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Article

Type II Photooxidation Mechanism of Biomolecules using Chloro(5,10,15,20-Tetraphenylporphyrinato)indium(III) as a Photosensitizer

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Foi determinado o mecanismo de fotooxidação de albumina de soro bovino (BSA), L-triptofano (Trp) e células vermelhas do sangue (RBC) por cloro(5,10,15,20-tetrafenilporfirinato) de índio(III) (InTPP). A velocidade de fotooxidação de Trp, BSA e RBC por InTPP foi diminuída na presença de NaN_3. A presença de D_2O aumentou a velocidade de fotooxidação de Trp e BSA e diminuiu a de RBC. Esta diminuição provavelmente está correlacionada com a redução da constante de associação entre InTPP e RBC na presença de D_2O. Não foi observada variação significativa na fluorescência das biomoléculas ou sobre a porcentagem de hemólise quando supressores de radicais (ferricianeto, manitol e superóxido dismutase) foram usados. Experimentos usando espectroscopia de ressonância paramagnética (EPR) mostraram que somente o $^1O_2$ foi gerado por InTPP. Foi proposto um modelo cinético para a fotooxidação de Trp e BSA. A concordância entre os resultados experimentais e este modelo corrobora a predominância do mecanismo via $^1O_2$ na fotooxidação das biomoléculas pelo InTPP.

The photooxidation mechanism of bovine serum albumin (BSA), L-tryptophan (Trp) and red blood cells (RBC) by chloro(5,10,15,20-tetraphenylporphyrinato)indium(III) (InTPP) was investigated. The photooxidation rate of Trp, BSA and RBC by InTPP was decreased in the presence of NaN_3. The presence of D_2O increases the photooxidation rate of Trp and BSA and decreases that of RBC. This decrease is probably related to a reduction of the binding constant between InTPP and RBC in the presence of D_2O. No significant change in biomolecule fluorescence or in the percent of hemolysis was observed when radical quenchers (ferricyanide, mannitol and dismutase superoxide) were used. Experiments using electron paramagnetic resonance (EPR) show that only $^1O_2$ was generated by InTPP. A mechanistic model based on the preferential oxidation of Trp and BSA by singlet oxygen is proposed. The agreement between the experimental data and the kinetic model gives additional support to the predominance of a mechanism via $^1O_2$ in biomolecule photooxidation by InTPP.

Keywords: chloro(5,10,15,20-tetraphenylporphyrinato)indium(III), photodynamic therapy, photooxidation mechanism, cancer, erythrocytes

Introduction

Photodynamic therapy (PDT) is a two-step therapeutic modality in which the topical or systemic delivery of photosensitizing drugs is followed by irradiation with visible light, at doses that are not in themselves harmful.[1] For treatment of cancer the photosensitizer is retained preferentially by the tumor and when excited by irradiation generates reactive oxygen species (ROS) that have a cytotoxic effect to the neoplasm.[2] These ROS can be generated by two mechanisms, known as type I and type II.[3,4]

In type I the photosensitizer in the excited triplet state can interact directly with the substrate and/or solvent, through an electron transfer reaction or hydrogen transfer, generating radical ions or neutral radicals, which quickly react with oxygen molecules producing ROS such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), capable of oxidizing a variety of biomolecules. In the type II mechanism, the photosensitizer in the excited triplet state can interact with ground state

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oxygen molecules ($^3O_2$) generating singlet oxygen ($^1O_2$) through an energy transfer process.[5] Studies in the literature suggest that the photooxidative mechanisms via singlet oxygen are often more efficient than radical processes due to the higher diffusibility of $^1O_2$ and the higher reaction rate constants with substrates.[6,7] Nonetheless, other studies show that photooxidations occur largely by a type I mechanism.[8,9] Some researchers have proposed that the type I mechanism is favored in polar solvents since the radicals generated through electron transfer would be stabilized in solvents with a high dielectric constant.[10,11] In contrast, the solubility and the lifetime of singlet oxygen are greater in lipophilic solvents and the type II mechanism is favored in hydrophobic systems. Recently, Vakrat-Haglili *et al.*[12] showed that the dye's environment determines the mechanism of photooxidation, yield, fate and efficacy of the species involved. Junqueira *et al.*[13] showed that the negatively charged micelle interface of sodium dodecyl sulfate (SDS) induces dimer formation of methylene blue and shifts a type II to type I mechanism. Other studies also have shown that some radicals such as superoxide and hydroxyl can be generated simultaneously with singlet oxygen by PDT.[14-16]

Tetraphenylporphines are compounds of interest in PDT due to their photophysical properties which favor singlet oxygen production.[17-19] Moreover the presence of indium(III) in the core of chloro[13^2-(dimethoxycarbonyl)-pheophorbidato methyl ester]indium (III) enhances *in vitro* and *in vivo* photosensitizing efficacies due to the heavy atom effect.[20,21] Chloro[13^2-(dimethoxycarbonyl)-pheophorbidato methyl ester]indium (III) encapsulated in liposomes of egg yolk phosphatidylcholine recently entered into phase II clinical trials for ocular photodynamic therapy in choroidal neovascular membranes.[22] Recent studies carried out in our laboratory have shown that the singlet oxygen quantum yield of chloro(5,10,15,20-tetraphenylporphyrinato)indium(III) (InTPP) (Figure 1) in dimethylsulfoxide (DMSO) (Φ_Δ = 0.72) was higher than that of 5,10,15,20-tetraphenylporphyrin (TPP) (Φ_Δ = 0.52) and that InTPP was an excellent photosensitizer in the photooxidation of tryptophan, bovine albumin and erythrocytes.[23] Even with this high value of Φ_Δ for InTPP, type I photosensitization pathways cannot be ruled out.[14] This work evaluates the main mechanism (type I or II) that acts in the photooxidation of tryptophan, bovine albumin and erythrocytes using InTPP or if both mechanisms proceed simultaneously in the photooxidation of the biomolecules and the cells. For this purpose, the influences of deuterium oxide and singlet oxygen quenchers were studied during the photooxidations. Electron paramagnetic resonance spectroscopy was also used to confirm if $^1O_2$ is the main reactive intermediate generated by InTPP for Trp and BSA photooxidation. An explicit mechanistic model for the photooxidation of the biomolecules is proposed based on the elementary steps reported by Rosenkranz *et al.*,[24] considering the experimental results for the main mechanism of photooxidation.

