiology A: Chemistry 167 (2004) 37–47
Journal of Photochemistry and Photobiology A:Chemistry
www.elsevier.com/locate/jphotochem

Type-I and type-II photoprocesses in the system photosense–ascorbic acid

Nina A. Kuznetsova*, Evgenya V. Pykhtina, Ludmila A. Ulanova, Oleg L. Kaliya

Organic Intermediates & Dyes Institute, 1/4 B. Sadovaya Street, Moscow 123995, Russia

Received 4 March 2004; accepted 24 March 2004

Available online 20 June 2004

Abstract

It was found significant difference in reactivity of undissociated ascorbic acid AH_2 and its monoanion AH^- towards singlet oxygen and in electron transfer to excited photosense. Kinetic analysis indicated that interaction of ascorbate with singlet oxygen in aqueous media results in quenching of the latter with rate constant $(k_q + k_r) = (6.8 \pm 1) \times 10^8\ mol^{-1}\,l\,s^{-1}$, where chemical quenching rate constant k_r is $1.4 \times 10^8\ mol^{-1}\,l\,s^{-1}$. The value of k_r for AH_2 is a factor of about 20 lower. The similar trend was observed for ascorbic acid oxidation in electron transfer to excited photosense. The corresponding rate constants were established for ascorbate AH^- and were found to be: for total quenching $(k_q + k_r)$ is of order $10^7\ mol^{-1}\,l\,s^{-1}$ and for radicals photogeneration $k_r = (2.5 \pm 0.5) \times 10^3\ mol^{-1}\,l\,s^{-1}$. Analysis of the photosense–ascorbic acid system had revealed the low efficiency of type I pathway, initiated by electron transfer from ascorbate to excited photosense, in comparison to efficiency of the route, mediated by singlet oxygen.

Keywords: Photodynamic therapy; Photosensitizer; Photosense; Ascorbic acid; Singlet oxygen; Electron spin paramagnetic resonance

1. Introduction

To date the photodynamic therapy (PDT) is clinic-approved method of cancer treatment. PDT involves the combination of visible light and a photosensitizer. Each component is harmless by itself, but in the presence of oxygen they induce cytotoxicity. The triplet state of the photosensitizer formed upon the light absorption can interact with ground-state dioxygen to yield cytotoxic singlet dioxygen (type-II mechanism) [1]. The photosensitizer triplet state, or biological substrate radicals formed by substrate interaction with the photosensitizer triplet state, can react with oxygen [2] to produce cytotoxic oxygen intermediates, such as superoxide, which are thought to provide alternative routes to photosensitized cell death (type-I mechanism). PDT is a highly selective method, as there is a preferential uptake of the photosensitizer by the diseased tissue.

Among photosensitizers for PDT the phthalocyanines are the more promising second-generation photosensitizers. Sulfonated aluminum phthalocyanine termed photosense ($AlPcS^n_{mix}$) has received special attention and now is approved for the wide clinical use in Russia. Photosense generates singlet oxygen with high quantum yield and is a typical type II sensitizer [3]. However, the photosensitizing activity of phthalocyanines is dependent on microenvironment (medium polarity, pH, presence of electron donors or acceptors, etc.) which may influence the relative importance of free radical (type I) or singlet oxygen (type II) reaction pathways.

It was suggested to use photosense with reductants (e.g. ascorbic acid) to increase type I radical route and hence photodynamic efficiency on the whole. Rosenthal and Ben-Hur were the first to propose [4] that photosense undergoes photoreduction in the presence of ascorbic acid giving anion radical which is oxidized further by oxygen with the superoxide formation according to the following scheme:

$$Ps \xrightarrow{h\nu} Ps^* \quad (1)$$

$$Ps^* + AH^- \rightarrow Ps^{\bullet -} + A^{\bullet -} + H^+ \quad (2)$$

$$Ps^{\bullet -} + {}^3O_2 \rightarrow Ps + O_2^{\bullet -} \quad (3)$$

Thus in the excess of ascorbic acid the radical mechanism of PDT may be sharply enhanced. Recently, the PDT procedure with photosense and sodium ascorbate was tested on mice and some positive results had been obtained [5].

On the other hand, ascorbic acid is known as efficient biological antioxidant and singlet oxygen quencher [6–8]. From this point of view ascorbate should interfere with type-II singlet oxygen route and decrease PDT efficiency. Suppression of PDT by ascorbic acid is noted in literature as well [9,10].

* Corresponding author. Tel.: +7-095-2549866.
E-mail address: jerrik@rol.ru (N.A. Kuznetsova).

1010-6030/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jphotochem.2004.03.029

So, there are controversial data about effect of ascorbate on PDT. One possible approach to understand what is happening in the complex biological assemblies is the analysis of the specific system. In the present paper we focus on mechanism and kinetics of oxidation processes photoinduced by photosense in the presence of ascorbic acid. The photooxidation of ascorbic acid by singlet oxygen in the system under investigation was considered, and formation of free radicals was studied by direct electron spin paramagnetic resonance (photo-ESR) method also.

2. Materials and methods

2.1. Chemicals

Photosense ($AlPcS^n_{mix}$) was from NIOPIK (Russia), ascorbic acid was of analytical grade. Reagents were used as supplied. The pH from 5.3 to 7.4 was adjusted with mixtures of KH_2PO_4–K_2HPO_4, for pH3.56, 2.86 and 1.68 potassium tartrate, mixture of citric acid with Na_2HPO_4 and potassium tetraoxalate were used, correspondingly. The ionic strength of the solutions was about 0.1 mol l^{-1}. The pH2 was adjusted by addition of sulfuric acid. Water was doubly distilled.

2.2. Instrumentation and methods

Singlet oxygen mediated photooxidation of ascorbic acid in the presence of photosense was studied in air-saturated buffer solutions at room temperature by following the disappearance of ascorbic acid absorbance at 265 nm (pH > 4.25) or at 245 nm (pH < 4.25). The photosense concentration was 5×10^{-6} mol l^{-1} in all experiments, concentration of ascorbic acid was varied from 1.5×10^{-3} to 5×10^{-5} mol l^{-1}. Typically a 3 ml of aqueous buffer solution, containing photosense and ascorbic acid, was added to the 1 cm path length cell and than irradiated in Q-band of sensitizer. The xenon lamp (150 W) was used as a light source. A 550 nm glass cut off filter GS 18 and water filter were used to filter off ultraviolet and far infrared radiation. An interference filter of 680 ± 25 nm was placed in the light path. The light intensity was measured with a power meter Spectra Physics 404 and was found to be 1.8×10^{15} photons s^{-1} cm^{-2}. Amount of quanta, absorbed by sensitizer, was estimated as overlap integral of the radiation source light intensity and the absorption of the sensitizer (the action spectrum) in the region of the interference filter transmittance. The quantum yields of ascorbic acid photooxidation were estimated with accuracy 15%.

