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Roles of hydrophilicities and hydrophobicities of dye and sacrificial electron donor on the photochemical pathway

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Abstract

Relative rates of the photosensitized production of singlet oxygen ($^1\text{O}_2$) and of superoxide ($\text{O}_2^{\bullet-}$) were determined using different couples of dyes and sacrificial electron donors (SEDs) of either high or low hydrophobicities. Such rates were also measured in the absence and presence of single unilamellar vesicles (SUVs) with 9DMPC:1DMPA mol ratio composition. The dyes aluminum phthalocyanine tetrasulfonate (AlPcS₄) and pheophorbide-a (PHEO) were used as hydrophilic and hydrophobic photosensitizers, respectively. Xanthine (X) and glutathione (GSH) were used as hydrophobic and hydrophilic SEDs, respectively. The presence of SUVs in the aqueous sample produces the physical separation or encounter of SEDs and photosensitizers according to their membrane binding constants. When both the SED and the photosensitizer are localized within the same phase, a strong decrease in the rate of $^1\text{O}_2$ formation, united to a strong increase in the rate of $\text{O}_2^{\bullet-}$ formation, is observed, relative to when both of these species are localized in different phases. The lipid phase is always present in the biological milieu. Thus, the use of a hydrophobic couple of both dye and SED (as in the case of X and PHEO), as well as a hydrophilic couple of both dye and SED (as in the case of GSH and AlPcS₄), should strongly favor the Type I mechanism over the Type II. Since only a small number of hydroxyl radicals are needed to initiate a chain reaction of phospholipid peroxidation, the latter could be more toxic to the tumor tissue than peroxidation by a much higher concentration of singlet oxygen molecules.

Keywords

superoxide; sacrificial electron donor; singlet oxygen; aluminum phthalocyanine tetrasulfonate; pheophorbide-a; Type I; Type II; lipids

1. Introduction

Photodynamic therapy (PDT) is a cancer treatment that uses a combination of red laser light, a photosensitizing agent, and molecular oxygen to produce the therapeutic effect [1, 2]. Porphyrins, phthalocyanines, chlorins and other dyes are currently being used in photodynamic treatment of tumors due to their large absorption coefficients in the 500–800 nm range. In the presence of dioxygen, these photosensitize the production of singlet oxygen and superoxide [1, 2]. Singlet oxygen production, the so-called Type-II pathway, is claimed to be the most important process that kills tumor cells [1, 2]. However, Type-I pathways,

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those involving photoreduction or photooxidation of substrates, have also been proposed as photocytotoxic events in PDT, especially in hypoxic environments [3]. However, the actual pathway should be a function of the hydrophobicity/hydrophilicity of both sensitizer and available sacrificial electron donors (SEDs) and thus their corresponding partition constants in lipid and aqueous environments. One consequence of a shift in pathway from Type II to Type I should be an increase in the production of superoxide-derived reactive oxygen species (ROS) with the consequent change in toxicity efficiency. For example, both superoxide and hydrogen peroxide can travel within tissues farther away than singlet oxygen to produce damage [4]. It has recently been proposed that hydrogen peroxide could act selectively against tumor cells as opposed to normal cells [5, 6]. The conjugated acid of superoxide, the hydroperoxyl radical, is more soluble in lipid membranes than the superoxide ion and also more reactive [7, 8]. In addition, only a small number of hydroxyl radicals are needed to initiate a chain reaction of phospholipid peroxidation. The latter is more toxic to the tumor tissue than peroxidation by a much higher concentration of singlet oxygen molecules [9]. Thus, the formation of these radicals in vivo may markedly increase the efficacy of the sensitizer.