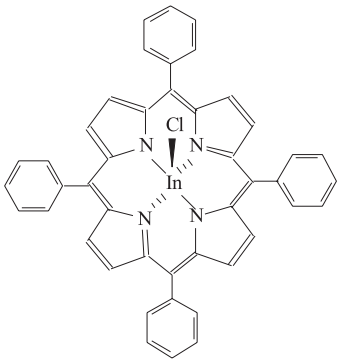


Figure 1. Molecular structure of chloro(5,10,15,20-tetraphenylporphyrinato) indium(III).

Experimental

Chemicals

Chloro(5,10,15,20-tetraphenylporphyrinato)indium(III) was purchased from Frontier Scientific, Inc. (Logan, UT, USA), with 95% purity. Bovine serum albumin, polyoxyethylene-sorbitan monolaurate (Tween® 20), dimethylformamide (DMF), potassium monobasic and dibasic phosphates, sodium chloride, potassium chloride, deuterium oxide, sodium azide, mannitol, dismutase superoxide, potassium ferricyanide, ethylenediaminetetraacetic acid (EDTA) and 2,2,6,6-tetramethyl-4-piperidone (TEMP) were purchased from Sigma Chemical Company (St. Louis, MO, USA) while Na_2HPO_4±4E_2O and tryptophan was from Merck (Whitehouse Station, NJ, USA). The red blood cells used in the hemolysis assays were donated by the Clinical Hospital of the University of Campinas, according to the Ethical Protocols for Human Experimentation of the Faculty of Medical Sciences of the University of Campinas.

InTPP solutions

Stock solutions of InTPP were prepared in DMF due to the low solubility of the photosensitizer in water. To obtain the desired concentration of InTPP in the biomolecules (Trp and BSA) photooxidation assays, aliquots of the stock solution were added to a photooxidation medium composed of phosphate buffer (pH 7.2, 0.07 mol L^{-1} K_2HPO_4 and 0.03 mol L^{-1} KH_2PO_4) and Tween® 20 (8.9 mmol L^{-1}). The final percentage of DMF in the photooxidation medium was maintained at 5%, v/v.

In the photohemolysis assays, aliquots of the InTPP stock solution were added to the photooxidation medium containing the erythrocyte solution (1.89×10^{10} cells L^{-1}), phosphate-buffered saline (PBS, pH 7.4, 171.1 mmol L^{-1} NaCl, 3.4 mmol L^{-1} KCl, 12.7 mmol L^{-1} Na_2HPO_4, 2.2 mmol L^{-1} KH_2PO_4), Tween® 20 (0.45 mmol L^{-1}) and DMF (1.6%, v/v).

Irradiation source

The irradiation system consisted of a mercury lamp (Phillips® HPLN 80W), a water jacket compartment (which absorbs infrared radiation emitted by the lamp), and a 400/600 nm bandpass filter (Oriel BG 38). The cuvette containing the solutions to be irradiated was placed 5 cm from the source lamp. The irradiance emitted by the mercury lamp, between 400 to 600 nm, was measured with a spectroradiometer (LI-1800, LI-Cor). The value measured was 62.7 W m^{-2}. All experiments were carried out in a dark room to prevent the influence of surrounding radiation.

The value of the irradiance absorbed by the photosensitizer solutions ($I_{photo,v}$), expressed as mol photons × m^{-2} × s^{-1} at each frequency, was calculated by equation 1:

$$I_{photo,\,v} = \frac{I_{o,v}\left[1 - \exp(-2.3A)\right]}{h\nu \times N_A} \quad (1)$$

where $I_{o,v}$ is the irradiance emitted by the mercury lamp at each frequency, A is the absorbance of InTPP, h is Planck's constant and N_A is Avogadro's constant. The total irradiance absorbed by the photosensitizer (I_{abs}) was calculated from: $I_{abs} = \Sigma_i\, I_{photo,v}$. To convert I_{abs} to mol photons × m^{-3} × s^{-1} the value of I_{abs} was multiplied by width of the cuvette (10^{-2} m).

Fluorescence measurements

Trp and BSA solutions were, respectively, excited at 281 and 279 nm, and fluorescence intensities were monitored at 357 and 326 nm with an ISS® PC1™-Photon Counting spectrofluorimeter from ISS (Champaign, IL, USA).

Evaluation of the mechanism involved in the photooxidation experiments

Trp and BSA photooxidation

Basically, the following photochemical reaction was utilized to determine photooxidation rate constants (k_p):

$$\text{Biomolecule} \xrightarrow{h\nu,\ \text{InTPP}} \text{Products} \quad (2)$$

The overall reaction is pseudo first-order with respect to biomolecules because the flux of photons and the concentration of photosensitizer are constant. Considering the concentration of the biomolecules to be proportional to their fluorescence intensity, the following expression is obtained:

$$\ln\left(\frac{F}{F_o}\right) = k_p t \quad (3)$$

where F is the fluorescence intensity of the biomolecules at time t, and F_o is the initial fluorescence before irradiation. Graphs of $\ln(F/F_o)$ *versus* irradiation time allow the determination of k_p.

