Journal of Fluorescence, Vol. 16, No. 3, May 2006 (© 2006)
DOI: 10.1007/s10895-005-0057-5

The Influence of Human Serum Albumin on The Photogeneration of Singlet Oxygen by *meso*-Tetra(4-Sulfonatophenyl)Porphyrin. An Infrared Phosphorescence Study

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Received October 3, 2005; accepted December 26, 2005
Published online: February 9, 2006

meso-Tetra(4-sulfonatophenyl)porphyrin ($TPPS_4$) is a water soluble photosensitizer, which is currently clinically tested as a PDT drug. In our contribution, we present IR spectral- and time-resolved phosphorescence data reflecting the influence of human serum albumin (HSA) on singlet oxygen photogeneration by $TPPS_4$. IR emission of $TPPS_4$ was studied in samples containing various concentrations of HSA in phosphate buffer. The observed changes in spectral and temporal behaviour of $TPPS_4$ and singlet oxygen phosphorescence caused by the addition of HSA are equivalent to the effect of nitrogen purging of HSA-free solutions of $TPPS_4$. The main feature induced by addition of HSA appears to be the occurrence of a long-lived (tens of microseconds) photosensitizer phosphorescence at 900 nm besides ordinary short-lived ($\approx 2\ \mu$s) one at 820 nm. It is accompanied by presence of a long-lived component of singlet oxygen emission with lifetime roughly corresponding to that of the long photosensitizer phosphorescence component. Moreover, the quantum yield of singlet oxygen phosphorescence decreases with increasing HSA concentration, while total quantum yield of $TPPS_4$ phosphorescence rises. These facts are explained by a shielding effect of HSA on bound molecules of $TPPS_4$ against quenching by oxygen which is analogous to oxygen removal by nitrogen purging.

KEY WORDS: $TPPS_4$; Singlet oxygen; Human serum albumin; Infrared phosphorescence; Photodynamic therapy.

INTRODUCTION

Porphyrin dyes are used in medicine for tumour detection and also for photodynamic therapy of cancer (PDT). Deep understanding of mechanisms of interaction between photosensitizers, proteins and oxygen is crucial for further progress in PDT of tumours. *meso*-Tetra(4-sulphonatophenyl)porphyrin ($TPPS_4$) is one of the drugs currently being tested for PDT. It is a water-soluble porphyrin exhibiting high quantum yield of singlet oxygen ($^1O_2$), equal to 0.62 in water [1]. Its photosensitizing properties were thoroughly explored in solutions [2–4]. Up to date, the investigation of $TPPS_4$ interaction with albumins has been based mainly on photosensitizer time-resolved triplet–triplet absorption [5–7].

Human serum albumin (HSA) is the most abundant serum protein consisting of 585 amino acid residues with molecular weight of approximately 66 kDa. HSA exhibits high affinity towards wide diversity of ligands that can be reversibly bound and thus distributed around human body [8]. According to Bartosova [9], HSA possesses one major binding site for $TPPS_4$ with binding constant of $3 \times 10^6\ M^{-1}$ and two to three sites of substantially lower affinity. Relatively little is known about the

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production of $^1O_2$ by $TPPS_4$ in the presence of HSA as well as about the chemical (photosensitized oxidation) and physical quenching of $^1O_2$ by HSA. The reaction constant of $(5 \pm 3) \times 10^8$ M^{-1} s^{-1} for oxidation of HSA by $^1O_2$ was determined by Davila [5].

The aim of this contribution is to investigate the production of $^1O_2$ by $TPPS_4$ at various HSA concentrations under aerobic and anaerobic conditions together with effects of $^1O_2$ interaction with HSA using direct time- and spectral-resolved measurements of both the photosensitizer and $^1O_2$ phosphorescence.

EXPERIMENTAL

Phosphate buffer (pH = 7.4, the pH value of human blood) solutions of $TPPS_4$ (Frontier Scientific Porphyrin Products) of concentration of 10 μM were used for all measurements. This concentration ensures that $TPPS_4$ is present in its monomeric form predominantly [10]. The samples differed in HSA (Sigma Aldrich) concentration (0, 1, 5, 50 μM). The sample preparation was carried out under dim light to avoid any photodegradation.