In this work, we have combined a hydrophilic (aluminum phthalocyanine tetrasulfonate, AlPcS₄) or a hydrophobic dye (pheophorbide-a, PHEO) with a hydrophobic (xanthine, X) or a hydrophilic (glutathione, GSH) SED, in the presence and absence of small unilamellar vesicles (SUVs), in order to study the behavior of these combinations on the relative photoproduction of singlet oxygen and superoxide. Although GSH is, and X is not, abundant in cells, X can be increased in mammal tissues by injection, as described elsewhere [10]. Also, purine catabolites, including X, accumulate in solid tumors that are exposed to hyperthermia [11].

2. Materials and methods

2.1 Materials

The dyes, AlPcS₄ and PHEO were obtained from Frontier Scientific and used as received (Fig. 1). GSH, 4-hydroxy-2,2,6,6-tetramethylpiperidine (4-OH-TEMP), sodium formate, DETAPAC, catalase, superoxide dismutase (SOD) and X were purchased from Sigma-Aldrich Chemicals and used as received. The compound 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) was purchased from Aldrich Chemicals. The hydroxylamine of TEMPOL, TEMPOL-H, was synthesized as described elsewhere by reduction of the corresponding nitroxyl radical precursor, TEMPOL, with hydrogen in the presence of platinum catalyst until less than 0.5% TEMPOL was detected as impurity utilizing EPR spectroscopy [12]. Dimyristoylphosphatidic acid (DMPA) and dimyristoylphosphatidylcholine (DMPC) were purchased from Avanti Polar Lipids. Fresh stock solutions of dyes were prepared in DMSO and used the same day. Fresh stock solutions of enzymes were prepared in distilled deionized water and also used the same day. The volume % of DMSO in all samples was kept below 1 %.

2.2 Small unilamellar vesicles (SUVs) preparation

The procedure described by New using probe sonication was followed [13]. In order to avoid lipid oxidation, DMPC and DMPA were used instead of egg yolk PC. A thin film of 9 DMPC: 1 DMPA mol ratio (100.0 mg), was prepared in an septum-stoppered Erlenmeyer flask, by evaporating the solvent from its chloroform solution, using first a nitrogen gas flow followed by high-vacuum application for 30 min. DMPA was used to increase liposome stability [13] as well as a model for biological membranes (negatively charged). Chelex-treated water (10.00 mL) was then added and the suspension strongly vortexed. The sample was deaerated for 20 minutes using high purity N₂. This was followed by sonication under a

N₂ atmosphere using a Branson 450 Probe sonifier and cooling with an ice bath. Samples were sonicated for 1.5 hours by switching off the sonicator for 30 seconds every 15 seconds of sonolysis. After sonolysis samples were left standing for 2 hours while heating at 30 (above the DMPC transition temperature). Samples were then submitted to ultracentrifugation (100,000 g for 20 minutes) to sediment undispersed lipid and multilamellar vesicles and to remove titanium particles. The clear supernatant of this suspension was carefully removed and stored under a N₂ atmosphere at room temperature.

Phospholipid content of the supernatant was determined using the Stewart assay as described by New [14].

2.3 Photoirradiation and EPR measurements

Air-saturated 20 mM phosphate buffer solutions (pH 7.4) were photolysed in Pyrex cells (1.00 cm × 1.00 cm) at the corresponding wavelength maximum of each dye. The latter were 675 nm for AlPcS₄ and 688 nm for PHEO in the absence of SUVs or 673 nm for PHEO in the presence of 3.0 mM SUVs. Solutions were irradiated with dye absorbances of 1.0 at the wavelengths mentioned above. Aliquots of the photolysed solution were transferred, after increasing irradiation periods to a quartz cell (60 × 10 × 0.25 mm) and placed in the EPR instrument cavity. A 1000W xenon arc lamp coupled to a Spectral Energy GM 252 high-intensity grating monochromator with a bandwidth of 20 nm was used as the irradiation source. EPR spectra were recorded on a Bruker ER-200D spectrometer at 100 kHz magnetic field modulation. EPR line intensities were determined from the peak-to-peak derivative amplitudes times the square of the peak-to-peak widths.

