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# Determination of the singlet oxygen quantum yield of bacteriochlorin *a*: a comparative study in phosphate buffer and aqueous dispersion of dimiristoyl-L-α-phosphatidylcholine liposomes

**Maryse Hoebeke* and Xavier Damoiseau**

*Centre of Oxygen R&D (CORD), Department of Physics, Institute of Physics, B5, University of Liège, 4000 Liège, Belgium. E-mail: M.Hoebeke@ulg.ac.be; Fax: +32 4 366 28 13; Tel: +32 4 366 36 51*

***Received 29th January 2002, Accepted 26th February 2002***
***First published as an Advance Article on the web 26th March 2002***

The production of singlet oxygen ($^1O_2$) by bacteriochlorin *a* (BCA) was studied in phosphate buffer and in dimyristoyl-L-α-phosphatidylcholine (DMPC) unilamellar liposomes. The comparative method used to measure $^1O_2$ production was a quantitative analysis of photooxidation reactions leading to the loss of absorbance of the water-soluble specific probe: anthracene-9,10-dipropionic acid. Rose Bengal, whose $^1O_2$ quantum yield ($\Phi^{RB}$) is well known in alcohols and phosphate buffer, was used as the standard for the quantification of the BCA singlet oxygen production. Our results confirm quantitatively that solubilization of BCA in liposomes leads to an increase in $^1O_2$ production. Indeed, the quantum yield of $^1O_2$ production by BCA ($\Phi^{BCA}$) is 0.05 in phosphate buffer and 0.33 in DMPC liposomes.

Furthermore, the diffusion characteristics of $^1O_2$ produced by BCA bound to liposome were also examined using the isotopic lifetime enhancement effect of $D_2O$. It was shown that $^1O_2$ spent at least 70% of its lifetime in the vesicular environment.

## Introduction

The aim of cancer therapy is to target selectively and destroy diseased tissue while sparing surrounding normal cells. Photodynamic therapy (PDT) involves administration of a sensitizer that preferentially accumulates in malignant tissues. Upon irradiation of the non-toxic sensitizer with light of appropriate wavelength, the tumour is mainly destroyed by the reactive oxygen species (ROS) or radicals yielded.[1,2] Singlet oxygen ($^1O_2$) is commonly considered as one of the most important cytotoxic agents involved in PDT.[3,4]

BCA is an attractive second-generation photosensitizer in view of its potential use in PDT.[5–9] Light-activated BCA can transfer energy from its triplet state: (a) directly to molecular oxygen to yield singlet oxygen (type II reaction) and (b) by interaction with the solvent or substrate *via* an electron or a proton transfer leading to the generation of radicals (type I reaction).[10] BCA is a hydrophobic sensitizer that strongly aggregates in polar media. It has also been shown that solubilization of BCA in liposomes induces dye monomerization.[11] Oxygen consumption measurements carried out with a Clarke electrode in water and in liposome solutions indicate that the production of $^1O_2$ is strongly influenced by the dye aggregation state since it seems that only the monomeric species is appreciably photoactive.[12] Indeed, monomerization of BCA by solubilization within liposomes increases the PDT activity and is accompanied by an enhancement of singlet oxygen production.[12] Electron paramagnetic resonance (EPR) experiments confirmed the latter results and, in addition, showed that the type I mechanism is not affected by the presence of liposomes.[12] Nevertheless, no quantitative determination of $^1O_2$ production was reported.

The efficiency of a type II photosensitized process is limited by the singlet oxygen quantum yield $\Phi(^1O_2)$ of the sensitizer. The aim of this study was to determine $\Phi^{BCA}$ in phosphate buffer and liposome solutions. The comparative method used to measure $^1O_2$ production was a quantitative analysis of the photooxidation reactions leading to the loss of absorbance of a $^1O_2$ specific probe. Rose Bengal,[13,14] whose $\Phi^{RB}$ is well known in alcohols and phosphate buffer, was used as the standard for the quantification of BCA singlet oxygen production. The value of $\Phi^{BCA}$ was found to be 0.05 in phosphate buffer. Our results also showed that BCA solubilization in liposomes increases the singlet oxygen production 6.6-fold in comparison to the aqueous value. These data confirm that singlet oxygen production is higher in media where BCA is in its monomeric form. In this respect, the PDT efficacy of BCA may be enhanced by liposomal delivery.