Absorption spectra were measured by Avantes Avaspec-1024 spectrometer. Infrared sensitive emission spectrometer equipped with Hamamatsu R5509 photomultiplier was used for measurements of $TPPS_4$ and singlet oxygen emission between 750 and 1350 nm with 5 ns time resolution. For detailed description of experimental set-up see Ref. [3]. One millilitre of fresh material was used for each phosphorescence measurement. Two thousand of excitation laser pulses of approximately 10 ns and 17 μJ at 420 nm were applied to obtain each kinetics. In HSA-photosensitized oxidation studies, 38 successive kinetics were measured at fixed wavelengths of 862, 1022 and 1278 nm and further processed.

RESULTS AND DISCUSSION

Typical absorption spectra of $TPPS_4$ in buffer exhibit Soret band maximum at 413 nm accompanied by Q_X and Q_Y maxima at 516, 553, 580 and 633 nm. As the concentration of HSA increases, the fraction of $TPPS_4$ bound to HSA increases as well. This is accompanied by bathochromic shifts of the respective absorption maxima to 421, 517, 553, 591 and 647 nm for 50 μM HSA. These observed absorption band shifts are exactly the same as those of $TPPS_4$–HSA complexes published by Andrade [10]. It is worth noting that even a very weak measurement light in absorption spectrometer was sufficient to induce photosensitized oxidation of HSA and thus only N_2 purged samples can provide reliable absorption spectra.

Table 1. Lifetimes (t_1, t_2) of Decays of Nitrogen-Purged $TPPS_4$ at Various HSA Concentrations

HSA concentration (μM)	t_1 (μs)	t_2 (μs)
0	1.7 ± 0.1	405 ± 20
1	1.8 ± 0.3	430 ± 20
5	1.9 ± 0.4	640 ± 50
50	1.9 ± 0.5	990 ± 120

Photosensitizer Phosphorescence

Phosphorescence of $TPPS_4$ in the absence of HSA was described in [3,4]. Under anaerobic conditions, the $TPPS_4$ phosphorescence comprises of two distinct components, each of them exhibiting monoexponential kinetics. The short component with emission maximum at 820 nm decays with lifetime of (1.7 ± 0.1) μs. The maximum of the long one is located at 900 nm and its lifetime is (405 ± 20) μs for 10 μM $TPPS_4$.

In the case of N_2 purged samples containing HSA, the $TPPS_4$ phosphorescence spectra and kinetics consist again of two components. The lifetime of the shorter one remains constant for all HSA concentrations (Table 1). Maximum of this component remains at the same wavelength as in HSA-free sample. While the spectral position of the long component maximum is constant in the whole HSA concentration range, its lifetime rises with increasing concentration of HSA from (405 ± 20) μs in HSA-free sample to (990 ± 120) μs in 50 μM HSA (see Table 1). This phenomenon can be explained by HSA preventing depopulation of the bound triplet $TPPS_4$ molecules by water, analogically to what has been published by Foley on phthalocyanine–HSA solutions [6]. The exchange between free and bound forms is substantially faster than their respective triplet lifetimes and therefore only single lifetime t_2 increasing with HSA concentration is observed.

$TPPS_4$ phosphorescence in the absence of HSA under aerobic conditions exhibits strictly monoexponential decay with lifetime of (1.9 ± 0.1) μs, which is in a very good agreement with earlier published (1.8 ± 0.1) μs [3] and (1.9 ± 0.2) μs [4]. Second exponential phosphorescence component of much longer lifetime arises after the addition of HSA. It is documented in Fig. 1, which shows typical time- and spectral-resolved emission of air saturated sample containing 5 μM HSA. These two components can be distinguished within entire spectral region of 750–1240 nm. Figure 2 presents typical $TPPS_4$

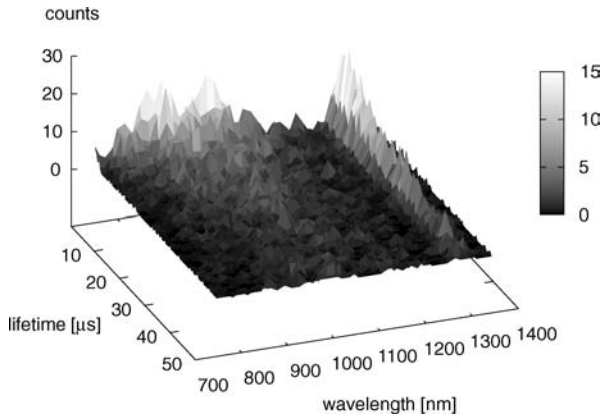

Fig. 1. Three-dimensional plot of time- and spectral-resolved phosphorescence of $TPPS_4$ (around 900 nm) and singlet oxygen (at 1280 nm) under aerobic conditions with 5 μM HSA. Raw data are shown for the sake of clarity.

phosphorescence decay at 1022 nm of 1 μM HSA sample together with its biexponential fit.

