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Generation of singlet oxygen by porphyrin and phthalocyanine derivatives regarding the oxygen level

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ABSTRACT

Background. The principle of photodynamic effect is based on the combined action of photosensitiser, molecular oxygen and light, which produce various reactive oxygen species and are associated with significant cellular damage. Singlet oxygen is one of the most serious representatives, which is characterised by powerful oxidising properties. Moreover, concomitant hyperbaric oxygen treatment can support these effects. Therefore, the subject of our study was to compare the yields of singlet oxygen for four different photosensitizers in dependency on the oxygen concentration.

Material and methods. Four different photosensitizers 5,10,15,20-tetrakis(1-methyl-4-pyridinio)porphyrin tetra(p-toluenesulfonate), tetramethylthionine chloride, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin zinc(II) and zinc phthalocyanine disulfonate were investigated to determine the yield of singlet oxygen in PBS by Singlet Oxygen Sensor Green reagent under different partial pressures of oxygen (0.4 and 36 mg/l).

Results. There were no noticeable shifts in the excitation and emission fluorescence spectra regarding the oxygen concentration. Concerning the same molar concentration of photosensitizers the production of singlet oxygen was highest for 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin zinc(II), where the rate of the fluorescence change was more than 3 times higher than that obtained for 5,10,15,20-tetrakis(1-methyl-4-pyridinio)porphyrin tetra(p-toluenesulfonate). On the other hand, zinc phthalocyanine disulfonate showed the lowest yield in singlet oxygen production.

Conclusions. Singlet oxygen production, within the range of oxygen concentrations achievable in tissues under normoxia or hyperoxia, does not depend on these concentrations. However, the singlet oxygen generation is significantly influenced by the type of photosensitizer, with the highest yield belonging to 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin zinc(II).

Introduction

The principal of the photodynamic effect (PDE) is the light absorption by a specific compound known as a photosensitizer. After absorption, the excited photosensitizer can release the excess energy by fluorescence or undergoes a transition to a triplet state via intersystem crossing. Close contact with surrounding molecules can subsequently lead to an electron transfer, forming free radicals or radical ions. Which, in turn, may finally interact with other molecules producing superoxide anion radicals, hydrogen peroxides and hydroxyl radicals (type I reaction) Alternatively, the excited photosensitizer can transfer the energy to a molecule of oxygen to form singlet oxygen (type II reaction). Although both reactions can co-occur, their proportion depends on the chemical structures of photosensitizers and substrate molecules and on the oxygen level [1, 2].

The production of reactive oxygen species (ROS) by PDE is the basis of photodynamic therapy (PDT), which is becoming an alternative method in treating oncological, cardiovascular, skin, and eye diseases. PDT is also used to treat chronic inflammation and drug-resistant bacterial infections [3]. Photosensitizers used in PDT represent diverse chemical compounds, including porphyrins, chlorophylls, bacteriochlorins, phthalocyanines, pheoforbides, purpurines, 5-aminolevulic acid (ALA), texaphirines. Porphyrin derivatives belong to the first generation of photosensitizers. This group shows high absorption in the Soret band and poor solubility in polar solvents, which disadvantages the use for clinical purposes [4, 5].

On the other hand, the second generation of photosensitizers, such as phthalocyanines, chlorins and purpurins, absorbs light in the red region allowing better tissue penetration. It also has a higher potential to accumulate in the target cells [5]. An increase in solubility of hydrophobic photosensitizers can be achieved, for example, by sulfonation. Further, the formation of a complex with zinc and aluminium increases the yield and lifetime of the photosensitiser's triplet state, and thus single oxygen can be promoted [6–9].