Liposomes, which are commonly used as models for photophysical studies of cell membranes, also constitute a simple model for investigating the effects of compartmentalization on the lifetime and the mobility of $^1O_2$.[15–17] With each region of the liposome being characterized by a singlet oxygen lifetime, observations and comparison of the decay of a $^1O_2$ probe in $H_2O$ and $D_2O$ liposomal solutions were used to examine the $^1O_2$ pathway. From our analysis of the results, it appears that singlet oxygen, site-specifically generated by BCA in the lipid bilayer, spends at least 70% of its excited lifetime in a hydrophobic environment.

## Experimental

### Chemicals

Bacteriochlorophyll *a* was purchased from Sigma or extracted from the anaerobic photosynthetic bacterium *Rhodospirillum rubrum*. It was purified according to the method of Omata and Murata.[18] The purity of bacteriochlorophyll *a* was evaluated using thin-layer chromatography (TLC). Using an eluent of 93% methanol and 7% phosphate buffer (pH 7, 10 mM), the bacteriochlorophyll *a* yielded a single blue spot on Machery–Nagel Nano-Sil $C_{18}$-100 TLC plates (Düren, Germany). From

this starting material, the photosensitizer bacteriochlorin *a* was obtained by saponification and acid hydrolysis of bacteriochlorophyll *a* as described previously.[5] BCA was stored under nitrogen in the dark at −20 °C until required.

Rose Bengal (RB) was obtained from Eastman Kodak (USA) and dimiristoyl-L-α-phosphatidylcholine (DMPC) from Sigma (USA). RB was purified by gel chromatography according to the method of Gandin *et al*.[13] Absolute methanol, chloroform, $D_2O$ and sodium azide were obtained from Merck. Anthracene-9,10-dipropionic acid (ADPA) was synthesized by a method previously described.[13]

### Preparation of unilamellar liposomes

To avoid reactions between the lipid radical chains and ROS, completely saturated DMPC phospholipids were used. Unilamellar liposomes were prepared using an extrusion procedure as described previously.[19,20] The final lipid concentration of the stock solution was $7.4 \times 10^{-3}$ M. The water-soluble ADPA singlet oxygen quencher was then added to solutions of DMPC unilamellar liposomes at the desired concentration. Finally, the preparation was incubated for 1 h at 25 °C with BCA in methanol. Complete incorporation of sensitizer molecules in the bilayers was ensured by using a lipid-to-dye ratio greater than 125.[11] Under our experimental conditions, the final methanol concentration was 2% or less.

### Measurement of the kinetics of substrate bleaching

In order to study the production and the diffusion of $^1O_2$ generated by BCA, the photo-oxidation of a specific $^1O_2$-scavenging agent was followed by absorption spectroscopy in the presence or the absence of $NaN_3$, a $^1O_2$ inhibitor. The absorption measurements were carried out on a Kontron double beam spectrophotometer (Uvikon 941 instruments) with 1 cm UV sealed cuvettes. Circulating thermostated water through a cuvette holder was used to control the temperature.

Singlet oxygen production was monitored by following the loss of absorbance at 358 nm of ADPA. This was used as a $^1O_2$ probe in phosphate buffer and in the aqueous phase of liposomal solutions. However, in phosphate buffer and in DMPC liposomes, the BCA absorption spectrum exhibits a double band peaking between 300 and 400 nm. To take into account the absorption spectrum modifications due to a BCA photobleaching process, spectra of the irradiated solutions were recorded in the following way. Two solutions containing the sensitizer alone and the sensitizer together with the probe ADPA respectively were irradiated simultaneously outside the spectrometer using a slide projector (Pradovit RA 150, Leitz, Germany). A cut-off filter (OG515, Schott, Germany) was used in order to eliminate light under 500 nm and consequently to avoid direct photodegradation of the $^1O_2$-scavenging agents. At regular intervals, the two cuvettes were placed in the cell holder and photo-oxidative loss of the specific $^1O_2$ probe was measured by monitoring the difference absorbance changes as a function of the irradiation time. Under these conditions, all the measurements were corrected for the BCA photodegradation process.

Deoxygenation of solutions was carried out using a system consisting of two vacuum pumps (Leibold pallet pump and diffusion pump THP 055) with 8 cycles of a freeze–thawing procedure using liquid nitrogen.