Photogeneration of free radicals was studied by the electron spin resonance (ESR) spectroscopy. ESR steady state measurements were performed with "Rubin" spectrometer over X-range (at 9.4 GHz) operating at 100 kHz field modulation. In these experiments the aqueous buffer solutions of photosense (5×10^{-5} mol l^{-1}) were saturated with ultra pure argon or oxygen during 30 min in the dark. Then the powder of ascorbic acid was added (concentration of ascorbic acid $\sim 1 \times 10^{-4}$–5×10^{-3} mol l^{-1}) and the investigated sample (~0.02 ml) under the stream of argon or oxygen, respectively, was put into ESR cell (0.1–0.15 cm path length). The cell containing the investigated solution was directly irradiated within the microwave cavity of ESR spectrometer using a halogen lamp H4, equipped with water and cut-off glass filters, in order to excite photosense in the Q absorption band. The intensity of filtered light, measured in front of the cell by a calibrated power meter Spectra Physics 404, was equal to 1×10^{17} photons s^{-1} cm^{-2} for wavelength range 640–700 nm. Quantum yields (Φ_R) of radical photogeneration were calculated as the ratio of initial rate of radical formation (W_{form}) to initial rate of light absorption (W_{abs}). The values of W_{form} were received from the initial slopes of the related kinetic curves. The amounts of radicals in the solutions were measured by peak-to-peak method using nitroxyle radical (TEMPOL) as standard and taking into account the ESR signal shapes. The rate of light absorption W_{abs} was estimated as overlap integral of the incident light intensity and the absorption of photosense. The error of radical species quantum yields was estimated to be of 25%.

The electronic absorption spectra were recorded with a Hewlett Packard HP 8453 spectrophotometer.

All experiments were carried out at ambient temperature.

3. Results

3.1. Electronic spectra

It is well documented that degree of sulfonation effects the aggregation of sulfosubstituted aluminium phthalocyanine in aqueous solution [11,12]. The effects of pH on aggregation and absorption spectrum profiles of $AlPcS_2$, $AlPcS_4$ and a mixed sulfonated sample were investigated [13–15] as well. Photosense is a mixture of differently sulfonated compounds $AlPcS^n_{mix}$ with average degree of sulfonation $n \sim 3$ and, as spectroscopic study shows, is very similar to the sample, investigated in [15]. It was found that the absorption spectra of photosense in buffer solutions with pH 3.56 and 7.4 are characteristic for the monomer species. Furthermore, the Beer–Lambert plot is seen to be linear over the entire concentration range studied (10^{-6}–10^{-5} mol l^{-1}), indicating no aggregation.

The Q-band absorption maximum dependence on pH for photosense 5×10^{-6} mol l^{-1} aqueous solution is shown in Fig. 1 for the pH range from 1.5 to 9, used for studies. The data obtained (Fig. 1) evidence that λ_{max} for photosense is situated about 679 nm in pH region from 1.68 to 5.5 and 675 nm at pH > 7.5 up to basic solutions. Such behavior points on existence of at least two pH-dependent spectroscopic forms of photosense at pH below 9 and is consistent with reported earlier for sulfosubstituted aluminum phthalocianines [14,16] acid–base transition with $pK_a = 6.7$.

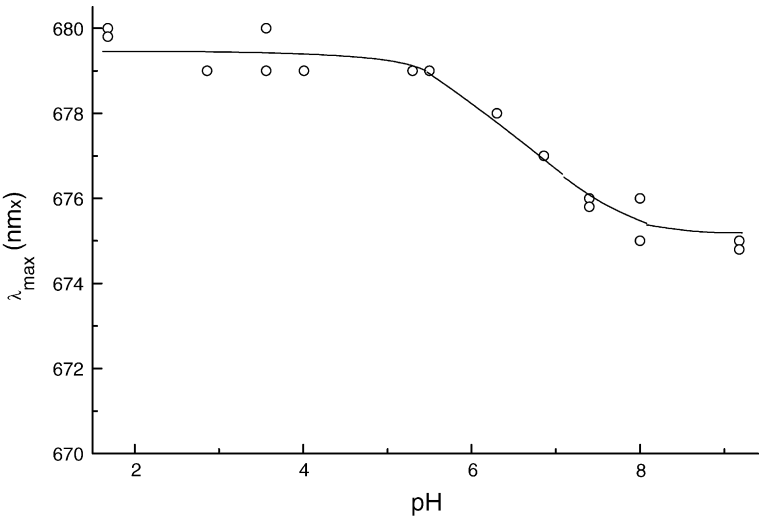


Fig. 1. The Q-band absorption maximum λ_{max} (nm) dependance on pH for photosense 5×10^{-6} mol l^{-1} aqueous solution.

The molar decadic extinction coefficients ϵ for photosense Q-band were 140 000 at pH 3.56 and 7.4, this value is in reasonable agreement with those reported in literature [13] (and references therein).

The following well established properties of ascorbic acid are relevant to this study. Ascorbic acid has two pK's, one at 4.25 [17] which represents the equilibrium $AH_2 \rightleftarrows AH^- + H^+$ and another at 11.78 [17] which represents $AH^- \rightleftarrows A^{2-} + H^+$. In basic solutions ascorbate undergoes auto-oxidation and is not sufficiently stable in the presence of oxygen. Therefore experiments in the current study were performed over the pH region from 2 to 7.4, where only monoanion AH^- and undissociated form AH_2 are in equilibrium. The peaks in electronic absorption spectra of AH^- and AH_2 species in aqueous media are at 265 and 245 nm, correspondingly, and the molar extinction coefficients used here are 14 500 l mol^{-1} cm^{-1} for AH^- at 265 nm [18] and 10 100 l mol^{-1} cm^{-1} for AH_2 at 245 nm [19].

3.2. Singlet dioxygen-mediated process

The fact of ascorbic acid oxidation by singlet oxygen is well-established [6–8]. Since $AlPcS^n_{mix}$ is known as efficient singlet oxygen sensitizer [3], under its excitation the sensitized oxidation of ascorbic acid by singlet oxygen is expected.

Actually, we have found that irradiation of air-equilibrated solutions of ascorbic acid in the presence of photosense with visible light modifies the respective absorption spectra. Fig. 2 depicts the absorption spectra of 0.75×10^{-3} mol l^{-1} solution of ascorbic acid in water upon irradiation at wavelengths 680 ± 25 nm in the presence of photosense, and

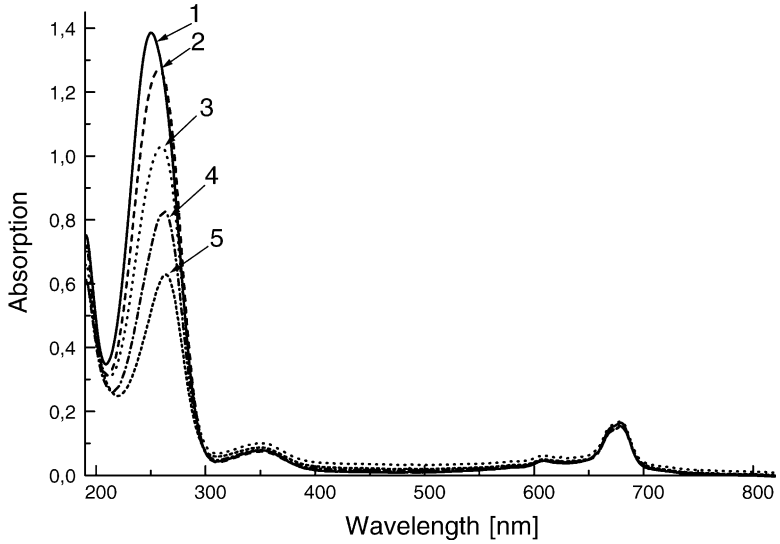


Fig. 2. Absorption spectrum of ascorbic acid (0.75×10^{-3} mol l^{-1}) and photosense (5×10^{-6} mol l^{-1}) in water and its evolution under irradiation with visible light of $\lambda = 680 \pm 25$ nm. Traces are: (1)-before irradiation, and (2)–(5) after 10, 25, 35, and 45 min irradiation.

