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Singlet oxygen dosimetry using uric acid as a chemical probe: Systematic evaluation

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ABSTRACT

The singlet oxygen generation of a photosensitizer compound (PS) is an important property for photodynamic treatments. The PS quantum yield of $^1O_2$ (Φ_Δ) is usually obtained by the time-resolved measurement of $^1O_2$ phosphorescence emission. However, the equipment employed is quite expensive. In the present study, the methodology previously proposed by us for Φ_Δ dosimetry using uric acid (UA) as a chemical-probe was systematically evaluated. The PS photo-excitation was achieved with a polychromatic light source (LED) that generates $^1O_2$ which attacks the UA. The level of UA oxidation is proportional to a parameter called chemical photodynamic efficiency, which is correlated to Φ_Δ by comparing it to a standard compound. The photobleaching reaction of the PS is considered in the calculation. Several parameters that influence this measurement have been investigated with different PS (phenothiazines, xanthenes, chlorins, and benzoporphyrin dyes) in water, ethanol, and aqueous P-123 polymeric surfactant. Excellent results were obtained when standard compounds belonging to the same class of the photosensitizer and the same solvent were used. The chemical method of $^1O_2$ evaluation with UA is reliable, exact, precise, easy to perform and low cost, although limited by the standard compound employed as Φ_Δ^{Std}.

1. Introduction

The evaluation of the efficiency of photosensitizer compounds (PS) to generate toxic species that damage biological tissues is essential to photodynamic therapy (PDT). Once the PS is promoted to the excited singlet state ($^1PS^*$), it can be deactivated by several mechanisms, including the intersystem crossing to triplet state ($^3PS^*$). From this electronic state, the compound can react with biological substrates, classified as type I photochemical reactions (electron transfer route), leading to free radicals, especially reactive oxygen species (ROS), or type II photochemical reactions (energy transfer mechanisms) toward molecular oxygen ($^3O_2$) to singlet oxygen ($^1O_2$). Both types of reactions produce highly reactive and toxic species that damage the immediate locale of the light-absorbing PS, i.e., the undesirable biological tissue [1–5]. The main PDT pathway is attributed to a type II reaction [3,6,7]; therefore, it is important to evaluate the ability of the PS under illumination to produce $^1O_2$.

The most employed measurement of singlet oxygen yield (Φ_Δ) is based on PS excitation using a LASER followed by time-resolved analysis of $^1O_2$ phosphorescence emission at near infrared, around 1270 nm. However, this method employs expensive equipment which many research groups cannot afford. One alternative methodology consists of the use of chemical trapping or scavenging of $^1O_2$ acceptors by reaction. This chemical probe is a substrate that undergoes oxidation during the PS illumination. Based on the level of oxidation of the substrate, Φ_Δ is obtained [8,9]. Several compounds can be used as a $^1O_2$ scavenger, such as diphenylisobenzofuran, tryptophan, *p*-nitrosodimethylaniline, anthracene-9,10-dipropionic acid, betacyanin, and bovine serum albumin, among others [8,10–12].

One very interesting chemical probe for $^1O_2$ quantification is uric acid (UA) (Scheme 1). This methodology was initially employed by Fischer and co-workers [11].

They proposed [11] irradiating the PS with LASER monochromatic light in the presence of UA and measuring the amount of reacted UA at a determined time monitored by UV electronic spectrophotometry. Based on this result, a scale named Photodynamic Activity (PA) was proposed from Eq. (1).

$$PA = \frac{\Delta Abs_{UA} \cdot 10^5}{E_0 \cdot t \cdot Abs_{\lambda irr}} \quad (1)$$

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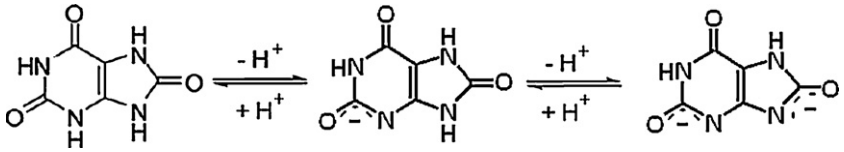

Scheme 1. Species of UA corresponding to its protolytic equilibrium $UA^0 \rightleftharpoons UA^- \rightleftharpoons UA^{=}$. Extracted from [13].

where ΔAbs_{UA} is the decrease in the absorbance of UA at 292 nm, E_0 is the fluency rate (W/m) of the LASER, t is the irradiation time, and $Abs_{\lambda irr}$ is the PS absorbance at the wavelength that the LASER emits. However, Eq. (1) is restricted to monochromatic light sources. Bonacin and co-workers [12] proposed the use of betacyanin (Bc) as a chemical probe and a light-emitting diode (LED) as a light source. For polychromatic light as emitted by LED, the photon flux absorbed by the PS (R) is calculated by Eq. (2):

$$R = \int Abs_{PS} E_{LED} d\lambda \quad (2)$$

where Abs_{PS} is the absorbance of the PS and E_{LED} is the LED light intensity. Eq. (2) takes into account the spectral overlap between the PS absorption and the light source emission. Parameter R can be related to the degradation of the Bc chemical scavenger through its rate constant, which is proportional to the $^1O_2$ formation; the oxidation of the Bc obeys first-order kinetic law. The proposed methodology is valid for photostable compounds, however, several photosensitizers in aerated solution suffer photobleaching under illumination.