The oxygen molecule is the third key component in PDT (besides photosensitiser and light). The type II oxygen-dependent reaction is primarily responsible for the biological PDT effect [10]. Oxygen partial pressure (pO_2) in normal tissues ranges from 30 to 60.0 mmHg (i.e. 1.2 to 2.4 mg/l of dissolved oxygen at 37°C, 0.9% salinity, and normal atmospheric pressure) [11, 12]. Hyperbaric oxygen (HBO) treatment causes a several-fold increase in the cerebral tissue pO_2 [13]. A recent study aimed at tissue oxygenation in the head and limbs revealed that transcutaneous pO_2 during HBO can be increased even 8–15 times [14]. Thus, our presented study aimed to investigate the significance of the different concentrations of oxygen on the production of singlet oxygen for four different photosensitisers.

Material and methods

Photosensitizers, hypoxia and hyperoxia

Four different photosensitizers were investigated to determine the yield of singlet oxygen in PBS under different partial pressures of oxygen. 5,10,15,20-tetrakis(1-methyl-4-pyridinio)porphyrin tetra(p-toluenesulfonate) (TmPyP) and tetramethylthionine chloride (methylene blue, MB) were purchased from Sigma-Aldrich (Sigma-Aldrich, MO, USA). Whereas Jiří Mosinger (Department of Inorganic Chemistry, Charles University in Prague, Czech Republic) and Jan Rakušan (Centre for Organic Chemistry Ltd, Rybitvi, Czech Republic) synthesised and donated 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin zinc(II) (ZnTPPS) and zinc phtalocyanine disulfonate (ZnPcS), respectively. Their synthesis was described previously [15, 16]. **Figure 1** shows chemical structures of these photosensitizers. Different partial pressures of oxygen related to dissolved oxygen concentrations of approximately 0.4 and 36 mg/l (measured by oximeter Greisinger 3630, Germany) were achieved by pure nitrogen and oxygen bubbling via injection needles into hermetically sealed cuvettes filled with 3 ml of PBS for 20 minutes.

Fluorescence spectroscopy

Photosensitisers' excitation and emission spectra were measured with the fluorescence spectrometer FLS980 (Edinburgh Instruments, UK). The resulting concentration of all photosensitizers in PBS was 1 μM. The emission spectra for ZnTPPS and TmPyP were obtained at 420 nm excitation (at excitation and emission bandwidths of 1 nm). In contrast, MB and ZnPcS were excited by