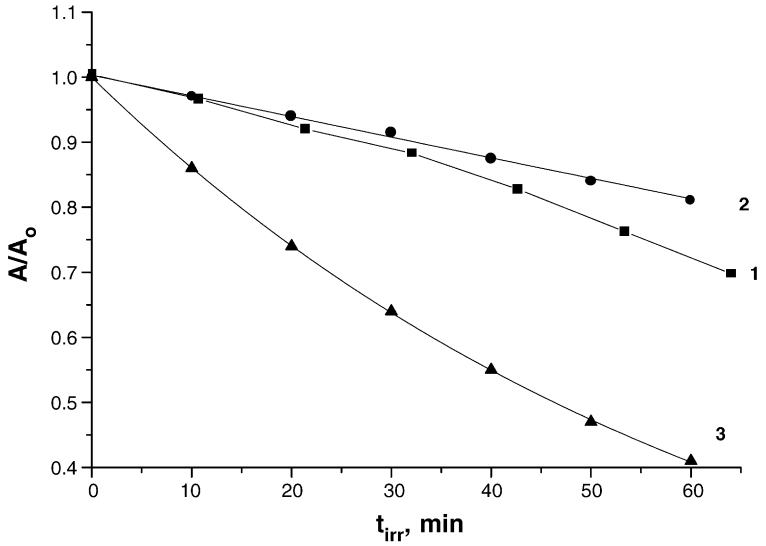


Fig. 3. Kinetics of sensitized by photosense oxidation of ascorbic acid (4×10^{-4} mol l^{-1}): (1)-in H_2O, (2)-in buffer of pH 3.56, (3)-in buffer of pH 7.4.

shows that ascorbic acid absorption decreases and peak wavelength shifts to the red from 250 nm to 263 nm with photolysis time. This picture is similar to one observed in paper [20] for variations of ascorbic acid spectra with concentration. Photoinduced spectral modifications were suppressed when N_2 bubbled solutions were tested or in the presence of 0.01 mol l^{-1} sodium azide (a known specific $^1O_2$ physical quencher). This evidence demonstrates the occurrence of an $^1O_2$-mediated mechanism of ascorbic acid photooxidation, sensitized by photosense.

Without light we have observed decrease of ascorbic acid absorbance due to auto-oxidation. At pH < 4 this process was negligible, in more basic solutions, however, the contribution of auto-oxidation in the total ascorbic acid degradation was more pronounced (5–12% at pH 5–7.4). Presented herein data are corrected for auto-oxidation.

The kinetic of sensitized by photosense oxidation of ascorbic acid in aqueous solution (Fig. 3 curve 1) shows acceleration of the oxidation rate as irradiation progresses. Above mentioned concomitant shift of ascorbic acid absorption maximum (Fig. 2) evidences that equilibrium between AH_2 and AH^- is shifted during photolysis to the ionized form. It was found also, that as ascorbic acid is consumed, the pH3.95 of its initial 1.5×10^{-3} mol l^{-1} solution is increasing following curve on Fig. 4. These data are consistent with higher reactivity of ascorbate monoanion than that of unionized AH_2 form.

Kinetics for ascorbic acid sensitized oxidation at controlled pH conditions (pH 3.56 and 7.4) are presented on

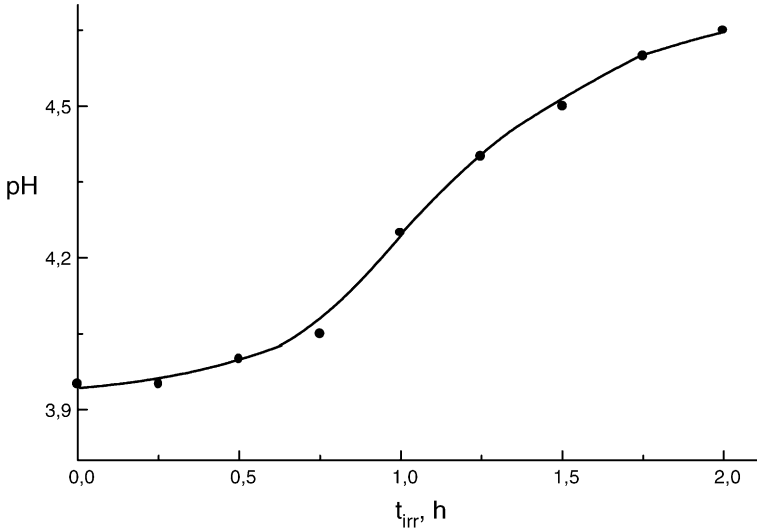


Fig. 4. The pH of air-saturated aqueous solution of ascorbic acid (1.5×10^{-3} mol l^{-1}) and photosense (5×10^{-6} mol l^{-1}) vs. irradiation time. Light of $\lambda = 680 \pm 25$ nm (Q-band excitation of photosense).

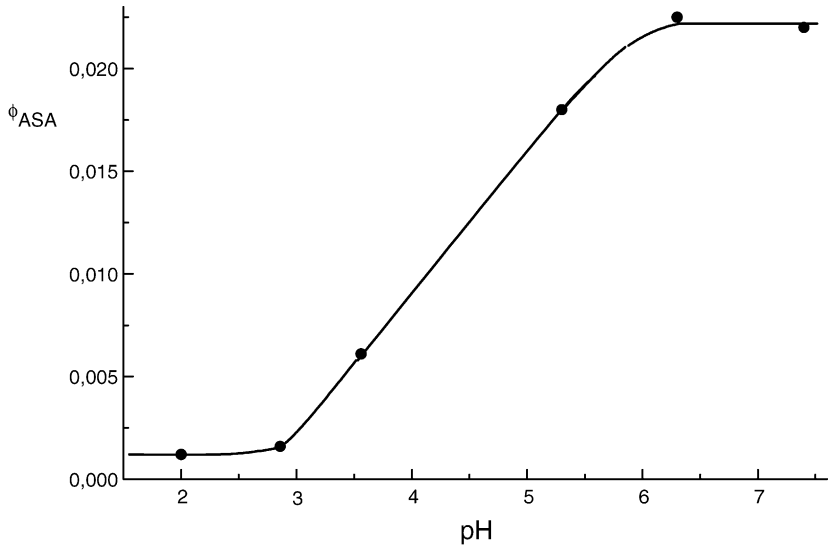


Fig. 5. Dependence of quantum yield on pH for oxidation of ascorbic acid (2×10^{-4} mol l^{-1}) sensitized by photosense.

Fig. 3. The obtained results evidence that rates of process under investigation are dependent on pH. Consequently the rate constant for singlet oxygen quenching by ascorbic acid will be dependent on pH.