### Light intensity evaluation

The total light intensity absorbed by the sensitizer in the solution depends on several factors: (i) the shape and the intensity of the dye absorption spectrum, (ii) the light source intensity $I_{source}(\lambda)$ and (iii) the transmission factor $T_{filter}(\lambda)$ of the optical filter used. When the cuvette containing the solution is irradiated with light, the intensity transmitted by the dye at wavelength $\lambda$ is given by the following Beer–Lambert law:

$$I_{trans}(\lambda) = I_0(\lambda)\, 10^{-\varepsilon(\lambda)cl} = I_0(\lambda)\, 10^{-Abs(\lambda)} \qquad I_0(\lambda) = I_{source}(\lambda)\, T_{filter}(\lambda) \tag{1}$$

where $l$, $\varepsilon(\lambda)$ and $c$ are the optical path length, the dye extinction coefficient and the dye concentration, respectively. The intensity absorbed by the dye at wavelength $\lambda$ is given by:

$$I_{abs}(\lambda) = I_0(\lambda) - I_{trans}(\lambda) = I_0(\lambda)\,(1 - 10^{-\varepsilon(\lambda)cl}) = I_0(\lambda)\,(1 - 10^{-Abs(\lambda)}) \tag{2}$$

Consequently, the total intensity absorbed by the dye in the solution is estimated by:

$$I_{abs} = \int_0^\infty I_0(\lambda)\,(1-10^{-Abs(\lambda)})\,d\lambda = \int_0^\infty I_{source}(\lambda)\,T_{filter}(\lambda)\,(1-10^{-Abs(\lambda)})\,d\lambda \tag{3}$$

The filter used here is a cut-off filter (OG515), with $T_{filter}(\lambda) = 0$ for $\lambda < 500$ nm. Furthermore, there is not any appreciable dye absorption above 900 nm. Consequently, the total intensity absorbed by the dye is estimated by the following equation:

$$I_{abs} = \int_{500}^{900} I_{source}(\lambda) T_{filter}(\lambda)(1 - 10^{-Abs(\lambda)})\,d\lambda \tag{4}$$

## Results and discussion

During PDT treatment, $^1O_2$ is probably the main ROS involved in the mechanism of cell killing.[3,4] Therefore, BCA is an attractive sensitizer since it has been demonstrated that BCA photosensitizes the production of $^1O_2$ and that $^1O_2$ is involved in killing cells in the presence of photoexcited BCA.[10,12] Furthermore, oxygen consumption and electron paramagnetic resonance experiments seem to indicate that solubilization of BCA in the bilayers of DMPC liposomes leads to an increase of singlet oxygen production relative to the same value in phosphate buffer. This increase is concomitant with the monomerization of the dye. However, EPR spin-trapping experiments using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and specific quenchers showed that the production of other ROS like $OH^\bullet$ and $O_2^{-\bullet}$ remains low in comparison with that of $^1O_2$.[12] This is why only the $\phi(^1O_2)$ value, which is an important parameter directly correlated to the BCA photosensitizing efficiency, was examined.

In the present investigation, the BCA singlet oxygen quantum yield ($\Phi^{BCA}$) was calculated in phosphate buffer and DMPC liposome solutions by the indirect method explained in the following sections.

### Singlet oxygen production during BCA irradiation in phosphate buffer

Fig. 1 shows the loss of absorbance of ADPA monitored at 358 nm during the photosensitizing reaction mediated by BCA or RB irradiated at $\lambda > 500$ nm. In both cases, the ADPA kinetics is monoexponential. The slopes of the ADPA decay were determined by linear regression fitting of the experimental data. In the absence of BCA, in the presence of BCA but in the dark, under vacuum or in the presence of $NaN_3$ ($5 \times 10^{-2}$ M), no decrease in ADPA was detected, thus confirming the specificity of ADPA destruction of $^1O_2$ (data not shown). Moreover, previous laser flash photolysis studies have shown that $NaN_3$ ($5 \times 10^{-2}$ M) and ADPA ($10^{-5}$ M) do not quench the BCA triplet state.[10–12]

The absolute value of $\Phi^{BCA}$ can be determined by comparing the decay slopes of ADPA in the presence of BCA and RB separately, using the absolute value of $\Phi^{RB} = 0.75$ in phosphate buffer. Indeed, in the presence of ADPA, $^1O_2$ generated by a