Lifetimes of the fast component were found around 2 μs independent of HSA concentration. On the other hand, lifetimes of the slow component increase from (15.0 $\pm$ 1.4) to (100 $\pm$ 90) μs with increasing HSA concentration (see Table 2).

This lifetime increase is accompanied by the increase of relative integral intensity of the slow component at the expense of the fast one. Since the fraction of $TPPS_4$ bound to the protein increases with increasing HSA concentration, the 2 μs component can be attributed to the phosphorescence of the free $TPPS_4$, while the other one corresponds to phosphorescence of $TPPS_4$ bound to HSA. The longer lifetime of bound $TPPS_4$ can be explained by

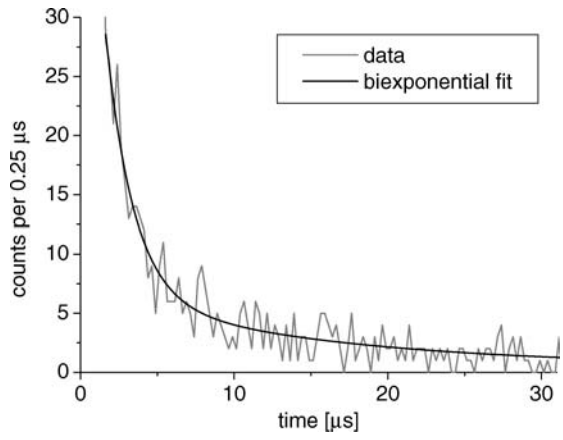

Fig. 2. Typical $TPPS_4$ phosphorescence decay of 1 μM HSA sample at 1022 nm together with its biexponential fit.

Table 2. The Lifetimes (t_1, t_2) of Biexponential Decays and Integral Intensities of the Short and Long Components (I_1, I_2) of Air-Saturated Samples at 1022 nm

HSA concentration (μM)	t_1 (μs)	t_2 (μs)	I_1 (a.u.)	I_2 (a.u.)
0	1.9 $\pm$ 0.1	—	0.21 $\pm$ 0.08	0
1	1.7 $\pm$ 0.3	15.0 $\pm$ 1.4	0.56 $\pm$ 0.05	0.50 $\pm$ 0.05
5	2.1 $\pm$ 0.5	25.1 $\pm$ 3.4	0.35 $\pm$ 0.06	1.29 $\pm$ 0.07
50	2.4 $\pm$ 2	100 $\pm$ 90	0.3 $\pm$ 0.1	3.7 $\pm$ 3.3

Note. The zero exposition-extrapolated values are presented to diminish the effect of photoinduced oxidation.

shielding of $TPPS_4$ by HSA against quenching by oxygen in accordance with [7,11].

Moreover, the spectral behaviour of the long component is exactly the same as that of N_2 purged samples, which proves that shielding of $TPPS_4$ molecules by HSA has very similar effect on $TPPS_4$ triplet properties as removal of oxygen by N_2 purging.

Singlet Oxygen Phosphorescence

Immediately after the excitation pulse the phosphorescence of singlet oxygen rises and past reaching its maximum it decays. The rise is attributed to gradual population of singlet oxygen via excitation energy transfer from the photosensitizer triplets. The decline occurs due to deactivation of singlet oxygen together with weakening of its photogeneration.