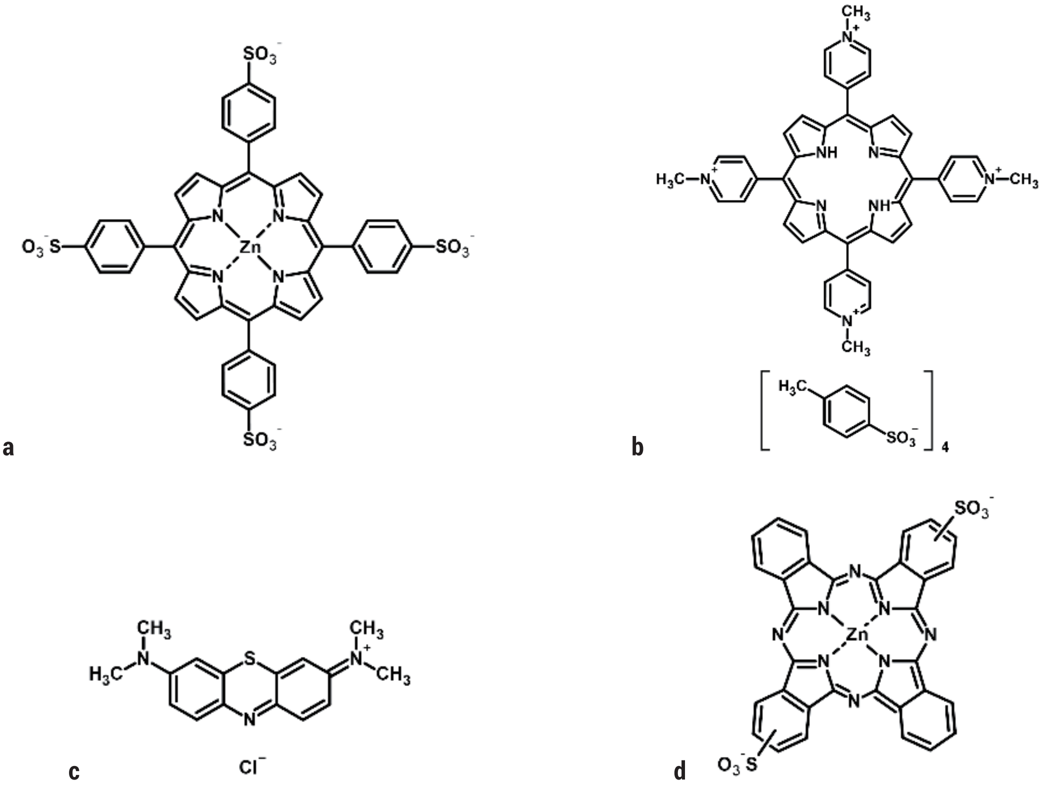


Figure 1. Chemical structure of photosensitizers: 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin zinc(II) (ZnTPPS, a); 5,10,15,20-tetrakis(1-methyl-4-pyridinio)porphyrin tetra(p-toluenesulfonate) (TmPyP, b); tetramethylthionine chloride (methylene blue, MB, c); zinc phtalocyanine disulfonate (ZnPcS, d)

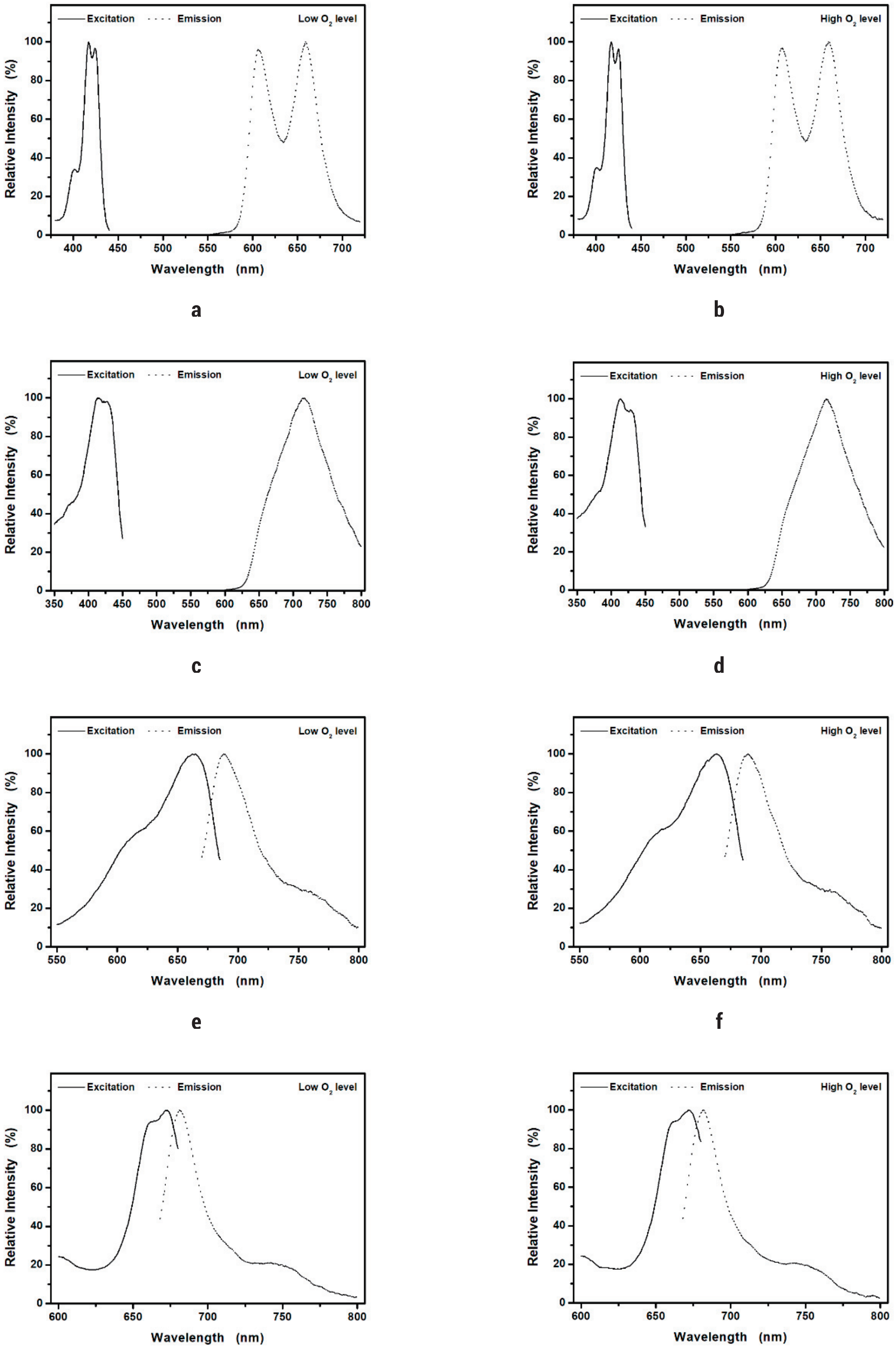
a wavelength of 663 nm (at excitation and emission bandwidths of 1 nm). The excitation spectra of photosensitizers were collected for their emission maxima, i.e. 607 nm for ZnTPPS, 715 nm for TmPyP, 689 nm for MB and 681 nm for ZnPcS.