2.4 Singlet oxygen determination

This was performed using an EPR method based on the reaction of ¹O₂ with a sterically hindered amine, 4-OH-TEMP in this case, to yield a nitroxyl radical, TEMPOL [15, 16]. This technique is very selective for ¹O₂ and neither superoxide nor oxygen reacts with 4-OH-TEMP [17]. Rates of TEMPOL formation, R_{TEMPOL,1O2}, were measured from the initial rates of increase in the line intensities following the procedure described by Ben-Hur et al., [18] where the rates of increase in the intensity of the two lowest field first derivative EPR lines of TEMPOL are measured during the photolyses. Air-saturated samples (2.00 mL) containing 100 μM DETAPAC, 600 μM sodium formate, 200 U/mL catalase, 200 U/mL SOD, dye (A = 1 at the dye maximum wavelength above 650 nm), in the presence or absence of SED (0 or 50 μM), in the presence or absence of 3 mM SUVs and 20 mM phosphate buffer (pH 7.4) were stirred protected from stray light in a quartz cell of 1.00 cm light path for 30 minutes previous to irradiation. Since the extinction coefficient of PHEO increases upon addition of SUVs (see below), the concentration of PHEO was adjusted to always produce an absorbance of 1 in the absence or presence of lipids. This was not needed for AlPcS₄. The reagents DETAPAC, catalase, SOD and sodium formate were added to avoid Fenton-type production of hydroxyl radicals and the subsequent degradation of 4-OH-TEMP and TEMPOL. This was followed by addition of 160 mM of 4-OH-TEMP, exposure to air with continuous magnetic stirring and photoirradiation at the dye maximum wavelength (± 20 nm) for 1 minute intervals with a radiant power of 8 mW. Since some of the produced TEMPOL may be photoreduced by the dye, especially in the case where SEDs are present, a small aliquot containing an excess of ferricyanide was added, after each 1 minute irradiation, to 350 μLs of the irradiated solution to produce a 1mM concentration of ferricyanide. This reagent is known to oxidize hydroxylamines or nitroxides efficiently [19]. Samples were then submitted to electron paramagnetic EPR analysis after each irradiation interval by transferring the irradiated ferricyanide-treated sample to an EPR flat quartz cell. A sample treated in the same manner was also submitted to EPR analysis just before

irradiation and this residual line intensity was subtracted from those observed after irradiation.

2.5 Superoxide determination

The procedure and sample composition described for the singlet oxygen determination was used with the exception that irradiation was performed in the presence and absence of 200 U/mL SOD and that 20 mM TEMPOL-H was used as a probe for superoxide instead of 4-OH-TEMP. Furthermore, no addition of ferricyanide was done. It has been demonstrated that TEMPOL is produced from its hydroxylamine, TEMPOL-H, via oxidation by $O_2^{\bullet-}$ [20]. Thus, initial SOD-inhibitable rates of TEMPOL ($R_{TEMPOL,O_2^{\bullet-}}$) we determined from the SOD-inhibitable rates of production of the one-electron oxidation product of TEMPOL-H instead of using the most used test for superoxide ion, i.e. the SOD-inhibitable ferricytochrome c reduction [21], since overlap exists between the Soret band of this molecule and those of the dyes under study here. Initial rates of TEMPOL formation were measured from the initial rates of increase in the line intensity of the lower field first derivative TEMPOL EPR peak in air-saturated solutions containing dye and 20mM TEMPOL-H. SOD-inhibitable rates of TEMPOL were obtained by subtracting initial average rates of line intensity increase in the presence of SOD to those obtained in the absence of this enzyme.

