Study of photodynamic reactions in human blood

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Abstract. Comparative studies of oxygen consumption, changes of photosensitizer fluorescence, and photodestruction of erythrocytes, and photodestruction of oxygen transport protein hemoglobin were performed during photodynamic reaction in whole and hemolyzed blood with phthalocyanines, chlorines, porphyrins, and methylene blue photosensitizers *in vitro* and in selected cases *in vivo*. The present work deals with the investigation of blood oxygen saturation SO_2 and photosensitizer fluorescence during and immediately after light irradiation in the photodynamic therapy process. It has been observed that SO_2 behavior strongly correlates with the type of photosensitizer. The decrease of photosensitizer fluorescence (photobleaching) during light irradiation can be followed by the recovery of the photosensitizer fluorescence immediately after interruption of the irradiation within 6–8 min. The levels of photodestruction of erythrocytes in whole blood and photodestruction of hemoglobin in hemolyzed blood in combination with the above photosensitizers reveal the influence of photodynamic reactions upon the ability of blood to transport oxygen. Maximal photohemolysis activity has been found with chlorine $p6$ photosensitizers. © *2000 Society of Photo-Optical Instrumentation Engineers.* [S1083-3668(00)00303-8]

Keywords: photodynamic therapy; oxygen consumption; photobleaching; photohemolysis.

1 Introduction

One of the most important objects of current investigation is human blood. This is a wonderful creation of nature and a part of the human body possessing characteristics of both biological tissues and fluids. We investigate the interaction of blood with photosensitizers and its participation in photodynamic reactions. The current study is part of a broader research effort aimed at developing an accurate and fast clinical system of photodynamic therapy (PDT) efficacy monitoring *in vivo*.

The efficiency of many modern photosensitizers used in PDT depends on the presence of dissolved oxygen in the irradiated tissues. The interaction of the photons, photosensitizer, and oxygen molecules results in the formation of the active forms of oxygen such as singlet oxygen and oxygen radicals. These reactive forms can damage different biomolecules, subcellular and cellular structure.[1] It is assumed that the destroying effect is mainly due to the formation of singlet oxygen (type II PDT process) resulting from the interaction of a light excited photosensitizer with molecular oxygen (triplet ground state). Hence, the effect will be proportional to the level of singlet oxygen formation, which in turn depends on light intensity, type of photosensitizer, its concentration, and molecular oxygen concentration.[2,3] The decrease in the last quantity is indicative of the level of singlet oxygen

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generation.[4] Direct destruction of photosensitizer in the course of PDT, defined as a photobleaching,[2,5] can exhibit itself as a decrease of photosensitizer concentration and or its photodynamic activity in tissue. Hence, one can estimate the biological effects due to photodynamic reactions by means of measuring the photosensitizer photobleaching and the oxygen consumption within the light-irradiated volume of the biological tissue during PDT. A complete PDT efficacy monitoring system includes explicit evaluation of tissue oxygenation, photosensitizer concentration, and light irradiation dose.[2,3] In the present comparative study with phthalocyanines, chlorines, porphyrins, and methylene blue photosensitizers, we concentrated on tissue oxygenation and photosensitizer fluorescence monitoring during PDT. We considered two processes characterizing tissue oxygenation status: oxygen consumption during PDT in whole blood and the influence of photodynamic reactions upon the ability of blood to transport oxygen. Several aspects of the latter were examined: functional study of the ability of PDT-deoxygenated whole blood to be reoxygenated and investigations of the levels of photodestruction of blood oxygen transport components including erythrocytes or red blood cells (RBCs), and oxygen transport protein hemoglobin, which is normally located inside RBCs.[8] Photohemolysis, the destruction of RBC's by the photodynamic reaction,[9–11] was investigated with whole blood. On the other hand, hemoglobin photodestruction is best examined under conditions of significant photohemolysis, as in hemolyzed blood. Concentration of the dissolved oxygen in living tissue will be proportional to hemoglobin oxygenation [blood oxygen saturation, % (SO_2)] inside erythrocytes.[2] It is defined by