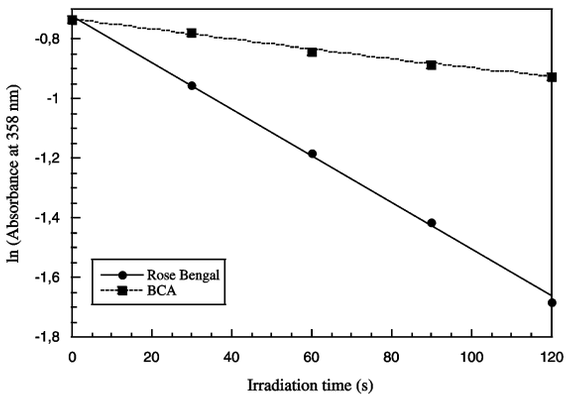


**Fig. 1** Photobleaching of ADPA ($7.5 \times 10^{-6}$ M) monitored at 358 nm in the presence of (●) RB ($10^{-5}$ M) and (■) BCA ($2 \times 10^{-5}$ M) in phosphate buffer.

sensitizer S undergoes the following decay processes in phosphate buffer.

(i) Light absorption by the sensitizer:

$$^1S + h\nu \longrightarrow {}^1S^* \tag{5}$$

(ii) Intersystem crossing:

$$^1S^* \longrightarrow {}^3S^* \tag{6}$$

(iii) Energy transfer:

$$^3O_2 + {}^3S^* \longrightarrow {}^1O_2 + {}^1S \tag{7}$$

(iv) Singlet oxygen deactivation by the solvent (rate constant $k_d = 2.5 \times 10^5$ s$^{-1}$):

$$^1O_2 \longrightarrow {}^3O_2 \tag{8}$$

(v) Physical quenching of $^1O_2$ by ADPA (rate constant $k_P$):

$$^1O_2 + \text{ADPA} \longrightarrow \text{ADPA} + {}^3O_2 \tag{9}$$

(vi) ADPA oxidation by $^1O_2$ (rate constant $k_R$):

$$^1O_2 + \text{ADPA} \longrightarrow \text{ADPAO}_2 \tag{10}$$

Upon continuous stationary irradiation, [$^1O_2$] is constant and the differential equations describing the [ADPA] and [$^1O_2$] kinetics are:

$$\frac{d[^1O_2]}{dt} = \Phi^S I_{abs} - k_R[\text{ADPA}][^1O_2] - k_P[\text{ADPA}][^1O_2] - k_d[^1O_2]$$
$$= \Phi^S I_{abs} - (k_R + k_P)[\text{ADPA}][^1O_2] - k_d[^1O_2] = 0 \tag{11}$$

$$\Rightarrow [^1O_2] = \frac{\Phi^S I_{abs}}{(k_R + k_P)[\text{ADPA}] + k_d} \tag{12}$$

$$\frac{d[\text{ADPA}]}{dt} = -k_R[\text{ADPA}][^1O_2]$$
$$= -k_R[\text{ADPA}]\frac{\Phi^S I_{abs}}{(k_R + k_P)[\text{ADPA}] + k_d} \tag{13}$$

In our experiments, the sum ($k_R + k_P$) is equal to $8.2 \times 10^7$ M$^{-1}$ s$^{-1}$ and the ADPA concentration was about $7.5 \times 10^{-6}$ M.[13] Thus, in eqn. (13) the term ($k_R + k_P$)[ADPA] may be neglected compared to $k_d$. The ADPA concentration decay is thus simply expressed by:

$$\frac{d[\text{ADPA}]}{dt} = -k_R[\text{ADPA}]\frac{\Phi^S I_{abs}}{k_d} = -k_S[\text{ADPA}] \tag{14}$$

$$\Rightarrow \ln[\text{ADPA}] = \ln[\text{ADPA}]_0 - k_S t \tag{15}$$

The kinetics of ADPA bleaching photosensitized by RB or BCA (Fig. 1) was indeed first order. This observation is in agreement with eqn. (15), confirming that $^1O_2$ is predominantly deactivated by the solvent. If the absolute value of $\Phi^{RB}$ in phosphate buffer is known, then $\Phi^{BCA}$ can be estimated from

$$\Phi^{BCA} = \Phi^{RB}\frac{k_{BCA}}{I^{BCA}}\frac{I^{RB}}{k_{RB}} \tag{16}$$

where $k_{BCA}$ and $k_{RB}$ are the first order rate constants of the substrate destruction by BCA and RB respectively. $I^{RB}$ and $I^{BCA}$ are the total intensities absorbed by RB and BCA in the solution, respectively. Analysis of the experimental data in Fig. 1 allows direct determination of $k_{BCA}$. However, some precautions have to be taken in the determination of $k_{RB}$ because it has been shown that RB, in its triplet state, can also react directly with ADPA [13] leading to ADPA destruction by the following process:

$$^3RB^* + \text{ADPA} \rightarrow {}^1RB + {}^3\text{ADPA}$$
$$^3\text{ADPA} + {}^3O_2 \rightarrow \text{ADPAO}_2 \tag{17}$$