The pH dependence for quantum yields of sensitized by photosense oxidation of ascorbic acid (Φ_{ASA}) follows a typical sigmoidal p*K*a curve (Fig. 5). It should be mentioned that pH-induced acid–base transitions have not much influence on singlet oxygen quantum yield of sulfonated aluminium phthalocyanine, particulaly at pH above 3 [14]. Therefore, it is not the photosense to be a cause of relationship shown on Fig. 5. In fact the curve of quantum yield versus pH dependence unambiguously points on $pK_a = 4.25$, which is relevant to $AH_2 \rightleftarrows AH^- + H^+$ process. At pH values above 4.25 the ascorbic acid is in its deprotonated form hence, it is more easily oxidizable. The pH dependence of the sensitized photolysis quantum yield demonstrates an insignificant contribution of the unionized form AH_2 in the process (Fig. 5).

Effect of pH on the total rate constant $k_Q = (k_q + k_r)$ for singlet oxygen quenching by ascorbic acid has been studied in D_2O containing acetonitrile (50% (v/v)) [21]. It was found that k_Q depends critically on the protonation state of substrate. However in H_2O neither pH dependence, nor even reliable data for quenching rate constants were established. The only report was [6], where a marked isotope effect was found: k_Q in H_2O was 3.3 times faster than k_Q in D_2O, and k_Q in H_2O was reported to be 8.3×10^6 mol^{-1} l s^{-1}. This value is surprisingly low, as we suppose, due to considerable amount of AH_2 inactive form in provided by ascorbic acid solute acidic conditions.

We have re-estimated the constant for singlet oxygen quenching by ascorbic acid employing controlled pH conditions. The kinetic analysis of the following conventional scheme was used:

$$^1O_2 \xrightarrow{k_d} {}^3O_2 \quad (4)$$

$$^1O_2 + ASA \xrightarrow{k_q} {}^3O_2 \quad (5)$$

$$^1O_2 + ASA \xrightarrow{k_r} \text{products} \quad (6)$$

where k_d, k_q and k_r are the rate constants for the decay of $^1O_2$, "physical" quenching of $^1O_2$ by ascorbic acid (ASA) and formation of oxidation products, respectively.

The expression describing the quantum yield of ascorbic acid photooxidation is

$$\Phi_{ASA} = \Phi_\Delta \frac{k_r[ASA]}{k_d + k_q[ASA] + k_r[ASA]} \quad (7)$$

Rewriting Eq. (7) as its double reciprocal and rearranging gives Eq. (8)

$$\frac{1}{\Phi_{ASA}} = \frac{1}{\Phi_\Delta}\left(\frac{k_r + k_q}{k_r} + \frac{k_d}{k_r[ASA]}\right) \quad (8)$$

The plots of $1/\Phi_{ASA}$ versus 1/[ASA] were employed for pH 7.4 and 3.56 conditions and according to Eq. (8), gave straight lines (Fig. 6) with the slopes 0.0060 ± 0.0002 mol l^{-1} for pH 7.4 and 0.0290 ± 0.0015 mol l^{-1} for pH 3.56. These slopes are equal to $k_d/\Phi_\Delta k_r$ with $k_d = 1/\tau_\Delta = 3.22 \times 10^5$ s^{-1} ($\tau_\Delta = 3.09 \times 10^{-6}$ s in water [22]). Using value of $\Phi_\Delta = 0.38$ for $AlPcS^n_{mix}$ [3], the constants k_r were calculated to be $(1.4 \pm 0.2) \times 10^8$ mol^{-1} l s^{-1} and $(2.9 \pm 0.3) \times 10^7$ mol^{-1} l s^{-1} for pH 7.4 and 3.56 conditions, correspondingly. At pH 7.4 the dominant species for ascorbic acid are AH^- (99.9%) with low concentration of AH_2 (0.1%) and negligible amount of A^{2-}. Hence constant $k_r = (1.4 \pm 0.2) \times 10^8$ mol^{-1} l s^{-1} is relevant to monoanion AH^-.

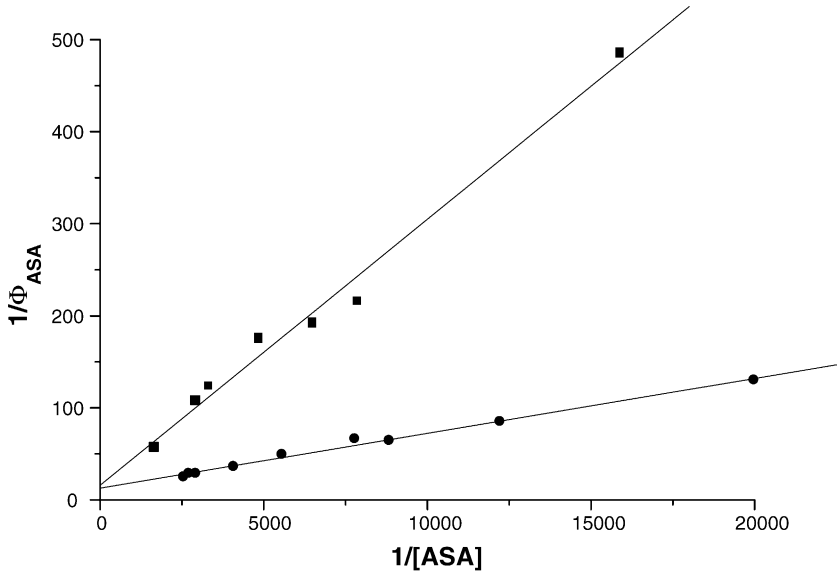


Fig. 6. Plots of $1/\Phi_{ASA}$ vs. 1/[ASA] for the photooxidation of ascorbic acid, sensitized by photosense, at pH 7.4 (●) and at pH 3.56 (■).

At pH 3.56 the species in equilibrium are AH_2 (80%) and AH^- (20%). The corresponding value of $k_r = (2.9 \pm 0.3) \times 10^7\,mol^{-1}\,l\,s^{-1}$ may be interpreted in terms of negligible reactivity of unionized form AH_2 in contrast to monoanion AH^-, and the latter being responsible for singlet oxygen quenching.

The experiments under comparable conditions show (Fig. 5) that the rate constant for singlet oxygen chemical quenching by AH_2 is a factor of about 20 lower than that for monoanion AH^-, and this is about $7 \times 10^6\,mol^{-1}\,l\,s^{-1}$.

In general for the pH region, where amount of A^{2-} is negligible (up to pH ≈ 10), the experimental rate constant k_r^{ASA} of singlet oxygen chemical quenching by ascorbic acid is related to $k_r^{AH_2}$ and $k_r^{AH^-}$ (rate constants of $^1O_2$ chemical quenching by undissociated form AH_2 and monoanion AH^-, correspondingly) by Eq. (9):

$$k_r^{ASA} = (1-\alpha)k_r^{AH_2} + \alpha k_r^{AH^-} \tag{9}$$

where α is degree of ascorbic acid first ionization. The α may be expressed as (10):

$$\alpha = \frac{K_a}{[H^+] + K_a} \tag{10}$$

where K_a represents the constant of ascorbic acid first ionization.