In the absence of HSA, it is reflected in the formula for singlet oxygen emission intensity:

$$I_{SO}(t) = b\{\exp(-t/t_{SO}) - \exp(-t/t_1)\} \qquad (1)$$

where t_{SO} represents the lifetime of singlet oxygen in the sample and t_1 corresponds to the lifetime of $^3TPPS_4$ [3]. In the presence of HSA, the situation becomes more complicated. As is shown earlier, $TPPS_4$ phosphorescence revealed existence of at least two distinct groups of photosensitizer triplets, which were ascribed to free and bound $TPPS_4$. Therefore, the singlet oxygen phosphorescence intensity has to follow time evolution in the form of linear combination of at least two expressions of Eq. (1) type with two different photosensitizer triplet lifetimes t_1 and t_2. Taking into account oxygen diffusion constant in water of 2 $\times$ 10^{-5} cm^2 s^{-1} [12], singlet oxygen travels 200 nm during its 4 μs lifetime. Its path is therefore substantially longer than the size of HSA molecules as well as the distance between them. Hence, it is reasonable to assume that each $^1O_2$ molecule interacts with both water

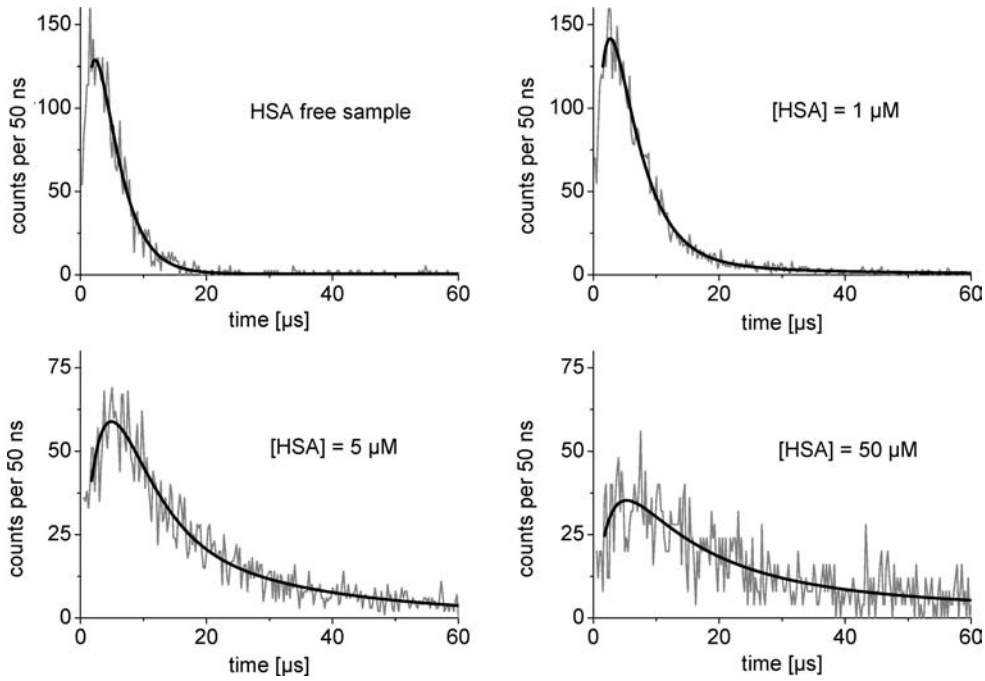

Fig. 3. Singlet oxygen phosphorescence kinetics at 1278 nm at various HSA concentrations and their fits by Eq. (2).

and HSA environment during its lifetime. This makes the question whether singlet oxygen was produced by free or bound $TPPS_4$ irrelevant and that is why the same effective value of t_{SO} for both components in Eq. (2) was used.

$$I_{SO}(t) = d\{\exp(-t/t_{SO}) - \exp(-t/t_1)\} + f\{\exp(-t/t_2) - \exp(-t/t_{SO})\} = I_f(t) + I_b(t) \quad (2)$$

Phosphorescence kinetics of singlet oxygen photogenerated by $TPPS_4$ in samples of different HSA concentrations are shown in Fig. 3. Equation (2) provides reliable fits of all singlet oxygen kinetics in the presence of HSA. The obtained lifetimes t_1 remain constant within our experimental accuracy in the whole HSA concentration range and fit well with the lifetime of $TPPS_4$ triplet in HSA-free samples under aerobic conditions determined from the photosensitizer phosphorescence. This further justifies the identification of the first component in Eq. (2) with singlet oxygen photogenerated by free $TPPS_4$ molecules. In the case of t_2 lifetimes, the correspondence with bound $TPPS_4$ triplet lifetimes is not so striking. Nevertheless, they both exhibit identical increase with HSA concentration.