Only 77% of the ADPA bleaching results from an interaction with $^1O_2$.[13] So, the slope of the ADPA decay measured from Fig. 1 was multiplied by 0.77 to obtain the real value of $k_{RB}$. In phosphate buffer, $\Phi^{BCA}$ was calculated to be 0.05 with a relative error estimated to be lower than 10%.

### Singlet oxygen production during BCA irradiation in DMPC liposome solutions

Fig. 2 shows the loss of absorbance of ADPA monitored at 358

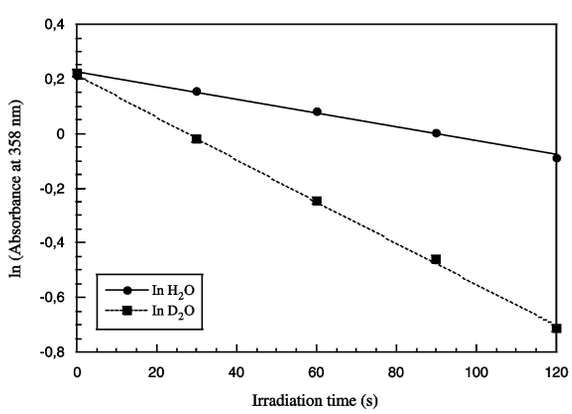


**Fig. 2** Photobleaching of ADPA ($2.25 \times 10^{-5}$ M ) monitored at 358 nm in the presence of BCA ($10^{-5}$ M) bound to DMPC liposomes (lipid concentration $2 \times 10^{-3}$ M) in $H_2O$ (●) and in $D_2O$ (■).

nm during the photosensitizing reaction mediated by BCA bound to liposomes and irradiated at $\lambda > 500$ nm. In all the experiments, the temperature was above the phase transition (23.9 °C) of the DMPC liposomes. The ADPA decay kinetics is monoexponential. The slopes of the ADPA decay were determined by linear regression fitting of the experimental curves. In the absence of BCA, in the dark or in $N_2$-saturated solutions, no decrease of ADPA was detected (data not shown).

Membrane-associated BCA exists only in the monomeric form.[10] Under our experimental conditions, the lipid-to-dye ratio is greater than 125 and the dye is located only within the membrane[11] while ADPA is in the aqueous phase outside the liposome. Singlet oxygen, which is site-specifically generated by BCA in the inner region of the membrane, can diffuse through the lipid phase and the aqueous phase. To take into account the heterogeneity of the environment,[15,16] the rate of $^1O_2$ deactivation by the solvent, $k_d$, is represented by

$$k_d = g^{H_2O} k_d^{H_2O} + g^v k_d^v \quad (18)$$

where $k_d^{H_2O}$ and $k_d^v$ are the partial constants for $^1O_2$ quenching in water and in the vesicle phase, respectively. $g^{H_2O}$ and $g^v$ are the corresponding weighting factors related by

$$g^{H_2O} + g^v = 1 \quad (19)$$

The rate of ADPA photobleaching due to irradiation of sensitizer S then becomes

$$\frac{d[ADPA]}{dt} = -k_R[ADPA]\frac{\Phi^S I_{abs}}{(k_R + k_P)[ADPA] + g^{H_2O} k_d^{H_2O} + g^v k_d^v} \quad (20)$$

With an ADPA concentration of $2.25 \times 10^{-5}$ M and using values $k_d^{H_2O} = 2.5 \times 10^5$ s$^{-1}$, $(k_R + k_P) = 8.2 \times 10^7$ M$^{-1}$s$^{-1}$, $k_d^v = 4 \times 10^4$ s$^{-1}$,[15,21] eqn. (20) can be simplified to

$$\frac{d[ADPA]}{dt} = -k_R[ADPA]\frac{\Phi^S I_{abs}}{g^{H_2O} k_d^{H_2O} + g^v k_d^v} \quad (21)$$