Singlet oxygen measurement

The singlet oxygen sensor green reagent (SOSG, Invitrogen by Thermo Fisher Scientific, MA, USA) was used to detect singlet oxygen production in PBS buffer under different oxygen levels. First, the SOSG was added to the PBS buffer to reach the final 3µM concentration in each cuvette. Then the cuvettes were placed in the temperature-controlled holder of the spectrofluorometer. A generation of singlet oxygen was monitored as an increase in fluorescence emission intensity at 530 nm. An external source equipped with a mercury lamp, an optical bandpass filter of 460–480 nm, and optical fibre attached to the cuvette holder was used to excite SOSG. Singlet oxygen production was initiated by manually opening the shutter of the spectrofluorometer lamp. ZnTPPS and TmPyP were activated by light with an excitation wavelength of 420 nm and a slit width of 2.6 nm, whereas MB and ZnPcS were activated by light with an excitation wavelength of 663 nm and a slit width of 3.3 nm. The different slit widths were chosen so that the reference detector showed the same number of registered photons (500,000 cps). The appropriate irradiances measured by the IL 1705 radiometer system using the SED033 sensor (International Light Technologies, USA) were 4.1×10^{-4} W/cm^2 for the light of 420 nm and 2.1×10^{-4} W/cm^2 for the light of 663 nm. The rate of change in fluorescence determined the quantification of the singlet oxygen production during the first 7 seconds and the difference in fluorescence values after 4 minutes of the sample irradiation.

Data analysis

The data presented illustrate either representative traces or means ± standard errors of at least four independent measurements. The one-way analysis of variance (ANOVA) was used to compare experimental groups.



a b c d e f g h

Figure 2. Normalized fluorescence spectra: ZnTPPS (a, b); TmPyP (c, d); MB (e, f); ZnPcS (g, h); under low (0.4 mg/ml, a, c, e, f) and high (36 mg/ml, b, d, f, h) oxygen concentration in PBS