N_2- or O_2-saturated phosphate buffer solutions (pH 7.2) containing InTPP (20 μmol L^{-1}), Trp (175 μmol L^{-1}) or BSA (116 μmol L^{-1}), plus Tween® 20 (8.9 mmol L^{-1}) and DMF (5% v/v), were irradiated for periods from 30 to 60 min. The effects of D_2O (10-50% v/v) or NaN_3 (0.01-2.0 mmol L^{-1}) and the influence of radical quenchers (potassium ferricyanide (50-2800 μmol L^{-1}), mannitol (20-2800 μmol L^{-1}) or dismutase superoxide (12.5-100.0 μg mL^{-1})) was investigated to analyze the involvement of type II and I mechanisms, respectively, in biomolecule photooxidations. Oxygen-saturated phosphate buffer solutions (pH 7.2) containing InTPP (1.1 or 20 μmol L^{-1}), Trp (150 μmol L^{-1}) or BSA (23 or 116 μmol L^{-1}), plus Tween® 20 (8.9 mmol L^{-1}), DMF (5%, v/v), and D_2O, NaN_3 or a radical quencher were irradiated for 30-60 min. Fluorescence intensities were measured at different times.

Hemolysis of RBC

Photohemolysis was carried out as described by Silva *et al.*[23] Typically, human blood of a single donor was collected 48 hours before the hemolysis assays in a tube containing EDTA, which was used as an anticoagulant. The tube was kept in a freezer until the moment of use. The serum was separated from the erythrocytes by centrifugation at 780 × g for 10 min. The RBC were washed with NaCl solution (0.85%, m/v) using three times the blood volume and then the cells were centrifuged at 1760 × g for 10 min to reduce the anticoagulant and serum residues. This procedure was repeated three times. RBC solutions (4 mL, 1.89×10^{10} cells L^{-1}) containing PBS (pH 7.4), Tween® 20 (0.45 mmol L^{-1}) and DMF (1.6%, v/v) were incubated in the dark with InTPP (6.4 μmol L^{-1}) for 30 min and then irradiated for 60 min using the photooxidation system previously described. InTPP was initially solubilized in DMF and later added to the solution containing PBS, Tween® 20 and DMF. The involvement of type II and I mechanisms in photohemolysis was evaluated, respectively, by effects

of D_2O (25-75%, v/v) or NaN_3 (50-150 μmol L^{-1}) and by the influence of radical quenchers (potassium ferricyanide (50-2800 μmol L^{-1}), mannitol (20-2800 μmol L^{-1}) or dismutase superoxide (12.5-100.0 μg mL^{-1})). Samples of 550 μL were collected at intervals of 10 min and centrifuged at 500 × g for 5 min. The supernatant was analyzed using a Hewlett Packard 8453A Diode Array Spectrophotometer at a wavelength of 542 nm to measure the oxyhemoglobin chromophore released from the erythrocytes as a result of the destruction of the RBC by the photodynamic action of the photosensitizer. The percentage of hemolysis achieved with the photodynamic action was calculated by equation 4:

$$\frac{100(A_1-A_2)}{A_T}\ \% \qquad (4)$$

where A_1 is the absorbance of the supernatant from the solution that contains the photosensitizer, A_2 is the absorbance of the control (without photosensitizer), and A_T is the absorbance of total hemolysis, which is calculated from the lysis of the erythrocytes with an ultrasound cell disrupter (Ney ULTRAsonik™ ultrasonic system).

Binding of sensitizers to erythrocytes

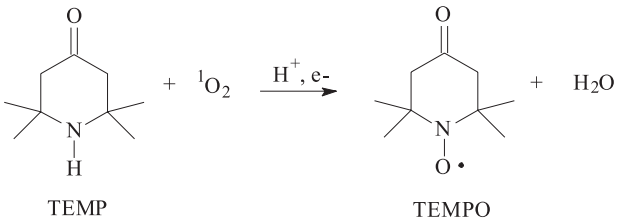
The binding constant of sensitizer to erythrocytes in the presence of D_2O was measured as described by Silva *et al.*[23] Typically, 1.0 mL of RBC at a total concentration ($[P]_{total}$) of 1.1×10^{11} cells L^{-1} in a solution containing 75% D_2O, PBS (pH 7.4), Tween® 20 (0.45 mmol L^{-1}) and DMF (1.6%, v/v), were incubated in the dark in the presence of photosensitizer concentrations ranging from 1.0 to 8.0 μmol L^{-1} for 30 min and then centrifuged at 500 × g for 5 min. The concentration of the free photosensitizer was determined spectrofluorimetrically ($\lambda_{excitation}$ = 436 nm and $\lambda_{emission}$ = 614 nm), based on a previously constructed calibration curve. Silva *et al.*[23] showed that the InTPP binding to erythrocytes is cooperative (there are four cooperative binding sites per cell). Then, we calculated the binding constant (K) of InTPP using the equation 5:[25]

$$\nu = \frac{nK[L]^n}{1+K[L]^n} \qquad (5)$$

where $\nu = [L]_{bound}/[P]_{total}$ = [sites occupied]/[sites total], n is the number of cooperative sites and [L] is the free concentration of the InTPP. $[L]_{bound}$ in equation 5 was calculated by: $[L]_{bound} = [L]_{total} - [L]$. The parameters n and K of equation 5 were evaluated using the software Origin® 7.5 (OriginLab Inc, Northampton, MA, USA).

EPR measurements

According to Lion *et al.*,[26] the presence of singlet oxygen - in any system - can be confirmed by the detection of a triplet EPR signal assigned to the stable nitroxide radical 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (TEMPO). TEMPO is generated by the reaction of TEMP with $^1O_2$ (Scheme 1).[26] Oxygen-saturated solutions of TEMP (38 mmol L^{-1}) were irradiated for 30 min at room temperature in the presence or absence of InTPP (20 μmol L^{-1}), in phosphate buffer (pH 7.2) containing Tween® 20 (8.9 mmol L^{-1}) and DMF (5%, v/v). The irradiation system was the same described earlier. EPR spectra were recorded with an ELEXSYS-CW Bruker spectrometer using a rectangular cavity operating in TE_{102} mode. Aliquots from irradiated solutions were promptly transferred to a flat quartz cell and measurements were carried out at room temperature with the following instrument settings: microwave power, 20 mW; microwave frequency, 9.685 GHz; field modulation frequency, 100 kHz; field modulation amplitude, 1 G; time constant, 21 ms; and scan time, 84 s.