Isotopic lifetime enhancement by solvent deuteration was used to determine the values of the weighting factors $g^{H_2O}$ and $g^v$ and to evaluate the average time spent by $^1O_2$ in the aqueous and membrane phases. The rate of ADPA bleaching in the DMPC–$D_2O$ system was compared to the corresponding rate in the $H_2O$–DMPC system (Fig. 2). The ratio of the rates of ADPA destruction in $H_2O$ and $D_2O$ is given by

$$\frac{g^{H_2O} k_d^{D_2O} + g^v k_d^v}{g^{H_2O} k_d^{H_2O} + g^v k_d^v} \quad (22)$$

and was estimated to be equal to 2.8 (Fig. 2).

Eqns. (19) and (22) form a system of 2 equations with two unknowns: $g^{H_2O}$ and $g^v$. By using $k_d^{D_2O} = 1.8 \times 10^4$ s$^{-1}$,[15] values of $g^{H_2O} = 0.24$ and $g^v = 0.76$ can be calculated.

Because some ADPA molecules are located near the interfacial region of the liposomes,[16] $^1O_2$, generated in the membrane of DMPC liposomes by BCA, can react directly with these particular molecular probes without diffusing through the external aqueous phase. Consequently, the $D_2O$ isotopic effect is probably reduced and $g^v$ underestimated. Taking into account experimental error, our results indicate that $^1O_2$ spends at least 70% of its lifetime in the vesicular environment. This value is consistent with previously published results for other sensitizers located in the inner region of the membrane.[15,16,22]

Taking into account the heterogeneity of the environment, $\Phi^{BCA}$ in DMPC liposomes is estimated by the following equation

$$\Phi^{BCA} = \Phi^{RB}\frac{k_{BCA}}{k_{RB}}\frac{I^{RB}}{I^{BCA}}\frac{g^{H_2O} k_d^{H_2O} + g^v k_d^v}{k_d^{H_2O}} \quad (23)$$

Using kinetic parameters and following the same procedure as in the previous section, a $\Phi^{BCA}$ value of 0.33 was obtained.

The results of this study lead to the following conclusions. We present, for the first time, quantitative measurements showing that $\Phi^{BCA}$ depends strongly on the solvent. Comparison of the $\Phi^{BCA}$ value in phosphate buffer (0.05) and in liposomes (0.33) confirms that solubilization of BCA in liposomes leads to an important increase of its $^1O_2$ production. The same kind of behavior, observed previously with chlorins and purpurins, was attributed to the lower solubility of these compounds in water and the corresponding aggregation effects.[23] The correlation between singlet oxygen production and the aggregation state may also be made for BCA. Indeed, previous experimental results indicate that solubilization of BCA in liposomes is concomitant with the monomerization of the molecules.[11,12]

With regard to the essential role of $^1O_2$ in PDT, this enhancement of $^1O_2$ production is potentially useful information for improving the therapeutic applications of BCA. Solubilization of BCA in any delivery system inducing the dye monomerization process might be a way to explore improvements in its efficiency in PDT. More particularly, liposomal delivery of the compound is a special way of investigating in view of previous results and those of the present work. Indeed, it has been shown that the survival of WiDr cells (derived from a primary adenocarcinoma of a rectosigmoid colon carcinoma) after PDT treatment was lower with a liposomal formulation of BCA than with treatment with BCA in cell culture medium.[12] Among the causes that could explain this effect, there are, of course, a higher singlet oxygen production but also other parameters like the modification of the cell uptake or location inside the cells when liposomes are used as drug carriers.

## Abbreviations

Singlet oxygen: $^1O_2$; anthracene-9,10-dipropionic acid: ADPA; singlet oxygen quantum yield: $\Phi(^1O_2)$; bacteriochlorin *a*: BCA; BCA singlet oxygen quantum yield: $\Phi^{BCA}$; dimyristoyl-L-α-phosphatidylcholine: DMPC; electron paramagnetic resonance: EPR; Rose Bengal: RB; RB singlet oxygen quantum yield: $\Phi^{RB}$; sensitizer: S; S singlet oxygen quantum yield: $\Phi^S$ ($^1O_2$); sensitizer S excited in its first singlet state: $^1S$; sensitizer S excited in its triplet state: $^3S^*$.

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