Results

Fluorescence spectroscopy

To compare the yields in the production of singlet oxygen for different photosensitizers it was necessary to find the optimal conditions, i.e. their excitation maxima. The porphyrin derivatives are known for their absorption maxima in the Soret band. The excitation peak of the synthesized zinc complex of porphyrin ZnTPPS was located at about 420 nm with two maxima at 417 and 425 nm and a shoulder at 401 nm (**Figure 2a-b**). The commercially available derivative TmPyP showed an excitation peak at about 2 nm shorter wavelength, with the maximum at 414 nm and a shoulder at 428 nm (**Figure 2c-d**). A much more significant spectral difference for these 2 porphyrins was found in the peak width. Whereas the full width at the half maximum (FWHM) for TmPyP was 58 nm, the excitation peak of ZnTPPS was significantly narrower with the FWHM of 20 nm. MB and non-commercial phthalocyanine derivative ZnPcS have their excitation spectra in the red region with a dominance around 660 nm (**Figure 2e-f and 2 g-h**, respectively). Compared to ZnPcS, the excitation spectrum of MB is wider. **Table 1** summarises the values of the excitation and emission maxima. In addition, there were not any noticeable shifts in the spectral characteristics regarding the difference in the oxygen concentration.

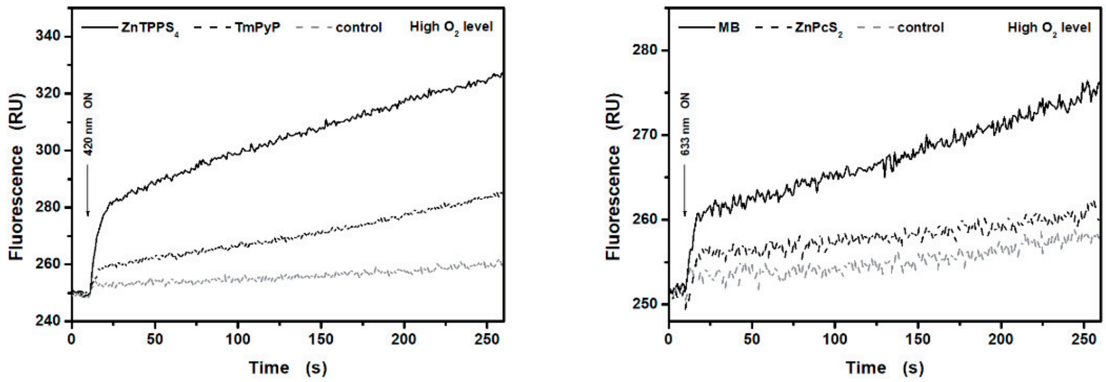
Singlet oxygen measurement

The generation of singlet oxygen by PDE using different photosensitizers at two different concentrations of oxygen in PBS was measured continuously for 4 minutes from the start of irradiation of the solution (**Figure 3**). The porphyrin

Table 1. Excitation and emission maxima of photosensitizers in PBS with low (0.4 mg/ml) and high (36 mg/ml) oxygen concentrations

Photosensitizer	Oxygen level	Excitation λmax (nm)	Emission λmax (nm)
ZnTPPS	Low	*401*, 417, 425	607, 659
	High	*401*, 417, 425	607, 659
TmPyP	Low	414, *428*	715
	High	414, *428*	715
MB	Low	*621*, 663	689, *758*
	High	*621*, 663	689, *758*
ZnPcS	Low	*663*, 672	681, *744*
	High	*663*, 672	681, *744*

Shoulders are in italics.



a

b

Figure 3. Representative traces of singlet oxygen production reflect the formation of high fluorescent SOSG endoperoxide from SOSG in the presence of singlet oxygen generated by: ZnTPPS and TmPyP during exposition to the light of 420 nm (a); MB and ZnPcS during exposition to the light of 663 nm (b)

Table 2. Singlet oxygen production quantification by different photosensitisers in PBS with low (0.4 mg/ml) and high (36 mg/ml) oxygen concentrations. Due to the significantly different response in SOSG fluorescence over time, the change in fluorescence intensity per unit of time in the first 7 seconds (ΔF/Δt) and the total change in fluorescence intensity (ΔF) after 4 minutes of irradiation were evaluated. The one-way analysis of variance (ANOVA) was used to compare the means of these data differing in oxygen concentration, and the p-value is the significance level of the analysis.