$$SO_2 = \frac{C_{HbO_2}}{C_{HbO_2} + C_{Hb}}, \tag{1}$$

where C_{HbO_2} and C_{Hb} are the concentrations of oxyhemoglobin and deoxyhemoglobin, respectively. Development of new methods of monitoring of PDT with oxygen mediated photosensitizers is important for scientific and clinical reasons. Specifically, we are interested in the *in vivo* processes for a particular photosensitizer. *In vitro* oxygen consumption is purported to be an irreversible process because of limited content of oxygen in the sample.[12,13] In living tissue, the blood oxygen level is a balance between arterial input and venous output, which are the parts of blood microcirculation supply system of tissue metabolism.[14] The photodynamic process causes damages microvasculature[15,16] and causes additional consumption of oxygen in the tissue and directly in the blood, thus shifting this balance. The decrease of blood oxygen saturation following PDT depends on irradiation photon density, photosensitizer type, its concentration in blood and surrounding tissue, and extent of blood microcirculation within the volume of light irradiation.[17] Measurements of fluorescence spectra of photosensitizers dissolved in blood reveal the processes of their binding with blood components. For monitoring of photosensitizer concentration or its photodynamic activity in the course of PDT we studied the rate and the level of photobleaching described by reduction of the photosensitizer fluorescence. Actually, the term ''photobleaching'' is used to designate chemical destruction of the photosensitizer molecule, but in this current paper the term will relate to the

measurable fluorescence decrease of the photosensitizer.[2] We assume that the fluorescence reduction is proportional to loss of photodynamic activity of the photosensitizer.[2] It could be accompanied by changes of the phototoxicity of the photosensitizer.[5,7] In turn, the photosensitizer phototoxicity depends on oxygen concentration in surrounding media.[18] On the other side, photobleaching itself can lead to a decrease of oxygen consumption.[6,7]

2 Materials and Methods

2.1 Materials

2.1.1 *Preparation of Blood*

We used heparinized, fully oxygenated whole blood from healthy donors and heart disease patients of the Moscow Medical Academy within 1–3 h after donation. The value of blood hematocrit was 42 ± 1. Before preparation of the sample, the blood was incubated with or without a photosensitizer for 30 min at 37°C. The control sample did not contain a photosensitizer. The concentration of some of the photosensitizers in the test sample K (mg/ml) was close to its maximal blood level for a typical PDT treatment. It was calculated as

$$K(mg/l) = K_w W / U, \tag{2}$$

where K_w is the recommended photosensitizer concentration per kilogram of body weight (mg/kg), W is the body weight (kg), and U is the total volume of blood (normally about 5000 ml).[8]

2.1.2 *The Methods of Sample Preparation In Vitro*

The sample preparation technique *in vitro* depends on the duration of the measurement. In these studies, fluorescence spectra measurement takes only 1 s, while to measure absorption spectra requires 5–12 min. Therefore for fluorescence studies, blood contained in a standard cuvette was adequate. The standard methods of sample preparation for absorption measurements did not work for the comparative studies of photodynamic reactions in blood because of the instability of its scattering and absorption properties. The whole blood in the cuvette undergoes the process of sedimentation and its optical properties cannot be treated as stationary for several hours after sample preparation. To overcome these difficulties we have developed new methods enabling one to work with thin layers of the native whole blood with cells fixed in space.[19]

2.1.2.1 *The Preparation of Blood Sample for Fluorescence Research*

For fluorescence measurement the blood sample was investigated in standard 1 mm cuvette before and after RBC precipitation. Before preparation of the sample, the blood was incubated with or without photosensitizer for 30 min at 37°C. The sample was put in the standard 1 mm cuvette and placed in a special light-proof holder. Then for preparation of the blood component sample we kept the cuvette with the whole blood in a refrigerator at 4°C for approximately 1.5 h for the spontaneous separation settling of blood into plasma and erythrocytes. Before use the sample was held about 30 min at room

temperature in the dark for warming. After that the prepared sample was ready for measurements within 4–6 h.

2.1.2.2 The 'Glass Slide' Method

0.08 ml of whole blood was deposited on a standard glass slide. Then the blood droplet was covered by another glass slide to achieve homogeneous distribution of the liquid between the slides. Such a ''sandwich'' is naturally held together by capillary forces. The thickness of the blood layer is $20 \pm 5 \ \mu$m. Then the prepared sample was left in the dark for ~40 min to complete the erythrocyte aggregation process at room temperature. The resultant sample yielded quite stationary optical properties for at least 2 h. The relative spread of the blood oxygen saturation from spectral measurements was 2%–3%. To estimate the errors and ensure reproducibility, we typically repeated the measurements on 15–20 samples. The ability to participate in the photodynamic reactions is retained in these samples for 24–28 h from the time of preparation.