Scheme 1.

Oxygen concentration measurements

The oxygen concentrations in the N_2- and O_2-saturated phosphate buffer solutions containing InTPP, Trp or BSA, plus Tween® 20 and DMF were measured before the photooxidation experiments using a Clark-type oxygen electrode connected to a computer-operated Oxygraph control unit (Hansatech Instruments, Norfolk, England) at 25 °C.

Results and Discussion

Determination of the mechanism (type I or II) for biomolecule photooxidation

Photooxidations were carried out under both aerobic and anaerobic conditions to evaluate the involvement of molecular oxygen in the degradation of Trp and BSA. Figures 2a and 2b show a significant reduction of fluorescence intensities (> 70%) for irradiated solutions of Trp and BSA in the presence of O_2. In contrast, photodegradation was negligible under anaerobiosis (reduction < 34%). These results clearly show that oxygen is essential for the photooxidations. The small amount of degraded Trp and BSA, under anaerobic conditions, probably occurred due to the incomplete removal of oxygen

from the solutions. Ericson *et al.*[27] reported that oxygen is not completely removed from solutions deoxygenated with N_2. Measurement of the oxygen concentration in the solutions used in these experiments shows that, in anaerobic conditions, the oxygen concentration (139.8 ± 0.8 μmol L^{-1}) was 5.2 times smaller than the oxygen concentration measured in aerobic conditions (723.7 ± 0.6 μmol L^{-1}).

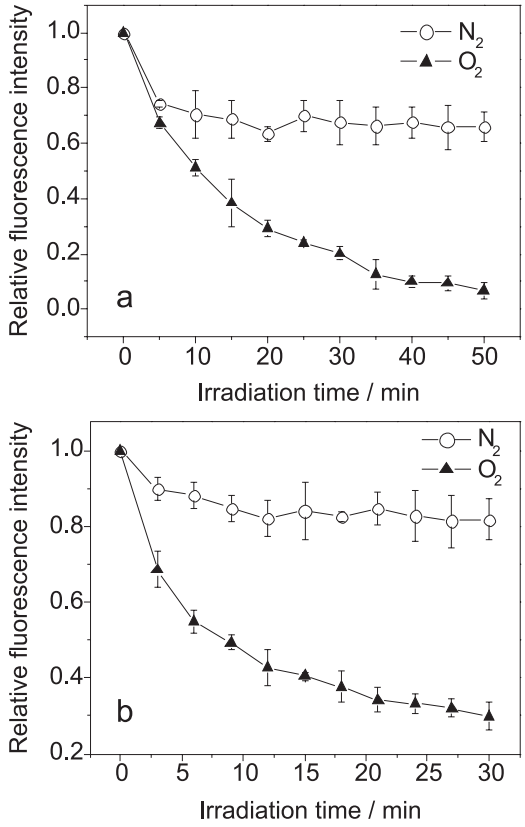


Figure 2. Evaluation of the involvement of molecular oxygen in the photooxidation of (a) Trp and (b) BSA. N_2- or O_2-saturated phosphate buffer solutions (pH 7.2) containing InTPP (15 μmol L^{-1}), and Trp (150 μmol L^{-1}) or BSA (150 μmol L^{-1}) plus Tween® 20 (8.9 mmol L^{-1}) and DMF (5%, v/v) were irradiated for several min. Fluorescence intensities of the solutions were measured at different times. Data represents mean ± SD of three independent experiments.

Figures 3a and 3b show that increasing the concentration of D_2O to 50%, photooxidation rate constants for Trp and BSA increase 3.9 and 13.9 times, respectively. It is known that the lifetime of singlet oxygen is approximately one order of magnitude higher in D_2O than in H_2O. Thus, these results suggest that the biomolecules are photooxidized by a type II mechanism. Conversely, in the presence of 2.0 and 2.6 mmol L^{-1} sodium azide, rate constants for Trp and BSA decrease, respectively, 4.2 and 3.3 times (Figures 4a and 4b). This decrease was higher at low concentrations of sodium azide, and lower at higher concentrations. It is known that azide quenches singlet oxygen by two processes (reversible and irreversible).[28] The latter is characterized by generation of superoxide and azide radicals which can oxidize biomolecules. Probably at high concentrations of azide (> 0.2 mmol L^{-1} for Trp and > 1.0 mmol L^{-1} for BSA photooxidations), the generation of radical species accounted for the slower decrease in the rate constants. Otherwise, when a low concentration of a quencher was used, a non-significant amount of radical species was generated and an abrupt decrease of rate constant was observed due to the quenching of singlet oxygen. These results are similar to data reported in other research and provide strong evidence to support the hypothesis that the photooxidation of Trp and BSA occurs by a type II mechanism.[10,29,30] However, these trials do not allow evaluation of the participation of other active oxygen intermediates during the photooxidation. Many studies have shown that radicals (*e.g.*, superoxide and hydroxyl) can be generated by PDT simultaneously with the singlet oxygen.[14-16] Thus, we decided to evaluate the influence of known ROS scavengers on the InTPP-sensitized photooxidation of Trp and BSA. Photooxidations were carried out in the presence either of potassium ferricyanide (electron-scavenger), mannitol ($^{\bullet}OH$ quencher) or dismutase superoxide ($O_2^{\bullet -}$ suppressor). No alterations in the decay of the biomolecule fluorescence intensities were observed (results not shown). These results show that Trp and BSA are not oxidized by electron transfer, or by hydroxyl or superoxide radicals.