Photosensitizer	Oxygen level	ΔF/Δt (RU·s^{-1}) in the first 7 seconds		ΔF (RU) after 4 minutes	
ZnTPPS	Low	4.0 ± 0.3	p = 0.588	96 ± 15	p = 0.257
	High	3.9 ± 0.2		83 ± 15	
TmPyP	Low	1.2 ± 0.2	p = 0.972	42 ± 5	p = 0.734
	High	1.2 ± 0.1		40 ± 5	
MB	Low	1.0 ± 0.1	p = 0.549	32 ± 5	p = 0.847
	High	1.1 ± 0.1		31 ± 6	
ZnPcS	Low	0.68 ± 0.06	p = 0.291	13 ± 4	p = 0.899
	High	0.75 ± 0.09		12 ± 2	

derivatives were excited by a light wavelength of 420 nm at 4.1 × 10–4 W/cm^2 irradiance, whereas MB and ZnPcS were by 663 nm at 2.1 × 10–4 W/cm^2 irradiance. SOSG was added into the buffer as a sensor of singlet oxygen generation, which undergoes a chemical structure change producing high fluorescent SOSG endoperoxide. From the kinetic measurements, we evaluated the rates of that fluorescence increase during the first 7 seconds and the total change in fluorescence intensity in 4 minutes (**Table 2**). Conerning the same molar concentration of photosensitizers, the singlet oxygen production was highest for ZnTPPS, where the rate of fluorescence change was more than 3 times higher than that obtained for TmPyP. A similar yield in the singlet oxygen production, as was observed for TmPyP, was achieved for MB, but the irradiance value was halved there. In addition, the MB rate was about 30 % higher than was calculated for ZnPcS. Relatively similar results of the singlet oxygen production were achieved if we evaluated the changes in fluorescence after 4 minutes of measurement. Surprisingly, we did not observe statistically significant changes in singlet oxygen production relative to the different oxygen concentrations 0.4 and 36 mg/l (**Table 2**).

Discussion

2 types of reactions mediate PDE. It depends on many factors, including the photosensitiser's chemical structure, light wavelength and intensity, oxygen concentration, composition, dielectric constant and pH of the treated medium [17]. It is assumed that the reaction of type II generating singlet oxygen is the most crucial process conditioning the efficiency of PDE in PDT [10]. Thus, our study was focused on whether it is possible to achieve higher singlet oxygen yields by increasing the oxygen concentration. Four different sensitizers passed this assessment. Nyman and Hynninen reported that the diamagnetic cation complex formation, for example, with Al3+, Zn2+ and Ga3+, increases the yield and lifetime of the triplet state of photosensitisers [9]. So the production of singlet oxygen can be supported. Therefore, we compared two porphyrin derivatives ZnTPPS and commercially available TmPyP. Our results showed that the production of singlet oxygen under similar conditions was about 3 times higher in the case of the zinc complex.

In the case of comparing two representatives of the second generation of photosensitizers with absorption maxima in the red region of the visible electromagnetic spectrum, the zinc complex of the synthesized phthalocyanine ZnPcS showed a yield of only about 30 % greater than the non-metal photosensitizer MB.