Recently we proposed an alternative methodology to evaluate the Φ_Δ of $^1O_2$ with UA and a polychromatic light source (LED) that considers the PS photobleaching reaction [13]. For each PS, the amount of photons absorbed during t time by the PS under irradiation from a polychromatic light source (N_{Abs}) is calculated, with the photobleaching taken into account, as in Eq. (3). The UA only absorbs at the UV region.

$$N_{Abs} = \frac{1}{N_a \cdot h \cdot c} \int_0^t \int_{\lambda_1}^{\lambda_2} P_{(\lambda)} \cdot I_0 (1 - 10^{-bc\varepsilon}) \cdot e^{-k_{PB} t} d\lambda dt \quad (3)$$

where h: Planck constant (6.626×10^{-34} J s), c: light velocity (2.997×10^8 m s^{-1}), N_a: Avogadro constant (6.022×10^{23} mol^{-1}), I_0 ($1–10^{-bc\varepsilon}$): absorbed light intensity, $bc\varepsilon$: PS absorbance, $P_{(\lambda)}$: potency (in mW) at each wavelength, and k_{PB}: photobleaching rate constant. The N_{Abs} parameter is proportional to the degradation rate constant of UA (k_1) by Eq. (4), which furnishes the term that we have named the Chemical Photodynamic Efficiency (γ_Δ). This γ_Δ is determined by the slope of the plot of k_1 *versus* N_{Abs}. In fact, γ_Δ not only represents the UA degradation caused by $^1O_2$, but also by the other ROS; however, $^1O_2$ is the main reactive species in PDT [3,6,7]. Considering that the reactive species is only $^1O_2$, the Φ_Δ of the PS is calculated by Eq. (5) using a standard photosensitizer with a known Φ_Δ^{Std} and measuring the Chemical Photodynamic Efficiency for the standard γ_Δ^{Std} and for the investigated PS (γ_Δ^{PS}).

$$\gamma_\Delta \propto \frac{k_1}{N_{Abs}} \quad (4)$$

$$\phi_\Delta^{PS} = \frac{\phi_\Delta^{Std}}{\gamma_\Delta^{Std}} \gamma_\Delta^{PS} \quad (5)$$

However, the characteristics of the chemical probe employed for Φ_Δ evaluation should be thoroughly investigated to guarantee that the measurement is reliable, exact, precise, easy to perform and can be widely applied. In the present work, we investigated the details of the method proposed by us [13] using UA/LED experiments for the $^1O_2$ measurement. Several variables of the method were systematically investigated using phenothiazinium, chlorophyll, benzoporphyrin, and xanthene derivatives in different solvents.

2. Materials and methods

2.1. Dyes and systems

The phenothiazinium derivatives (PD) employed were methylene blue (MB) and *o*-toluidine blue (TBO), which are both cationic dyes employed in Microorganism Photodynamic Inactivation and PDT [14–19] (Fig. 1A).

The chlorophyll derivatives (CD) used were chlorophyll *a* (Mg-Chl), pheophytin (Pheo), pheophorbide (Pheid), and zinc chlorophyllide (Zn-Chld). The charges of each structure are due to the neutral pH employed in all experiments (Fig. 1B). These compounds are usually hydrophobic and show high Φ_Δ [13,20–24].

Benzoporphyrin derivatives (BD), a class of chlorin photosensitizer, were successfully applied for PDT, especially BPDMA®, the active drug of Visudyne®. During the synthesis of benzoporphyrin compounds two regioisomers are obtained, A- and B-ring isomers. A-ring compounds were employed to prepare monoacid derivatives, here named B3A$^-$ and BPDMA; B-ring compounds were used to prepare the monoacid derivative, here named BPDMB$^-$, and the ester derivative, here named TetraesterB; the structures of which are shown in Fig. 1C [25–32] with their charges in solutions with neutral pH [33,34].

The xanthene derivatives (XD) studied were eosin Y (EOS), erythrosin B (ERY), and bengal rose (RBB), all in dianionic protolytic form at neutral pH (Fig. 1D) [35,36].

Surfactant P-123 has been employed with success in PDT drug formulation to avoid self-aggregation phenomena of chlorins [24] and benzoporphyrins [29,37] in water.

The working solutions were phosphate buffer (7.5×10^{-3} mol L^{-1} of Na_2HPO_4, pH 7.25) in water at ionic strength controlled by 0.10 mol L^{-1} of NaCl in: (i) water and (ii) P-123 aqueous solutions (2% m/v, above its critical micellar concentration), and (iii) ethanol with pH around 9.0 by the addition of a small amount of NaOH (without buffer or NaCl). The analyzed systems were: PD and XD in water and in ethanol, CD and BD in ethanol and aqueous P-123.

2.2. Chemicals

Phenothiazinium derivatives (PD): Methylene blue (MB, B. Herzog), and *o*-toluidine blue (TBO, B. Herzog) exhibited high purity, as demonstrated by ^1H NMR analysis.

Chlorophyll derivatives (CD): Mg-Chl was extracted from spinach according to the description of Svec [38]. Other derivatives were synthesized from Mg-Chl following the procedures described in the literature [39–42]. The CD were purified using thin layer circular chromatography – Chromatotron (Harrison Research, model 8924) with silica as a stationary phase and characterized by UV–Vis and ^1H NMR (Varian, Gemini 300MHz). The stock solutions of CD were prepared in DMSO at ~10^{-3} mol L^{-1}. The working solutions