This gives expression (11):

$$k_r^{ASA} = \frac{k_r^{AH_2}[H^+] + k_r^{AH^-}K_a}{[H^+] + K_a} \tag{11}$$

With $k_r^{AH_2} = 7 \times 10^6\,mol^{-1}\,l\,s^{-1}$, $k_r^{AH^-} = 1.4 \times 10^8\,mol^{-1}\,l\,s^{-1}$ and $K_a = 5.6 \times 10^{-5}\,mol\,l^{-1}$ [17] the experimental rate constant k_r^{ASA} can be calculated from Eq. (12):

$$k_r^{ASA} = \frac{7 \times 10^6[H^+] + 7.84 \times 10^3}{5.6 \times 10^{-5} + [H^+]} \tag{12}$$

Eq. (12) is valid to pH below ≈10. In more basic solutions A^{2-} form of ascorbic acid appears in considerable amount. As species A^{2-} are more oxidizable and extremely unstable in the presence of oxygen, we failed to gain any information about its interaction with singlet oxygen.

The plots shown in Fig. 6 gave intercepts $= (k_q + k_r)/\Phi_\Delta k_r$. The intercepts were found to be 12.8 ± 2 for pH 7.4 plot and 15.6 ± 11.3 when pH 3.56 conditions were employed. The latter had low accuracy, however at pH 7.4 the total constant for singlet oxygen quenching $(k_q + k_r)$ was estimated from the intercept of the plot. When $\Phi_\Delta = 0.38$ and $k_r = (1.4 \pm 0.2) \times 10^8\,mol^{-1}\,l\,s^{-1}$, the $(k_q + k_r)$ for monoanion of ascorbic acid was calculated to be equal $6.8 \pm 1 \times 10^8\,mol^{-1}\,l\,s^{-1}$. This value is 4.2 or 2.2 times higher then reported for D_2O solution [23] or D_2O containing acetonitrile (50% (v/v)) [21], accordingly. In general the tendency is in line with found by Chou and Khan [6] solvent deuterium isotope effect on the rate constant for singlet oxygen quenching by ascorbic acid.

The total rate constant $(k_q + k_r)$ for ascorbate ion AH^- is five times larger than the rate constant k_r for photooxidation alone thus, most of the singlet oxygen is scavenged by a physical quenching process. Consequently, monoanion of ascorbic acid quenches singlet oxygen more rapidly, than reacts with it.

3.3. Type I radical pathway

To evaluate relative importance of type I mechanism in PDT with photosense in the presence of ascorbic acid, the generation of radicals was investigated using ESR measurements.

The ESR spectra of radicals, generated during photoirradiation in the argon- and oxygen-saturated buffer solutions of photosense ($5 \times 10^{-5}\,mol\,l^{-1}$), containing ascorbic

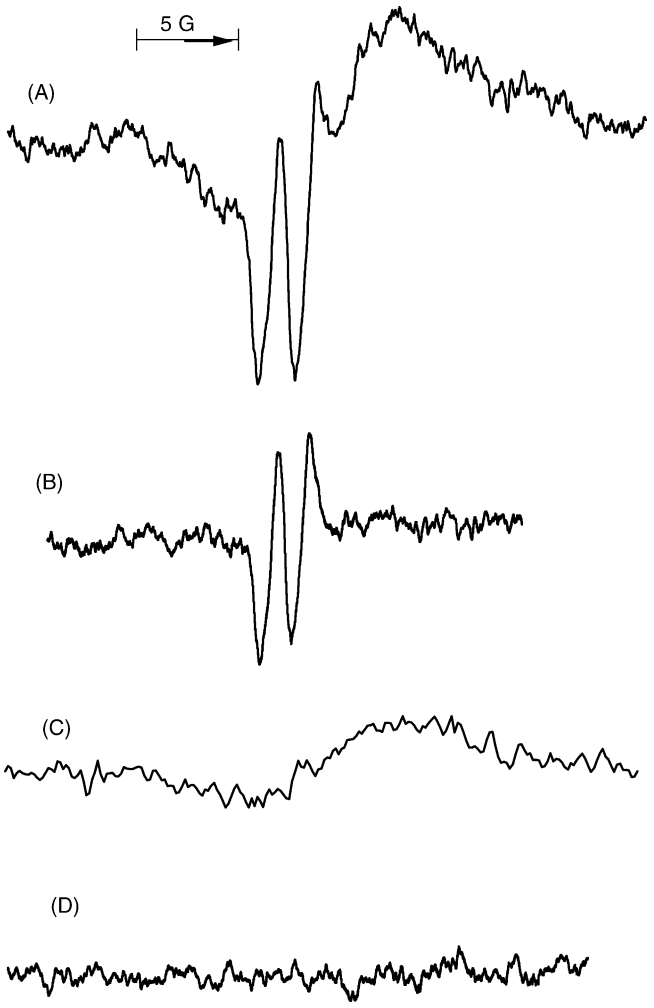
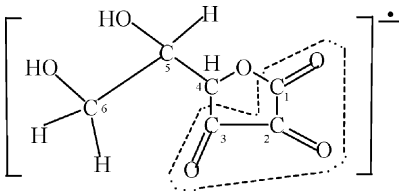


Fig. 7. ESR spectra of radicals obtained during photoexcitation of 5×10^{-5} mol l^{-1} photosense in argon- (A, C) and oxygen-saturated (B, D) aqueous buffer solutions of ascorbic acid (5×10^{-3} mol l^{-1}) at pH 7.4 (A, B) and 3.56 (C, D). The ESR conditions: a nominal nonsaturating microwave power of 10 mW; field modulation 1 G.

acid (5×10^{-3} mol l^{-1}) at pH 7.4 and 3.56 are depicted on Fig. 7.

In argon-saturated solution at pH 7.4 the two types of radicals were formed during photoirradiation (Fig. 7A). The doublet with $g = 2.0051 \pm 0.0005$ and a hyperfine coupling constant of $a^{H} = 1.87 \pm 0.08$ G is in good agreement with corresponding signal in ESR spectrum reported for ascorbate radical [24–26]. The ascorbate radical exists as the anion radical $A^{\bullet -}$ with the unpaired electron spread over a highly conjugated tricarbonyl system [26,27].



The second type of radical species was revealed as wide singlet with $g = 2.0025 \pm 0.0007$ and $\Delta H = 4.7 \pm 0.8$ G (Fig. 7). By analogy with [28,29] the singlet signal was attributed to the photosense anion-radical $Ps^{\bullet -}$.

Control experiments ensured that no signal was observed without light or in the absence of ascorbic acid, indicating that the radical species were formed in photoinduced electron transfer between photosense and ascorbate monoanion.

When oxygen-saturated conditions were employed to pH 7.4 buffer solution of photosense–ascorbic acid, only the doublet signal of $A^{\bullet -}$ was observed (Fig. 7B), its intensity increased significantly during the photoexcitation of photosense with visible light. No wide singlet attributable to photosense anion-radical was found in ESR spectrum. These data were interpreted as quenching of electron transfer process by a competitive energy transfer from reactive excited triplet state of photosense to oxygen, which results in singlet oxygen formation. Singlet oxygen oxidizes further ascorbate monoanion to give superoxide anion and ascorbate radical [23]. Another reason for photosence anion-radical absence is its rapid oxidation by oxygen, leading to superoxide-anion formation.