EPR experiments using TEMP as a singlet oxygen trapping agent were carried out to confirm if $^1O_2$ is the main reactive intermediate generated by InTPP for biomolecules photooxidation.[26,31] The reaction of TEMP with singlet oxygen produces a stable nitroxide radical (TEMPO) (Scheme 1) readily detectable by EPR. Irradiation of an oxygen-saturated phosphate buffer (pH 7.2) solution containing InTPP (20 μmol L^{-1}) and TEMP (38 mmol L^{-1}) led to the generation of a triplet EPR signal (Figure 5a) characteristic of a nitroxide radical (TEMPO in this case).[16,26] The intensity of the signal increased rapidly during irradiation of the solution containing InTPP (Figure 5a). In the absence of the photosensitizer, no increase of the signal was observed (Figure 5b). A small triplet signal was present before the irradiation of TEMP due to impurities of TEMPO. In conclusion, these results show that the photooxidation of TEMP proceeds via a type II mechanism, confirming that Trp and BSA are also oxidized by a type II process.

Determination of the mechanism (type I or II) for the RBC photooxidation

Figure 6a shows the results of photohemolysis experiments carried out in phosphate-buffered saline with

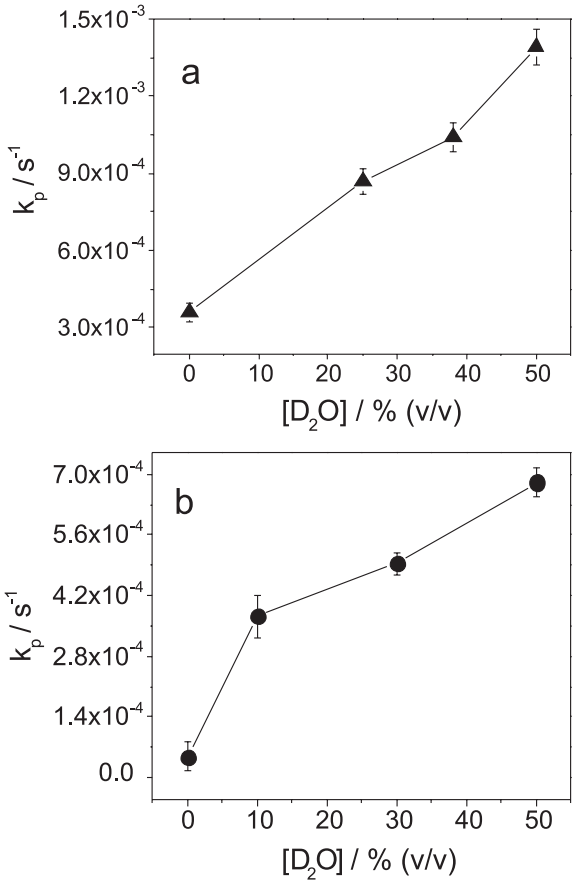


Figure 3. Influence of D_2O on the photooxidation rate constants of (a) Trp and (b) BSA. Oxygen-saturated phosphate buffer solutions (pH 7.2) containing InTPP (20 μmol L^{-1} in (a) and 1.1 μmol L^{-1} in (b)), Trp (150 μmol L^{-1}) or BSA (23 μmol L^{-1}) and different concentrations of D_2O (0-50%, v/v) plus Tween® 20 (8.9 mmol L^{-1}) and DMF (5%, v/v) were irradiated for 30-60 min. Fluorescence intensities were measured at different times and k_p values were obtained from graphs of ln(F/F_o) *versus* irradiation time. Data represents mean ± SD of three independent experiments.

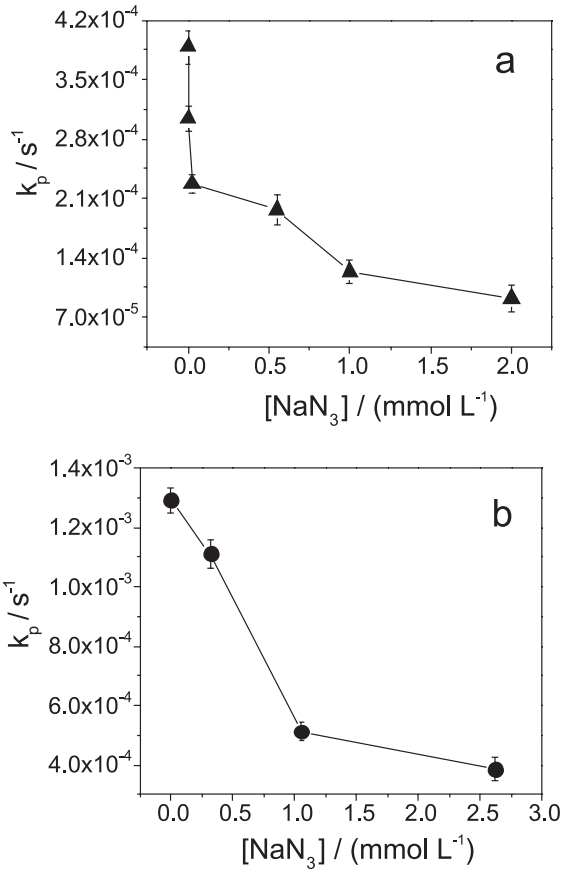


Figure 4. Influence of NaN_3 on the photooxidation rate constants of (a) Trp and (b) BSA. Oxygen-saturated phosphate buffer solutions (pH 7.2) containing InTPP (20 μmol L^{-1}), Trp (150 μmol L^{-1}) or BSA (116 μmol L^{-1}) and different concentrations of NaN_3 (0-2.0 mmol L^{-1}) plus Tween® 20 (8.9 mmol L^{-1}) and DMF (5%, v/v) were irradiated for 30-60 min. Fluorescence intensities were measured at different times and k_p values were obtained from graphs of ln(F/F_o) *versus* irradiation time. Data represents mean ± SD of three independent experiments.