2.6 X photooxidation

Samples were prepared and photolysed as indicated above followed by HPLC analysis of X and X and uric acid, UA. HPLC analyses were performed using two YMC Pack Pro C₁₈ HPLC (4.6 × 250 mm) columns and gradient elution going from 100% ammonium acetate at pH 7.4 to 1:1 ammonium acetate (pH 7.4):methanol in 15 minutes followed by 100% methanol. The flow rate of elution was 1 mL/min. The columns were washed daily with methanol for 1 hour and then with water for 40 minutes and stored in methanol:water (1:4, v/v). The column was re-equilibrated to initial conditions for 60 min before injection. A Waters 1500 series HPLC system, equipped with a Waters 2487 multiwavelength absorbance detector was used at a wavelength of 267 nm. UA and X peaks were detected by comparing their retention times with those of standard samples of X and UA.

2.7 Membrane binding constants

A spectroscopic titration technique was used as described previously [22, 23]. The intensity changes of the absorption maxima of the dyes or SEDs upon partitioning into the SUV lipid environment were used. The absorbance value in the absence of lipid, A_{aq} , and those in the presence of increasing concentrations of liposomes in the suspension, A_{obs} , were measured. A_{obs} was corrected for light dispersion by the SUVs before used. The variable parameter $\epsilon = A_{obs}/A_{aq}$ was shown to depend linearly on the inverse of the lipid concentration as described in Eq. (1). The binding constant, K_b , as well as the

$$\epsilon - 1 = \epsilon_b - 1 - \frac{\epsilon_b - 1}{K_b [\text{lipid}]} \quad (1)$$

value of ϵ at complete binding, ϵ_b , can be evaluated in this way.

Overlap of absorption peaks of GSH and xanthine with those of the lipid precluded the use of the method described above to obtain K_b values for those compounds. In addition, the ϵ value of AlPcS4 did not varied appreciably with lipid addition and TEMPOL-H and 4-OH-TEMP can not be detected by absorption spectroscopy. In those cases, K_b was determined using ultracentrifugation as described by Sikkema et al.[24] Solutions containing a

concentration of the compound to be partitioned near to that used in the sample, i. e. 50 μ M GSH, 7 μ M AIPcS4, 100 μ M TEMPOL-H and 5 mM 4-OH-TEMP, in the presence of 3 mM SUVs and 20 mM phosphate buffer (pH 7.4) were stirred in a light-protected sample bottle for 30 minutes previous to centrifugation. After equilibration, the liposomes were spun down in an Optima LE-80K ultracentrifuge (Beckman-Coulter) for 30 min at $150,000 \times g$ and the supernatant submitted to analysis. By this method all liposomes were pelleted, as was assessed using the Stewart assay. Concentrations of AIPcS4, GSH and xanthine in the supernatant were determined at their corresponding wavelength maxima. TEMPOL-H concentration in the supernatant was determined after oxidizing the extracted aliquot with a ten-fold concentration of sodium ferricyanide and determining the TEMPO radical produced using EPR spectroscopy, as described above. The concentration of 4-OH-TEMP in the supernatant was analyzed by GC chromatography (6890 Agilent) using FID detection. Liposome binding constants were determined using equation (2), where A_{woL} and A_{wL} are the

$$K_b = \frac{(A_{woL} - A_{wL})}{(A_{wL})[SUV]} \quad (2)$$

absorbances, EPR line intensities or GC integrated peak areas of the partitioning compound in the absence and in the presence of SUVs, respectively.

3. Results and discussion

3.1 Membrane binding constants

Increasing the SUV concentration in a sample containing a constant PHEO concentration produces an increase in the PHEO absorbance at 670 nm due to PHEO partition into the lipid phase and shifting of its aggregation equilibrium to its monomer [25], Fig. 2(a). A plot of $\epsilon - 1$ vs. $(\epsilon - 1)/[\text{lipid}]$ produces 2 different linear regions, thus implying that 2 different lipid-PHEO binding equilibria exist, i. e. one at low (corresponding to the continuous line) and another at high lipid concentration (corresponding to the dotted line), Fig. 2(b). The plot at high lipid concentration was used to determine the K_b value of PHEO since we used a concentration of lipid closer to this region of lipid concentration, i. e. 3 mM. The corresponding K_b value obtained from the inverse of the high lipid concentration slope is shown in Table 1. Binding constants of X, AIPcS4, GSH, TEMPOL-H and 4-OH-TEMP were determined by ultracentrifugation. Multiplying these K_b values by the lipid concentration (3.0 mM), the concentration ratios of the lipid-bound dyes or SEDs to the unbound substrates are obtained. Thus, values of the % of substrate bound to the lipids can be obtained from those ratios, Table 1. As expected, the hydrophobic molecules PHEO and X are almost completely attached to the SUVs while GSH and AIPcS4 are localized extensively at the aqueous phase. The piperidine derivatives TEMPOL-H and 4-OH-TEMP are essentially equally available at both phases.