In contrast to the situation in pH 7.4 solutions, at pH 3.56 in argon-saturated buffer solution of photosense–ascorbic acid the ascorbate radicals were not observed, weak signal of photosense anion-radical was found in ESR-spectra during the photoirradiation (Fig. 7C). In oxygen-saturated solution at pH 3.56 no ESR signals, induced by light, were observed (Fig. 7D).

Magnetic resonance parameters of free radicals detected under Q-band excitation of photosense in argon- and oxygen-saturated buffer aqueous solution containing ascorbic acid are represented in Table 1.

To estimate the quantum yields of radicals photogeneration the kinetics of $A^{\bullet -}$ and $Ps^{\bullet -}$ species under visible light irradiation were studied. The initial linear plots of $A^{\bullet -}$ kinetic at pH 7.4 in argon- and oxygen-saturated solutions are shown in Fig. 8. Presented for oxygen-saturated solutions data are corrected for auto-oxidation. It is seen (Fig. 8) that under the anaerobic conditions the rate of ascorbate radical photogeneration is significantly greater then in the presence of oxygen. Fig. 9 represents the growth of $Ps^{\bullet -}$ concentration, photoinduced in the presence of ascorbic acid at pH 7.4 and 3.56. Under the anaerobic conditions the rate of $Ps^{\bullet -}$ photogeneration is higher at pH 7.4 then at pH 3.56. The quantum yields of radicals formation are summarized in Table 1.

Under argon at pH 7.4 conditions a similar values (5×10^{-4}) were obtained for the quantum yields of $Ps^{\bullet -}$ and $A^{\bullet -}$ formation (Table 1). This finding is a confirmation of radicals appearance in the same, e.g., electron transfer, elementary step.

For oxygen-saturated solutions at pH 7.4 the quantum yield of ascorbate radical is about two orders of magnitude less than relative quantum yield of ascorbic acid photooxidation by singlet oxygen. In this context the most reasonable

Table 1
Magnetic resonance parameters and quantum yields of free radicals detected during Q-band photoexcitation of photosense (5×10^{-5} mol l^{-1}) in buffer aqueous solution in the presence of 5×10^{-3} mol l^{-1} ascorbic acid

pH	Saturating gas	Radical	Peak-to-peak width (G)	Coupling const. a^H (G)	g-factor	Quantum yield
7.4	Argon	$A^{\bullet -}$	–	1.87 ± 0.08	2.0051 ± 0.0005	5×10^{-4}
7.4	Argon	$Ps^{\bullet -}$	4.7 ± 0.8	–	2.0025 ± 0.0007	5×10^{-4}
7.4	Oxygen	$A^{\bullet -}$	–	1.87 ± 0.08	2.0051 ± 0.0005	5×10^{-5}
3.56	Argon	$Ps^{\bullet -}$	6.3 ± 0.7	–	2.0025 ± 0.0007	1.5×10^{-5}
3.56	Oxygen	No radical	–	–	–	–

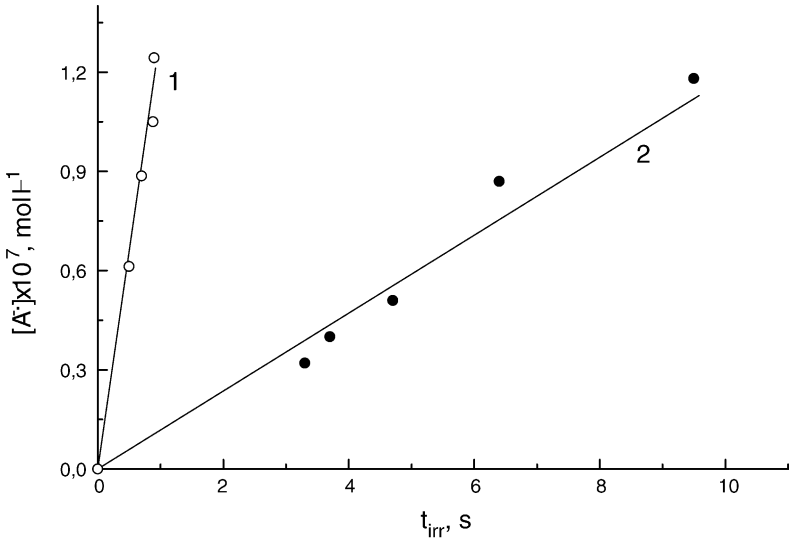


Fig. 8. The $A^{\bullet -}$ concentration during the photoexcitation of 5×10^{-5} mol l^{-1} photosense in argon (1) and oxygen-saturated (2) aqueous buffer solutions (pH 7.4), containing ascorbic acid (5×10^{-3} mol l^{-1}).

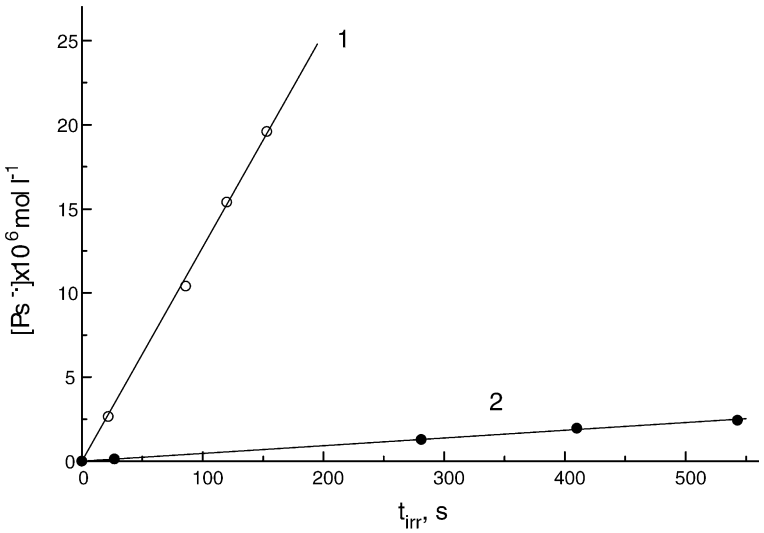


Fig. 9. The $Ps^{\bullet -}$ concentration during the photoexcitation of 5×10^{-5} mol l^{-1} photosense in argon-saturated aqueous buffer solutions of pH 7.4 (1) and pH 3.56 (2), containing ascorbic acid (5×10^{-3} mol l^{-1}).

assumption is that only partial ascorbic acid photooxidation leads to $A^{\bullet-}$ radicals with main rout proceeding as addition of the singlet oxygen to the double bond of ascorbate with formation of diamagnetic intermediates.

For argon-saturated solutions at pH 3.56 the quantum yield of $Ps^{\bullet-}$ was equal to 1.5×10^{-5}. This result in comparison to pH 7.4 anaerobic conditions is in line with low reactivity of unionized form of ascorbic acid in photoreduction of excited photosense.

The absence of $A^{\bullet-}$ signal in ESR spectrum at pH 3.56 anaerobic conditions requires a special consideration. It is worth noting that pH 3.56 solution contains a significant amount (about 20%) of monoanion AH^- in equilibrium with AH_2 form of ascorbic acid, and former participates in electron transfer to excited photosense. The absence of $A^{\bullet-}$ signal in ESR spectrum is most likely due to well-known rapid decay of ascorbate free radical in acidic medium [27].