Although simple, this method has a substantial disadvantage of ill-defined blood layer thickness. Hence correct quantitative comparisons, for example of the dynamics of oxygen consumption of different photosensitizers, is seriously hindered. Another method described below was developed for more accurate comparative studies of photodynamic reactions in blood.

2.1.2.3 The Gel Method

The washed erythrocytes or whole blood with or without the photosensitizer were added to 1.8% solution of the ultralow-temperature type of agarose in PBS buffer and poured into a cuvette of known thickness. (This method was not successful with polyacrilamyde gel because phthalocyanines inhibited the gel polymerization process.) The sample was then placed in the refrigerator at 4°C for 5–10 min to solidify the gel via the polymerization process. The relative scatter in measurements of the blood oxygen saturation by means of spectra measurements was less than 1% and was independent of the opt-probe position on the sample. For one experiment we usually repeated measurements with 5–10 samples. The gel samples are then capable of photodynamic reactions for 2–3 d.

2.1.3 Photosensitizers

We studied four groups of the Russian produced water-soluble photosensitizers (Table 1). They are Phthalocyanines, Chlorines, Porphyrins and Methylene Blue. PBS buffer was used as a solvent.

2.1.4 In Vivo Studies

The 75 C57B1/DBA (BDF$_1$) breeding female mice bearing Erlich carcinoma were used in the current work. All mice were 10–12 weeks old, with average body weight of 20–22 g. The photosensitizer Photosence (Table 1) has been injected intravenously at a dose of 4 mg/kg. The photosensitizer has been dissolved in physiological solution (0.9% NaCl) for intravenous injection. Two PDT sessions have been applied in 6 and 28 h, respectively, after the photosensitizer administration.

2.2 Methods

2.2.1 Absorption Measurements

2.2.1.1 Monitoring of the Dynamics of Photodynamic Oxygen Consumption

Blood oxygen saturation (SO_2) was calculated from the relationship between the scatter-corrected transmission spectra (range 520–590 nm) of oxy- and deoxy-hemoglobin. The statistical errors in evaluated blood oxygen saturation for in vitro measurements depended on the experimental sample preparation method described above in Secs. 2.1.2.2 and 2.1.2.3, with reproducibility within 3%–4%. The spectra measurements in vitro have been conducted in a two-beam spectrophotometer with integrating sphere (HITACHI U-3400, Japan). Measurement of one spectrum took 5–12 min. As an independent check, we determined the whole blood gas composition of selected PDT irradiated samples with the electrode measurements of the gas composition in a blood gas analyzer (CIBA-CORNING 238, England). The agreement with the spectral measurements was 15%.

For the spectral measurements in vivo during PDT we used the fiber optic spectrometer LESA-5 (Biospec Lab., Moscow, Russia).[22,23] The light from a halogen lamp was delivered to the skin tissue through a quartz fiber. The light delivery and detection spectrometer fibers were on the same side of the tissue to measure the diffuse reflectance. After passing through the tissue (blood vessels of derma), diffuse light was collected by the receiving fiber. The detector fiber's proximal end served as the input slit of the spectrometer. The spectrometer was PC controlled. Custom software allowed acquisition and display of spectra in real time (time resolution of 0.1 s per spectrum, spectral resolution of 7 nm). Subsequent spectral analysis yields absorption spectra after scatter correction of the measured diffuse reflectance.[24] The displayed blood oxygen saturation was calculated from the absorption spectra in real time. The reproducibility in evaluated blood oxygen saturation for the in vivo measurements was about 10%.

2.2.1.2 The Effect of Photodynamic Reactions on Oxygen Transport Ability of Blood

The examination of the blood capability to transfer oxygen included functional study of reoxygenation of PDT-deoxygenated whole blood, and investigations of the levels of photodestruction of erythrocytes (photohemolysis), and photodestruction of hemoglobin. To test the RBC's functional ability for reoxygenation after uniform irradiation in standard capillaries, we withdrew blood that was irradiated and deoxygenated and tried to restore its full oxygenation by gentle mechanical shaking in aerobic surroundings.