3.2 Singlet oxygen production

Photoirradiation of a sample containing PHEO and 160 mM 4-OH-TEMP, at 675 nm, the maximum absorption wavelength of PHEO, in the absence of SUVs, produces an increase in the TEMPOL EPR signal with irradiation time which rapidly levels off after 1 min of irradiation, Fig. 3. Photoirradiation of an identical sample, but in the absence of SUVs and in the presence of 50 μ M of the hydrophobic SED, X, produces a smaller rate of increase in the TEMPOL EPR signal with irradiation time than that observed for an identical sample but in the absence of X, Fig. 3. The latter observation should be due to a partial inhibition of the photosensitized formation of 1O_2 by X either by serving as SED and/or by physical

quenching of $^1\text{O}_2$. It is well-known that irradiation of PHEO with red light in air-saturated aqueous solutions generates singlet oxygen [26]. In fact, irradiation at 675 nm of a solution containing PHEO and 160 mM 4-OH-TEMP produces decreasing amounts of TEMPOL with an increase in concentration of the efficient $^1\text{O}_2$ deactivator, NaN_3 . A plot of the inverse in the rate of TEMPOL formation versus NaN_3 concentration is a straight line, Fig. 4. The ratio of the slope to the intercept of that line is equal to $k_{\text{N}_3-}/\{k_{\text{TEMPOL}} [4\text{-OH-TEMP}]\}$, [16, 27] where k_{N_3-} is the rate constant for the quenching of singlet oxygen by azide and k_{TEMPOL} is the rate constant for the reaction of 4-OH-TEMP with singlet oxygen ($4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ in water)[16] From the value of this ratio, $4.4 \times 10^2 \text{ M}^{-1}$, $k_{\text{N}_3-} = 2.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ is calculated. The latter rate constant is within the range of values reported in the literature (10^8 to $10^9 \text{ M}^{-1} \text{ s}^{-1}$)[28] thus indicating that the species producing TEMPOL is actually singlet oxygen. If the same mixture is photoirradiated in the presence of 3.0 mM SUVs, the rate in which the TEMPOL EPR signal is produced is strongly diminished relative to that in the absence of SUV, Fig. 3.

Furthermore, X is also photooxidized by PHEO to uric acid, as detected by liquid chromatography of the samples (uric acid is the photosensitized oxidation product of xanthine[29]). The amount of uric acid detected in the SUV-containing sample is more than 3 times larger than in the sample without SUVs, Table 2, thus indicating that the decrease in singlet oxygen production is largely caused by X photosensitized oxidation. Furthermore, singlet oxygen detection inhibition correlates with superoxide production enhancement (see below). Thus, the hypothesis of X acting as an SED instead of as physical deactivator of $^1\text{O}_2$ is better supported. In summary, when both X and PHEO are localized within the lipid membrane, the concentration $^1\text{O}_2$ detected decreases drastically as compared to the aqueous phase due to, most probably, a more efficient photoelectron transfer from X to the excited state of PHEO.

When AlPcS4 is used as the dye, a plot of the inverse in the rate of TEMPOL formation versus NaN_3 concentration is also a straight line (data not shown) and the ratio of the slope of that line to its intercept $5.0 \times 10^2 \text{ M}^{-1}$. Thus, a value of $k_{\text{N}_3-} = 3.2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ is calculated. The latter rate constant is, again, within the range of values reported in the literature (10^8 to $10^9 \text{ M}^{-1} \text{ s}^{-1}$)[28]. Thus, the species producing the nitroxide, TEMPOL, is in fact singlet oxygen.