Typical values of cellular pO_2 are in the range 9.9–19 mm Hg (i.e. about 0.4–0.8 mg/l) [18]. During hyperbaric oxygen therapy, these values may increase several times. However, our measurements in PBS did not show that increasing the oxygen concentration from 0.4 to 36 mg/l would lead to higher singlet oxygen production in the presence of various light-activated photosensitizers. Several experimental and clinical studies have shown that hyperbaric oxygen increases the efficacy of PDT in cancer [19, 20]. On the other hand, protoporphyrin IX precursors at higher oxygen concentrations did not induce significant enhancement in phototoxicity of human squamous carcinoma cells [21]. Consistent with our current and previously obtained results [22], the yield of singlet oxygen via PDT at typical physiological pO_2 values is already reaching its maximum. With certain simplification, each oxygen molecule could be considered a cube with a size equal to the molecule's size (i.e. approximately 0.3 nm). The total number of these cubes per volume of 1 l is 3.7×10^{25} ($1 / (0.3 \times 10^{-8})^3$). At the oxygen concentration of 0.4 mg/l, the number of oxygen molecules per 1 l is 7.5×10^{18} ($4 \times 10^{-7} / (32 \times 1.66 \times 10^{-27})$). Thus, there are 4.9×10^6 ($3.7 \times 10^{25} / 7.5 \times 10^{18}$) cubes per one molecule of oxygen in a space which corresponds to a mutual distance between two neighbouring oxygen molecules of approximately 50 nm ($(4.9 \times 10^6)^{1/3} \times 0.3$). At the photosensitizer concentration of 1 μM, the mutual distance between two neighbouring photosensitiser molecules is, on average, longer. When considering the homogenous distributions of photosensitizer and oxygen molecules, the maximum mutual distance between photosensitizer and oxygen molecule is halved (i.e. 25 nm), and the average distance equals 12.5 nm. In the case of an oxygen concentration of 36 mg/l, similar calculations give the average mutual distance of 2.5 nm. The excited triplet state of photosensitiser can transfer energy to the triplet state of the oxygen molecule because both electrons involved in the energy transfer process have the same spin. Therefore, mutual distance remains a crucial factor. According to the Förster theory [23], resonance energy transfer is inversely proportional to the sixth power of the distance. However, depending on the interacting molecules, this type of energy transfer can be maximally effective at mutual distances up to 5 or more nanometers [24]. In our simplification, we have omitted the fact that an oxygen molecule dissolved in water forms an induced dipole, which can electrostatically interact with the charged photosensitizer and thus significantly shorten their mutual distance. A relatively long lifetime of the photosensitiser triplet state, together with a high diffusion rate of oxygen, can also contribute to shortening distance. TMPyP and MB triplet states decay with a lifetime of about 2 μs in an air-saturated aqueous solution [25, 26]. The sulfonated zinc derivatives of phthalocyanines are known for their long triplet lifetimes and high singlet oxygen quantum yield [27]. In water, these lifetimes can reach up to 190 μs [28]. The average distance travelled by a diffusing molecule in a time t is given by $(2 \times D \times t)^{1/2}$, where D is the diffusion coefficient of the molecule in the medium [29]. Since typical diffusion coefficients in water at 298 K are about 2×10^{-9} m^2/s [30], each dissolved molecule can move 90 nm in 2 μs.

According to the above facts, photosensitiser selection can achieve PDT enhancement under normoxic conditions. Furthermore, a complex with metal ions such as Zn2+ can increase singlet oxygen production. MB is a tricyclic phenothiazinium, and it is used in medical practice to primarily treat methemoglobinemia, carbon monoxide or cyanide poisoning, and malaria [31, 32]. Moreover, our results confirmed that MB could also be a promising photosensitiser inducing higher singlet oxygen production. MB was already applied in the PDT for anticancer treatment [33] It was reported as an ideal photosensitizer for its adequate hydrophilic/lipophilic balance, high purity, stable composition, low cost, and strong absorption in the red region of the spectrum [34].

In conclusion, PDT represents an alternative treatment modality that can be very effective if it deals with specific issues such as photosensitiser selection, light dosage, and, most importantly, tissue hypoxia [35]. The use of oxygen carriers can achieve an increase of the oxygen pressure level in a tissue, improvement of blood flow, application of hyperbaric oxygen therapy, combining other therapies with PDT and fractionation of light, reducing oxygen consumption [35, 36]. Such approaches will be effective if the therapy aims at a hypoxic tissue, e.g. a developed (solid) tumour. However, our measurements showed that these solutions are unnecessary if PDT is used under normoxic conditions when the oxygen level is already sufficient to induce the maximum yield of singlet oxygen. According to our calculation, the lower oxygen availability can also be compensated by a longer interaction of the excited photosensitiser with the oxygen molecule, e.g. due to the longer triplet lifetime of the photosensitiser.

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Conflict of interest statement

The authors declare no conflict of interest.

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