In the case of air-saturated pH 3.56 buffer solutions, where all negative factors are operating (oxygen quenches reactive excited triplet state of photosense, ascorbic acid is in inactive AH_2 form) the concentrations of both $A^{\bullet-}$ and $Ps^{\bullet-}$ radicals were under the limit of determination.

To evaluate the rate constant of electron transfer from AH^- to triplet photosense we had studied the dependence of quantum yield of radicals formation (Φ_R) on ascorbate concentration. The scheme for processes following photosense excitation in deoxygenated aqueous media (pH 7.4) in the presence of ascorbate, is shown below.

$$Ps \xrightarrow{h\nu} {}^1Ps^* \tag{14}$$

$${}^1Ps^* \xrightarrow{k_d^S} Ps \tag{15}$$

$${}^1Ps^* \xrightarrow{k_{ST}} {}^3Ps^* \tag{16}$$

$${}^3Ps^* \xrightarrow{k_d^T} Ps \tag{17}$$

$${}^3Ps^* + AH^- \xrightarrow{k_q} Ps + AH^- \tag{18}$$

$${}^3Ps^* + AH^- \xrightarrow{k_r} Ps^{\bullet-} + A^{\bullet-} + H^+ \tag{19}$$

where k_d^T, k_q and k_r are the rate constants for photosense triplet state decay, its "physical" quenching by ascorbate monoanion and for formation of radicals $Ps^{\bullet-}$ and $A^{\bullet-}$, respectively.

From this scheme with the steady-state condition ($d[{}^3Ps^*]/dt = 0$) finally we get:

$$\frac{1}{\Phi_R} = \frac{k_q + k_r}{\Phi_T k_r} + \frac{k_d}{\Phi_T k_r} \times \frac{1}{[AH^-]} \tag{20}$$

The plot of $1/\Phi_R$ against $1/[AH^-]$ is a straight line (Fig. 10) with the slope $2.7 \pm 0.3\,mol\,l^{-1}$. By using for photosense $\Phi_T = 0.45$ [13] and $k_d = 1/\tau_0$ (τ_0 - life time of photosence triplet in the absence of ascorbate, $\tau_0 \approx 400$–$450\,\mu s$ [30]) the constant k_r was calculated to be $k_r = (2.5 \pm 0.5) \times 10^3\,mol^{-1}\,l\,s^{-1}$). The intercept of the plot (Fig. 10) is of low accuracy. We used it to estimate the order of the total constant ($k_q + k_r$) of triplet photosense quenching by ascorbate monoanion, which was found to be of $10^7\,mol^{-1}\,l\,s^{-1}$.

The value of rate constant for excited triplet state of photosense quenching by ascorbate was reported earlier as $6.5 \times 10^7\,mol^{-1}\,l\,s^{-1}$ [13], that is higher, than found in this work. Anyway evidence is that rate constant of radicals $Ps^{\bullet-}$ and $A^{\bullet-}$ formation is negligible in comparison with total ${}^3Ps^*$ quenching by ascorbate. Hence, obtained here results on both quantum yields and rate constants clearly show, that efficiency of type I pathway, initiated by direct electron transfer between triplet photosense and ascorbate, is low.

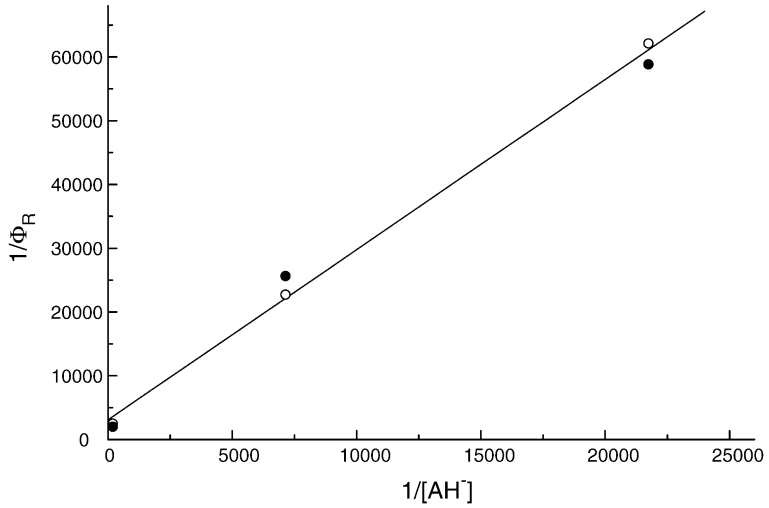


Fig. 10. Plot of $1/\Phi_R$ as a function of $1/[AH^-]$ for ascorbate radical (solid circles) and $Ps^{\bullet-}$ (open circles) formation during the photo-excitation of photosense ($5 \times 10^{-5}\,mol\,l^{-1}$) in aqueous buffer solution of ascorbic acid at pH 7.4.

Table 2
Rate constants for $^1O_2$ and photosense triplet quenching by AH^- and AH_2 forms of ascorbic acid

Quencher	Rate constants for $^1O_2$ quenching, $mol^{-1}\,l\,s^{-1}$		Rate constants for photosense triplet quenching, $mol^{-1}\,l\,s^{-1}$	
	k_r	$k_q + k_r$	k_r	$k_q + k_r$
AH^-	$(1.4 \pm 0.2)\times10^8$	$(6.8 \pm 1)\times10^8$	$(2.5 \pm 0.5)\times10^3$	$\sim10^7$
AH_2	$\sim7 \times 10^6$	–	–	–

4. Discussion

Influence of series of biological reductants on the relative significance of type I and type II mechanisms of PDT with some phthalocyanine sensitizers have been tested by flash-photolysis [15] or ESR [28,29] techniques. We have undertaken the study of photosense–ascorbic acid system and have found that both oxidation of ascorbic acid by singlet oxygen and by electron transfer to photosense triplet state are strongly dependent on pH of solution: at pH $<$ 4.25 ascorbic acid is in unreactive AH_2 form, while at pH $>$ 4.25 it exists mostly as monoanion AH^- with high reductive power. The rate constants for $^1O_2$ and photosense triplet quenching by AH^- and AH_2 forms of ascorbic acid are summarized in Table 2. Similar difference in reactivity of AH_2 and AH^- was reported earlier for ascorbic acid oxidation by $HO_2^\bullet$-radical [31]. Although pH-dependence of ascorbic acid reactivity is important for its applications, in the case of photodynamic therapy ascorbic acid works at physiologycal pH range (from pH 6 to pH 9), where it exists as monoanion and possesses high reactivity.

To rationalize our results with therapeutic response of PDT on increase of ascorbic acid concentration in tumor tissue, which, as was mentioned above, in some cases is and in some cases is not enhanced, we should consider influence of ascorbic acid on type I and type II mechanisms of PDT with photosense.