increasing D_2O concentrations. There was a marked decrease in the rate of photohemolysis when the concentration of D_2O is increased from 25 to 75%, v/v. For example, the irradiation time to inactivate 50% of the cell population increased from 29 to 34 min when the D_2O concentration increased from 0 to 25%, and to 44 and 51 min when 50 and 75% of D_2O were used. The same situation was observed by De Polis *et al.*[32] If the photohemolysis proceeds by a singlet-oxygen mechanism, it would be expected to be faster in D_2O than in H_2O because the lifetime of singlet oxygen is greater in the former solvent.[33] However, the rate decrease observed should not be taken as an argument against a singlet-oxygen mechanism. As discussed by Valenzeno *et al.*[34] the D_2O effects on photohemolysis using other sensitizers are somewhat variable, ranging from no effect to an enhancement of over 200%. We have shown recently that the binding constant between RBC and InTPP is $(2.40 \pm 0.05) \times 10^7$ L mol^{-1}, in aqueous solutions.[23]

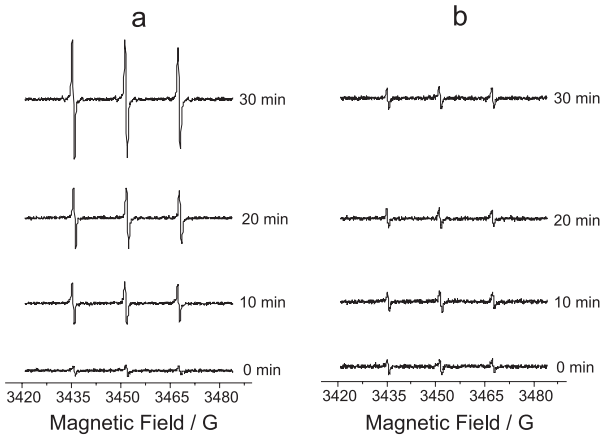


Figure 5. Indirect detection of singlet oxygen by electron paramagnetic resonance. EPR spectra of TEMPO generated by photooxidation of 2,2,6,6-tetramethylpiperidone (38 mmol L^{-1}) at room temperature in the (a) presence and (b) absence of InTPP (20 μmol L^{-1}), in oxygen-saturated phosphate buffer (pH 7.2) containing Tween® 20 (8.9 mmol L^{-1}) and DMF (5%, v/v). Irradiation times of solutions are indicated on the figures.

Therefore, InTPP has a high affinity for the erythrocyte membrane due to its lipophilicity.[23] It is thus expected that the InTPP molecules enter into the hydrophobic region of the lipid bilayer together with oxygen molecules where the singlet oxygen will be generated. However, the presence of D_2O in the photooxidation medium decreased the InTPP affinity for the erythrocytes membrane. The binding constant between RBC and InTPP was decreased to $(0.92 \pm 0.06) \times 10^7$ L mol^{-1} when 75% D_2O was used. Rosen and Klebanoff reported that D_2O may influence photochemical reactions by exchanging a deuterium for hydrogen in a C-H or an O-H bond that is involved in the reaction.[35] The D_2O may alter the observed reaction rate if a C-D or an O-D bond is involved in the rate-limiting reaction step. Besides, the exchange of deuterium for hydrogen at the binding sites can reduce the affinity of InTPP to bind to RBC. This smaller affinity probably contributed to decrease the photohemolysis in the D_2O solvent. In this situation, the increase of the D_2O concentration in the photohemolysis medium decreases the rate of the hemolysis and increases the time needed to inactive the RBC.

The effect of sodium azide as a singlet oxygen quencher was also investigated in the photohemolysis. Figure 6b shows that the presence of NaN_3 in the photohemolysis medium affected the ability of InTPP to inactive the RBC. For example, after 60 min of irradiation the average hemolysis percentage of erythrocytes was reduced from 83 to 66% when the azide concentration was increased from 0 to 50 μmol L^{-1}, and to 55 and 33% for 100 and 150 μmol L^{-1} of azide. These results suggest that the RBC are photooxidized by a type II mechanism.

Erythrocyte photohemolysis was also carried out in the presence of radical quenchers (potassium ferricyanide, mannitol or dismutase superoxide). No alterations in the hemolysis percentage were observed (results not shown). These results show that erythrocytes are not oxidized by electron transfer, or by hydroxyl or superoxide radicals.

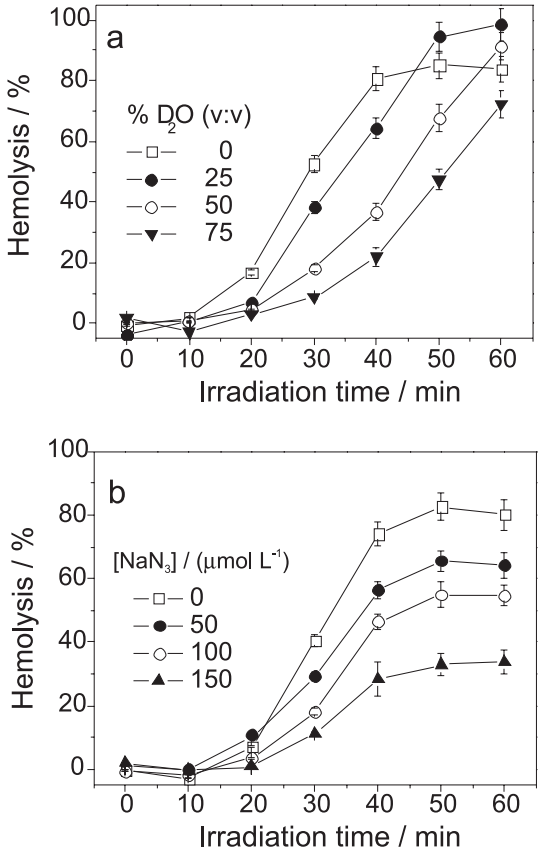


Figure 6. Influence of (a) D_2O and (b) NaN_3 on the photohemolysis of human red blood cells. RBC solutions (1.89×10^{10} cells L^{-1}) in PBS (pH 7.4) containing InTPP (6.4 μmol L^{-1}), Tween® 20 (0.45 mmol L^{-1}), DMF (1.6%, v/v) and different concentrations of D_2O (25-75%, v/v) or NaN_3 (50-150 μmol L^{-1}) were irradiated for 60 min. The absorbance of the oxyhemoglobin chromophore released from the erythrocytes as a result of the destruction of the RBC was measured at different times and the percent hemolysis values were obtained from equation (4). Data represents mean ± SD of three independent experiments.