The prolonging of effective lifetime of singlet oxygen with HSA concentration can be interpreted in this way: Water is well known for rapid quenching of singlet oxygen [13]. On the other hand, singlet oxygen lives much longer in majority of organic environments. Increasing HSA concentration changes effective singlet oxygen environment from water-like towards the more organic one. The observed $^1O_2$ lifetime increase indicates that the above-mentioned prolonging of $^1O_2$ lifetime prevails any shortening of $^1O_2$ lifetime due to chemical quenching by HSA.

Table 3. Lifetimes and Integral Intensities Obtained by Fitting $^1O_2$ Kinetics by Eq. (2)

HSA concentration (μM)	t_1 (μs)	t_2 (μs)	t_{SO} (μs)	$\int_0^\infty I_f(t)dt$	$\int_0^\infty I_b(t)dt$
0	1.8 ± 0.5	—	3.6 ± 0.5	4.2 ± 1.8	0
1	1.8 ± 0.2	24 ± 4	4.2 ± 0.3	5.2 ± 0.3	1.0 ± 0.2
5	2.1 ± 0.5	31 ± 3	8.8 ± 2.0	3.2 ± 0.4	2.6 ± 0.5
50	2.5 ± 0.8	47 ± 19	12.4 ± 4.9	3.4 ± 0.4	2.9 ± 0.5

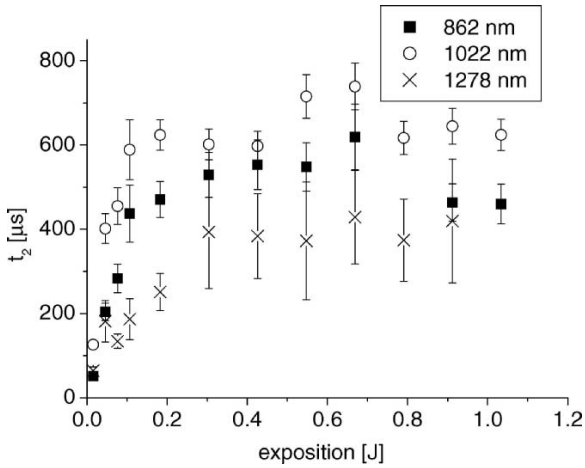

Fig. 4. The dependence of t_2 determined from phosphorescence at 862, 1022 and 1278 nm on the exposition of 50 μM HSA air-saturated sample.

Overall integral intensity of singlet oxygen phosphorescence (equal to the sum of respective integral intensities displayed in Table 3) remains constant for all studied HSA concentrations. Since the effective lifetime of singlet oxygen rises with HSA concentration, it means that the quantum yield of $^1O_2$ production decreases.

Interaction of HSA with Singlet Oxygen

HSA is oxidized by singlet oxygen photogenerated during phosphorescence measurements. Although the reaction rate of this oxidation is rather small $(5 \pm 3) \times 10^8$ M^{-1} s^{-1} [5], resulting changes are reflected in the growth of $TPPS_4$ phosphorescence lifetimes t_2. To investigate this phenomenon, we have analysed 38 successively measured kinetics at 862, 1022 and 1278 nm. No detectable changes were observed for 1 and 5 μM HSA samples. On the other hand, significant increase of t_2 appeared in 50 μM HSA (see Fig. 4). As the oxygen is consumed during HSA oxidation by singlet oxygen, total oxygen concentration in the sample decreases. It is reflected in the quintuple increase of t_2. The dependences presented in Fig. 4 represent quantification of HSA oxidation by singlet oxygen.

CONCLUSIONS

$TPPS_4$ phosphorescence lifetime under anaerobic conditions rises with increasing concentration of HSA due to HSA preventing water-induced depopulation of triplets of the $TPPS_4$ molecules bound to the protein.

Under aerobic conditions, spectral and temporal analysis of phosphorescence of triplet $TPPS_4$ as well as that of singlet oxygen revealed an additional HSA shielding of the protein-bound photosensitizer triplets from quenching by oxygen. The presented data reflect also photosensitized oxidation of HSA.

ACKNOWLEDGMENTS

This work was supported by project MSM00-21620835 from the Ministry of Education of the Czech Republic.

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