The decrease in the rate of production of singlet oxygen with addition of SEDs, in the absence or presence of SUVs and in the presence of the hydrophilic or the hydrophobic dye is summarized in Fig. 5(a). Even though the photochemical production of singlet oxygen varies with the identity of dye used, certain interesting patterns are observed in this figure. First of all, this graph shows that the presence of an SED (X or GSH) decreases the rate of singlet oxygen formation for all combinations of dye, lipid and SED. However, irradiation of mixtures of X plus PHEO has an opposite behavior of that detected when X plus AlPcS4 mixtures are irradiated, i. e. the presence of lipid decreases further the rate of singlet oxygen production in the case of PHEO while it decreases less in the case of AlPcS4 relative to the absence of the lipid. Thus, X is more available to interact with the excited state of PHEO at the membrane site while X is less accessible to AlPcS4 when SUVs are present. In contrast, addition of SUVs in mixtures of GSH and PHEO increase the yield of singlet oxygen as compared to the absence of SUVs while the presence of SUVs in samples containing AlPcS4 and GSH does not affect the yield of singlet oxygen produced as compared to the absence of lipids. This can be explained by the less accessibility of PHEO to GSH when PHEO is immersed in the lipid membrane while GSH is at the aqueous phase. The fact that both AlPcS4 and GSH are both hydrophilic explains why the presence of SUVs does not alter the rate of TEMPOL production since both the SED and the dye will only encounter each other in the aqueous phase.

3.3 Superoxide production

If a sample containing PHEO and 20 mM TEMPOL-H is photoirradiated at 675 nm, the maximum absorption wavelength of PHEO, an increase in the SOD-inhibitable rate of TEMPOL formation, $R_{\text{TEMPOL},\text{O}_2^{\bullet-}}$, with irradiation time is observed. A much larger rate of increase in $R_{\text{TEMPOL},\text{O}_2^{\bullet-}}$ is observed if 50 μM of X is included in the mixture, Fig. 6. However, if the same mixture is photoirradiated in the presence of 3.0 mM SUVs, $R_{\text{TEMPOL},\text{O}_2^{\bullet-}}$ is enhanced, and much more in the presence of X, Fig. 6, relative to the absence of SUVs. That this difference is not due to an increase in PHEO absorptivity when immersed in the lipid membrane is evidenced by two facts: (1) the absorbance of the dye in the sample is measured in the presence of lipid, when the sample contains lipid, and that is set to $A=1$, (2) the observed behavior detected here for superoxide production is the opposite to that observed for the production of singlet oxygen, Fig. 3. Thus, X is promoting the photosensitized formation of superoxide, a Type I pathway, and more extensively when both dye and X are immersed in the membrane. Therefore, again, when both X and PHEO coincide in the same lipid phase, the Type I product formation is strongly enhanced relative to when both of these species are in the aqueous phase. This observation is consistent with the decrease in the yield of $^1\text{O}_2$ when both X and PHEO are in the lipid phase, relative to the absence of the lipid phase. Increases in the rates of $\text{O}_2^{\bullet-}$ production (relative to the absence of SED) for all combinations of dye and SED, and in the presence or absence of SUVs, are shown in Fig. 5(b). Again, samples containing the hydrophilic dye AIPcS4 and X show a strong decrease of superoxide production if SUVs are added to the system as opposed to the PHE + X system. However, if GSH is used as SED, a decrease in the rate of superoxide production by the excited state of PHEO is observed if SUV is added as opposed to samples containing PHEO + X due to, as explained above, the inaccessibility of GSH to PHEO molecules immersed in the membrane. In a similar fashion to the trend detected for the decrease in $R_{\text{TEMPOL},^1\text{O}_2}$ in samples containing AIPcS4 + GSH, addition of lipid has no measurable effect on $R_{\text{TEMPOL},\text{O}_2^{\bullet-}}$ since the electron transfer reaction occurs in the aqueous phase only. In fact, decreases observed in $R_{\text{TEMPOL},^1\text{O}_2}$ roughly correlate with increases in $R_{\text{TEMPOL},\text{O}_2^{\bullet-}}$, Fig. 7 (linear correlation factor of 0.84), thus indicating that those decreases in singlet oxygen production are mostly due to the pathway shift to superoxide production.