Comparing the photooxidation of BSA and RBC by InTPP

The rate constant for the pseudo first-order photooxidation of the BSA using 6.4 μmol L^{-1} of InTPP is $k_p = (2.0 \pm 0.15) \times 10^{-4}$ s^{-1}. This result was obtained from a graph of $\ln(F/F_o)$ *versus* irradiation time (graph not shown). The half-life of BSA calculated from k_p is (57.8 ± 3.5) min. The time to inactivate 50% of the cell population of RBC, obtained with 0% D_2O (Figure 6a), is (29.2 ± 1.9) min. The association constant for BSA is $(1.15 \pm 0.07) \times 10^5$ L mol^{-1} and the binding sites are independent.[23] For erythrocytes, the association constant is $(2.40 \pm 0.05) \times 10^7$ L mol^{-1} and there are four cooperative binding sites per cell.[23] These results suggest that the higher photodynamic activity of InTPP with RBC may be associated with a higher affinity of InTPP for RBC than for BSA.

Kinetic model

As the photooxidation of Trp and BSA occurred via a singlet oxygen-mediated mechanism, we propose a mechanistic model consisting of ten elementary reactions (Table 1) for biomolecule photooxidation mediated by InTPP. The kinetic steps of this mechanism are similar to those proposed by Rosenkranz *et al.*[24] for the photodynamic inactivation of lysozyme.

P_o, ^1P, and ^3P, denote respectively the ground, excited singlet, and excited triplet states of the dye. O_2, and $^1O_2$, are the ground (triplet) and the excited singlet states of molecular oxygen. S is the substrate (biomolecule) and S-O_2 the product of photooxidation. In their work, Rosenkranz

Table 1. Elementary reactions of the mechanistic model for biomolecule photooxidation mediated by InTPP

$P_o \longrightarrow {}^1P$	$I_{abs} = 8.2 \times 10^{-8}$ mol photons $m^{-3}s^{-1}$	*calculated	(1)
${}^1P \longrightarrow P_o$	$k_2 = 7.3 \times 10^7\ s^{-1}$	calculated	(2)
${}^1P \longrightarrow {}^3P$	$k_3 = 1.2 \times 10^9\ s^{-1}$	ref. [36]	(3)
${}^3P \longrightarrow P_o$	$k_4 = 6.3 \times 10^5\ s^{-1}$	Own measurement	(4)
${}^3P + O_2 \longrightarrow P_o + O_2$	$k_5 = 3.0 \times 10^8$ L $mol^{-1}\ s^{-1}$	calculated	(5)
${}^3P + O_2 \longrightarrow P_o + {}^1O_2$	$k_6 = 1.0 \times 10^9$ L $mol^{-1}\ s^{-1}$	estimated	(6)
${}^1O_2 \longrightarrow O_2$	$k_7 = 5.0 \times 10^5\ s^{-1}$	ref. [24]	(7)
$S + {}^1O_2 \longrightarrow S{-}O_2$	$k_8 = 0.4 \times 10^7$ L $mol^{-1}\ s^{-1}$	ref. [37]	(8)
$S + {}^1O_2 \longrightarrow S + O_2$	$k_9 = 4.0 \times 10^7$ L $mol^{-1}\ s^{-1}$	ref. [37]	(9)
${}^3P + S \longrightarrow P_o + S$	$k_{10} = 2.7 \times 10^7$ L $mol^{-1}\ s^{-1}$	ref. [37]	(10)

*I_{abs} calculated for the InTPP concentration of 20 μmol L^{-1}.

et al.[24] showed that the enzymatic activity of solutions of lysozyme containing acridine orange decreased by an overall pseudo first-order process during irradiation with visible light. Applying quasi-stationary conditions for $[{}^1O_2]$ and $[{}^3P]$, the rate constant (k_p) for the process is given by the following expression:[24]

$$k_p = I_{abs}\phi_T \times \frac{k_6[O_2]}{k_4 + (k_5 + k_6)[O_2] + k_{10}[S]} \times \frac{k_8}{k_7 + k_8[S] + k_9[S]} \quad (6)$$

where k_p is a constant when the sum $k_7 + k_8[S] + k_9[S]$ is approximately independent of time.[24] This is always the case when $k_7 >> k_8[S] + k_9[S]$. ϕ_T is the triplet quantum yield of the photosensitizer and it represents the relation between the constants k_3 and k_2,: $\phi_T = k_3/(k_3+k_2)$.[19] Kinetic simulations of the InTPP-sensitized photooxidation of Trp were run with the software Gepasi 3.21.[38,39] Theoretical k_p values calculated by software Gepasi were obtained from plots of $\ln[Trp]/[Trp]_o$ *versus* irradiation time. [Trp] and $[Trp]_o$ are, respectively, the concentrations of tryptophan during at different time points and that before irradiation. The Trp concentrations at different times were provided by the software. We also considered that quenching of the excited singlet state (k_s) of the photosensitizer occurred by radiative (fluorescence decay 'f') and by non-radiative processes (internal conversion 'ic' and intersystem-crossing 'isc'). The rate constant for step (2), k_2, was calculated using the experimental values of k_f, k_{isc} and ϕ_f (fluorescence quantum yield) for InTPP.[36] The following equations were used: $k_2 = k_s - k_{isc}$, $k_3 = k_{isc}$ and $k_s = k_f/\phi_f$.[19] The constant k_4 was obtained from our own measurement of the triplet lifetime.[23]