It was firstly supposed by Rosenthal [4] and then generally accepted that ascorbic acid has to enhance type I radical route of PDT by donating the electron to photosensitizer in excited state. However, the following consideration shows that in oxygenated conditions, even with elevated ascorbic acid concentration, the electron transfer type I mechanism is not of great significance. Ascorbic acid tends to accumulate strongly in tumor. After ascorbic acid administration its level in tumor increases further, up to about $10^{-4}\,mol\,l^{-1}$. Assuming air-equilibrated biological level for oxygen $10^{-4}\,mol\,l^{-1}$, with bimolecular rate constants for photosense triplet state quenching by oxygen and ascorbate $2.3 \times 10^9\,mol^{-1}\,l\,s^{-1}$ [13] and $10^7\,mol^{-1}\,l\,s^{-1}$, correspondingly, ratio of rates of relative quenching processes will be about 200. It means that quenching of the photosense triplet by oxygen in the presence of ascorbate is dominating. Hence, processes (2) and (3) are not of great significance in air-saturated media, and singlet oxygen formation prevails.

It is known [32], however, that PDT mainly results in vascular occlusions, interrupting the supply of tumor by oxygen. Under hypoxic conditions in the biological environment direct electron transfer from ascorbate to the triplet state of photosense can prevail and gives free radicals: photosense anion-radical and ascorbate radical.

The most reasonable explanation of ascorbic acid ambivalent effect on the PDT therapeutic response seems to be as follows. In the presence of oxygen photosense behaves as type II sensitizer, meanwhile ascorbate monoanion scavenges singlet oxygen successfully in competition with other intracellular targets. Therefore, suppression of PDT by ascorbate, observed in some cases [9,10], may be accounted for in terms of its antioxidant properties. However, when concentration of ascorbate is elevated, these processes lead to abundant formation of ascorbic acid hydroperoxides [7,33]. Decomposition of hydroperoxides to the free radicals is catalyzed by iron ions, available in biological media:

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO^\bullet + OH^-$$

It may be supposed that formation of radicals from ascorbic acid hydroperoxides under certain conditions may increase efficiency of biomolecules damage in radical chain reactions. So ascorbic acid in elevated concentrations switches primary type II singlet oxygen to radical type I mechanism, the latter operates efficiently both in oxygenated and hypoxic conditions and in this way increases PDT therapeutic response, as was observed in [5].

5. Conclusions

Photosense under excitation sensitizes oxidation of ascorbate anion AH^- via both type I (prevails in the absence of oxygen) and type II (dominates in oxygenated conditions) pathways. Upon irradiation of photosense–ascorbic acid combination in aqueous media the radicals were produced in the process, which was found to depend on both solution pH and oxygenation. In the presence of oxygen formation of superoxide anion is negligible in comparison with efficient singlet oxygen photogeneration.

Acknowledgements

This work was supported by the Russian Foundation for Basic Research (Grant N 02-03-32168).

References

[1] I.J. MacDonald, T.J. Dougherty, J. Porphyrins Phthalocyanines 5 (2001) 105–129.
[2] E. Ben-Hur, A. Carmichael, P. Riesz, I. Rosenthal, Int. J. Radiat. Biol. 48 (1985) 837–846.
[3] N. Kuznetsova, N. Gretsova, V. Derkacheva, S. Mikhalenko, L. Solovyova, O. Yuzhakova, O. Kaliya, E. Lukyanets, Zh. Obshch. Khim. 72 (2002) 325–331.
[4] I. Rosenthal, E. Ben-Hur, Int. J. Radiat. Biol. 62 (1992) 481–486.
[5] A.V. Butenin, G.N. Vorozhtsov, O.L. Kaliya, E.A. Lukyanets, B.Yu. Kogan, G.A. Meerovich, N.L. Torshina, Yu.M. Luzhkov. Pat. RF 97100800 (20 January 1997).
[6] P.-T. Chou, A.U. Khan, Biochem. Biophys. Res. Commun. 115 (1983) 932–937.
[7] B.M. Kwon, C.S. Foote, J. Am. Chem. Soc. 110 (1988) 6582–6583.
[8] R.S. Bodannes, P.C. Chan, FEBS Lett. 105 (1979) 195–196.
[9] H. Singh, J. Vadasz, Biochem. Biophys. Res. Commun. 76 (1977) 391–397.
[10] S. Kimura, Y. Takahashi, J. Nutr. Sci. Vitaminol. 27 (1981) 521–527.
[11] P.C. Martin, M. Gouterman, B.V. Pepich, G.E. Renzoni, D.C. Schindele, Inorg. Chem. 30 (1991) 3305–3309.
[12] R. Edrei, V. Gottfreid, J.E. van Lier, S. Kimel, J. Porphyrins Phthalocyanines 2 (1998) 191–199.
[13] J. Davila, A. Harriman, Photochem. Photobiol. 50 (1989) 29–35.
[14] D. Phillips, Prog. React. Kinet. 22 (1997) 175–300.
[15] G. Ferraudi, G.A. Arguello, H. Ali, J.E. van Lier, Photochem. Photobiol. 47 (1988) 657–660.
[16] R.B. Ostler, A.D. Scully, A.G. Taylor, I.R. Gould, T.A. Smith, A. Waite, D. Phillips, Photochem. Photobiol. 71 (2000) 379–404.
[17] Handbook of Chemistry and Physics, 56th ed. CRC Press: Boca Raton, FL, 1976.
[18] G.R. Buettner, J. Biochem. Biophys. Meth. 16 (1988) 27–40.
[19] T. Sadat-Shafai, C. Ferradini, R. Julien, J. Pucheault, Radiat. Res. 77 (1979) 432–439.
[20] M.K. Shukla, P.S. Mishra, J. Mol. Structure 377 (1996) 247–259.
[21] R.H. Bisby, C.G. Morgan, I. Hamblett, A.A. Gorman, J. Phys. Chem. Part A 103 (1999) 7454–7459.
[22] S.Yu. Egorov, V.F. Kamalov, N.I. Koroteev, A.A. Krasnovsky Jr., B.N. Toleutaev, S.V. Zinukov, Chem. Phys. Lett. 163 (1989) 421–424.
[23] M. Rougee, R.V. Bensasson, C. R. Acad. Sci. 302 (1986) 1223–1226.
[24] I. Yamazaki, H.S. Mason, L. Piette, J. Biol. Chem. 235 (1960) 2444–2449.
[25] C.C. Felix, K. Reszka, R.C. Seaby, Photochem. Photobiol. 37 (1983) 141–147.
[26] G.P. Laroff, R.W. Fessenden, R.H. Schuler, J. Am. Chem. Soc. 94 (1972) 9062–9073.
[27] B.H.J. Bielski, A.O. Allen, H.A. Schwarz, J. Am. Chem. Soc. 103 (1981) 3516–3518.
[28] M. Shopova, T. Gantchev, J. Photochem. Photobiol., Part B: Biol. 6 (1990) 49–59.
[29] T.G. Gantchev, J.E. van Lier, Photochem. Photobiol. 62 (1995) 123–134.
[30] J.R. Darwent, I. McCubbin, D. Phillips, J. Chem. Soc. Faraday Trans. II 78 (1982) 347–357.
[31] D. Cabelli, B. Bielski, J. Phys. Chem. 87 (1983) 1809–1812.
[32] M. Ochsner, J. Photochem. Photobiol. Part B: Biol. 39 (1997) 1–18.
[33] B.-M. Kwon, C.S. Foote, S.I. Khan, J. Am. Chem. Soc. 111 (1989) 1854–1860.