In summary, addition of a lipid phase to the aqueous sample produces the physical separation or encounter of SEDs and dyes according to their membrane binding constants. When both the SED and the photosensitizer are localized within the same phase a strong decrease in the rate of $^1\text{O}_2$ formation, united to a strong increase in the rate of superoxide formation, is observed relative to when both of these species are localized in different phases. Table 3 summarizes the different scenarios encountered in this work and the observed results regarding singlet oxygen and superoxide production. Since the lipid phase is always present in the biological milieu, the use of a hydrophobic couple of both dye and SED (as in the case of X and PHEO), as well as a hydrophilic couple of both dye and SED (as in the case of GSH and AIPcS4), will strongly favor the Type I mechanism over the Type II. Since millimolar amounts of GSH are available inside cells, this work predicts that using a hydrophilic dye inside the cells, such as AIPcS4, should promote superoxide, and therefore H_2O_2 , production, as opposed to a hydrophobic dye with no hydrophobic SED present.

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Abbreviations

PDT	photodynamic therapy
ROS	reactive oxygen species
SUVs	small unilamellar vesicles
AlPcS₄	aluminum phthalocyanine tetrasulfonate
PHEO	pheophorbide-a
X	xanthine
GSH	glutathione
4-OH-TEMP	4-hydroxy-2,2,6,6-tetramethylpiperidine
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl
TEMPOL-H	the hydroxylamine of TEMPOL
DMPA	dimyristoylphosphatidic acid
DMPC	dimyristoylphosphatidylcholine

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HIGHLIGHTS

Role of sacrificial electron donor (SED) in the photochemistry of dyes was studied.

- Study was carried out in the absence or presence of small unilamellar vesicles.
- If SED and dye are at the same phase, a strong decrease in $^1\text{O}_2$ formation is observed.
- If SED and dye are at the same phase, a strong increase in $\text{O}_2^{\bullet-}$ formation occurs.
- If SED and dye are at different phases, superoxide production is strongly inhibited.

Fig. 1.
Dyes used in this work.

Fig. 2.

(a) Spectroscopic titration used in determining K_b for PHEO with 9DMPC:1DMPA SUVs. Sample contained 2 μM PHEO and from 0 to 1.00 mM SUVs in 20 mM phosphate buffer, pH 7.4 (b) Determination of K_b using data from (a).

Fig. 3.

Dependence of the TEMPOL yield upon irradiation of samples on the presence of SUVs and X. Samples contained 160 mM 4-OH-TEMP, 100 μ M DETAPAC, 600 μ M sodium formate, 200 U/mL catalase, 200 U/mL SOD, PHEO ($A = 1$), 0 or 50 μ M X, 0 or 3 mM 9DMPC: 1DMPA SUVs and 20 mM phosphate buffer (pH 7.4).

Fig. 4. Determination of the rate constant for the quenching of singlet oxygen by N_3^- . Sample composition is the same as that described in Fig. 3 but with NaN_3 addition.

Fig. 5.