We assumed that singlet oxygen was formed exclusively by energy transfer from the triplet state of the photosensitizer to ground state molecular oxygen. The proportion of ^3P quenched by O_2 is approximately equal to one because $k_5 + k_6 >> k_{10}$. Quenching of ^1P by O_2 was not considered because of the very short lifetime of the first excited singlet state of InTPP.[36] Then, k_5 was calculated using the following equations:[7]

$$\phi_\Delta = \phi_T \times k_6/(k_5 + k_6) \quad (7)$$

where ϕ_Δ is the quantum yield of singlet oxygen. According to the literature, values for k_6 are found in the range 1-3 $\times 10^9$ L $mol^{-1}\ s^{-1}$, therefore, we assumed $k_6 = 1 \times 10^9$ L $mol^{-1}\ s^{-1}$.[5] Singlet oxygen quantum yields ($\phi_\Delta = 0.72$) were obtained by laser flash photolysis experiments using pheophorbide-a as a reference.[23] An oxygraph apparatus was used to determine the concentration of oxygen ($[O_2] = 723.7 \pm 0.6$ μmol L^{-1}) as described earlier. The remaining constants in Table 1 were obtained from the literature.[24,37]

Comparing results with the kinetic model

Figure 7a shows a linear relation between $\ln(F/F_o)$ and the irradiation time for Trp in the presence of different concentrations of InTPP, which indicates that the sum $k_7 + k_8[S] + k_9[S]$ is approximately independent of time, probably due to the fact that $k_7 >> k_8[S] + k_9[S]$. The substitution of I_{abs} for $k_1[P_o]$ in equation 1 provides a modified expression for k_p:

$$k_p = k'_p \times [P_o] \quad (8)$$

where

$$k'_p = k_1\phi_T \times \frac{k_6[O_2]}{k_4 + (k_5 + k_6) + k_{10}[S]} \times \frac{k_8}{k_5 + k_7[S] + k_9[S]} \quad (9)$$

Therefore, a linear relation between k_p and $[P_o]$ must be maintained if photooxidations occur by the mechanism suggested in Table 1.

For the photooxidation of Trp, experimental k_p values increase linearly with [InTPP] (Figure 7b). The same results were observed for the photooxidation of BSA (data not shown). Theoretical values of k_p (for Trp) were determined by kinetic simulations (using the software Gepasi 3.21) and plotted for comparison with the experimental data (Figure 7b). The theoretical values are in good agreement with experimental data, validating the photooxidative mechanistic model consisting of ten elementary reactions (Table 1, where $^1O_2$, in step (8), is the only species responsible for the consumption of Trp). Therefore, kinetic modeling also confirms that InTPP photosensitized oxidation of Trp occurs in accordance with a type II process and Trp or BSA photooxidation occurs by the same elementary steps proposed by Rosenkranz *et al.*[24] for the photodynamic inactivation of lysozyme. To our knowledge, this is the first work showing that the photooxidation of biomolecules by photosensitizer may occur by the same elementary steps as for the inactivation of lysozyme.

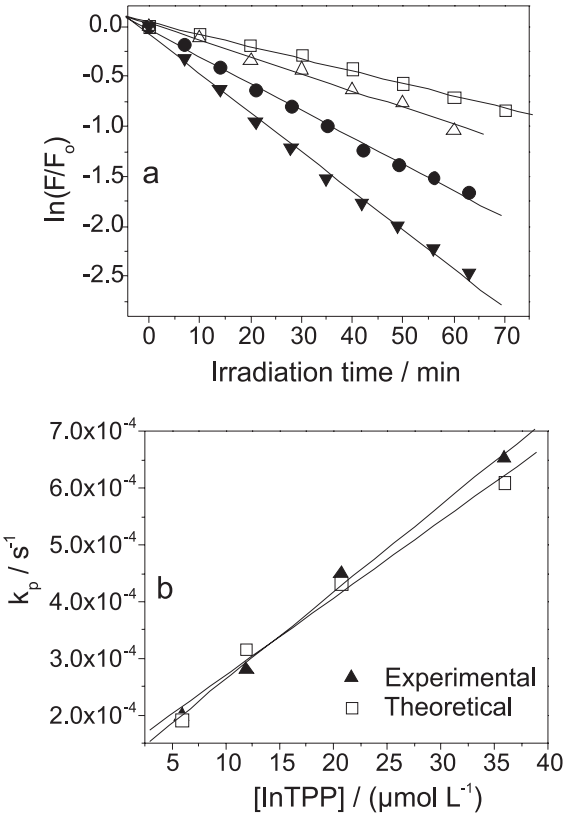


Figure 7. Comparison of experimental data with the kinetic model. (a) $\ln(F/F_o)$ *versus* irradiation time of Trp solutions ($[Trp]_o$ = 175 μmol L^{-1}, phosphate buffer pH 7.2, 5% (v/v) DMF and 8.9 mmol L^{-1} of Tween® 20) in the presence of InTPP at (□) 5.9, (Δ) 11.9, (●) 20.7 and (▼) 35.9 μmol L^{-1}. (b) Pseudo first-order rate constants for the Trp photooxidation (175 μmol L^{-1}) simulated (□) by Gepasi 3.21 kinetic software for different concentrations of InTPP (5.9, 11.9, 20.7 and 35.9 μmol L^{-1}) and obtained (▲) from experimental results.

Conclusions

In this work, we showed that a type II photodynamic mechanism explains the InTPP-mediated photooxidation of Trp and BSA, because the photooxidation rate constants (k_p) increased in the presence of deuterium oxide and decreased in the presence of sodium azide. The same mechanism also explains red blood cells photooxidation since the hemolysis percentage was reduced in the presence of sodium azide. Evidence in favor of a type II mechanism was also found when potassium ferricyanide, mannitol or dismutase superoxide were used as radical suppressors, because no variations on k_p values and percent hemolysis were observed. EPR studies corroborated the proposal that singlet oxygen was the principal oxidant generated by InTPP during the photochemical reactions, due to the detection of a triplet EPR signal assigned to TEMPO. Finally, kinetic simulations for the photooxidation of Trp were performed taking into account a series of 10 proposed elementary reactions (Table 1). Theoretical k_p values obtained from the simulations were in good agreement with experimental data, confirming, once more, that photooxidations mediated by InTPP occur by a type II mechanism.

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