Decrease in $R_{\text{TEMPOL},\text{O}_2}$ (a) and increase in $R_{\text{TEMPOL},\text{O}_2\bullet-}$ (b) upon addition of SEDs to samples containing PHEO or AlPcS4 in the presence or absence of 3 mM 9DMPC:1DMPA SUVs and 20 mM phosphate buffer (pH 7.4). Samples corresponding to (a) contained 160 mM 4-OH-TEMP, 100 μM DETAPAC, 600 μM sodium formate, 200 U/mL catalase, 200 U/mL SOD, dye ($A = 1$), 0 or 50 μM X or GSH, 0 or 3 mM 9DMPC:1DMPA SUVs and 20 mM phosphate buffer (pH 7.4). Samples corresponding to (b) contained 20 mM TEMPOL-H, 100 μM DETAPAC, 600 μM sodium formate, 200 U/mL catalase, 200 U/mL SOD, dye ($A = 1$), 0 or 50 μM X or GSH, 0 or 3 mM 9DMPC:1DMPA SUVs and 20 mM phosphate buffer (pH 7.4). Capital letters in the horizontal axis of this graph represent the composition of the corresponding irradiated sample: A, AlPcS4; P, PHEO; X, 50 μM xanthine; G, 50 μM GSH; L, 3 mM 9DMPC:1DMPA SUVs.

Fig. 6.

Dependence of the SOD-inhibitable TEMPOL yield upon irradiation of samples on the presence of SUVs and X. Samples contained 20 mM TEMPOL-H, 100 μ M DETAPAC, 600 μ M sodium formate, 200 U/mL catalase, 0 or 200 U/mL SOD, PHEO ($A = 1$), 0 or 50 μ M X, 0 or 3 mM 9DMPC:1DMPA SUVs and 20 mM phosphate buffer (pH 7.4).

Fig. 7.
Correlation between the decreases in $R_{\text{TEMPOL},1\text{O}_2}$ and increases in $R_{\text{TEMPOL},\text{O}_2^{\bullet-}}$ depicted in Fig. 5.

Table 1Lipid binding constants, K_b , of compounds used in this work

Compound	K_b/M^{-1}	% of substrate at the lipid phase
PHEO ^a	$(32 \pm 6) \times 10^3$	91
AlPcS ₄ ^b	34 ± 1	9
X ^b	$(1.8 \pm 0.1) \times 10^3$	84
GSH ^b	26 ± 2	7
TEMPOL-H ^b	$(3.6 \pm 2) \times 10^2$	52
4-OH-TEMP ^b	$(4.0 \pm 0.2) \times 10^2$	54

^a K_b measured using spectroscopic titration.^b K_b measured using ultracentrifugation. Samples contained 3 mM 9DMPC:1DMPA as the lipid phase.

Table 2

Xanthine and uric acid concentrations after 5 minutes of irradiation at 699 nm in the presence and absence of lipids^a

[Lipid]/mM	[xanthine]/ μ M	[uric acid]/ μ M
0.0	48 $\pm$ 3	6 $\pm$ 2
3.0	31 $\pm$ 4	22 $\pm$ 3

^aSamples were prepared containing 50 μ M X, with or without 3 mM lipid (9DMPC:1DMPA), 100 μ M DETAPAC, 600 μ M sodium formate, 200 U/mL catalase, 200 U/mL SOD, PHEO (A=1 at 673 nm) and 20 mM phosphate buffer (pH 7.4).

Table 3Summary of behaviors encountered in this work for different scenarios.^a

Scenario	Superoxide production	Singlet oxygen production
hydrophobic dye + hydrophobic SED + lipid	strongly increased	strongly decreased
hydrophilic dye + hydrophilic SED + no lipid	strongly increased	strongly decreased
hydrophilic dye + hydrophilic SED + lipid	strongly increased	strongly decreased
hydrophobic dye + hydrophilic SED + no lipid	increased	decreased
hydrophobic dye + hydrophobic SED + no lipid	increased	decreased
hydrophilic dye + hydrophobic SED + lipid	increased	decreased
hydrophilic dye + hydrophobic SED + lipid	slightly increased	slightly decreased
hydrophobic dye + hydrophilic SED + lipid	slightly increased	slightly decreased

^a As compared to the absence of SED