The photohemolysis level in the irradiated whole blood in vitro was determined as the relative change (%) of the total transmission and diffuse reflectance of the photosensitized blood sample relative to a photosensitizer-free sample, at the isosbestic wavelength of 805 nm[25,26] during the first 2 h after irradiation. The sample without photosensitizer was assumed to exhibit zero level of photohemolysis under the light irradiation. Since the erythrocytes are the main light scattering particles in human blood,[26] our measurements are particularly sensitive to changes in light transmission and reflection because of reduction of scattering due to lysis of RBCs. This

Table 1 Photosensitizers examined in this study (synthesized in Russia).

Photosensitizers	Composition	Absorption maximum in red range (nm) Solution in PBS buffer	Extinction coefficient (cm^{-1}/mol/l)	Molecular weight (Daltons)
Phthalocyanines	percentage of groups with various degree of sulfonation			
(a) Aluminum phthalocyanine (Photosence) (PhSn)	(a) tetra - 30, tri - 50, di - 20	(a) 675	(a) 100 000	(a) 862
(b) Zinc phthalocyanine I (PcZn1)	(b) tetra - 76, tri - 23, di - 1.5	(b) 665	(b) 98 500	(b)–(e) ~870 [a]
(c) Zinc phthalocyanine II (PcZn2)	(c) tetra - 7, tri - 12, di - 75	(c) 670	(c) 28 300	
(d) Zinc phthalocyanine III (PcZn3)	(d) tetra - 28, tri - 50, di - 22	(d) 675	(d) 39 600	
(e) Zinc phthalocyanine IV (PcZn4)	(e) tetra - 46, tri - 44, di - 10	(e) 675	(e) 46 000	
Chlorines				
(a) Chlorine p6 I (Chp6-1)	(a) trisodium salt Clp6	(a) 664	(a) 11 100	(a) 648
(b) Chlorine p6 II (Chp6-2)	(b) trisodium salt 3-devinyl-3 formyl Clp6	(b) 695	(b) 11 780	(b) 650 [b]
Porphyrins				
(a) Hematoporphyrin derivative (photogem) (PhG)	(a) 85% - oligomers, 15% monomers	(a) 630	(a) 4400	(a) 598–2920 [c]
(a) Uroporphyrin III (UPh)	(b) the mixture of isomers I and III (I/III=4/1)	(b) 625 (675)	(b) 4170 (400)	(b) 818 [d]
Methylene Blue		600–665		373,9
(MB)		(665)	(72 000)	

[a] Reference 15.
[b] Reference 20.
[c] Reference 16.
[d] Reference 21.

method allows one to detect rather pronounced (more than 5%), fast developed (1–2 h after irradiation) hemolysis. The reproducibility in evaluated photohemolysis for *in vitro* measurements was about 6%–25%.

For the photosensitizers exhibiting significant level of photohemolysis, we also examined the hemoglobin photodestruction level as a result of photodynamic reaction in hemolyzed blood. We used fully oxygenated hemolyzed blood. Determination of hemoglobin photodestruction was made from the relative change (%) of the sample absorption (no scattering in this case) at isosbestic wavelength of 568 and 586 nm[27] immediately after irradiation, relative to the sample without photosensitizer. Once again, the latter was assumed to display zero level of hemoglobin photodestruction under the light irradiation. The intent here was not to identify the hemoglobin derivatives resulting from photodynamic reaction, but rather to determine the relative changes of hemoglobin concentra-

tion in the sample during and after PDT. The reproducibility in evaluated hemoglobin photodestruction for *in vitro* measurements was about 3%–5%.

2.2.2 Fluorescence Measurements

Two sets of fluorescence studies have been conducted. First, we compared fluorescence spectra of selected photosensitizers in PBS and in blood to determine effect of solvent environment. The second study investigated photobleaching, and was carried out for the samples of whole blood, its components (i.e., separated RBSs and blood plasma), and photosensitizer solutions in PBS. For all these experiments *in vitro* we also used the fiber optic spectrometer LESA-5 (Biospec Lab., Moscow, Russia)[22,23,28] (see Sec. *2.2.1.1*). A He−Ne laser was used instead of a halogen lamp for excitation of fluorescence, and as the PDT light source. The sample fluorescence induced
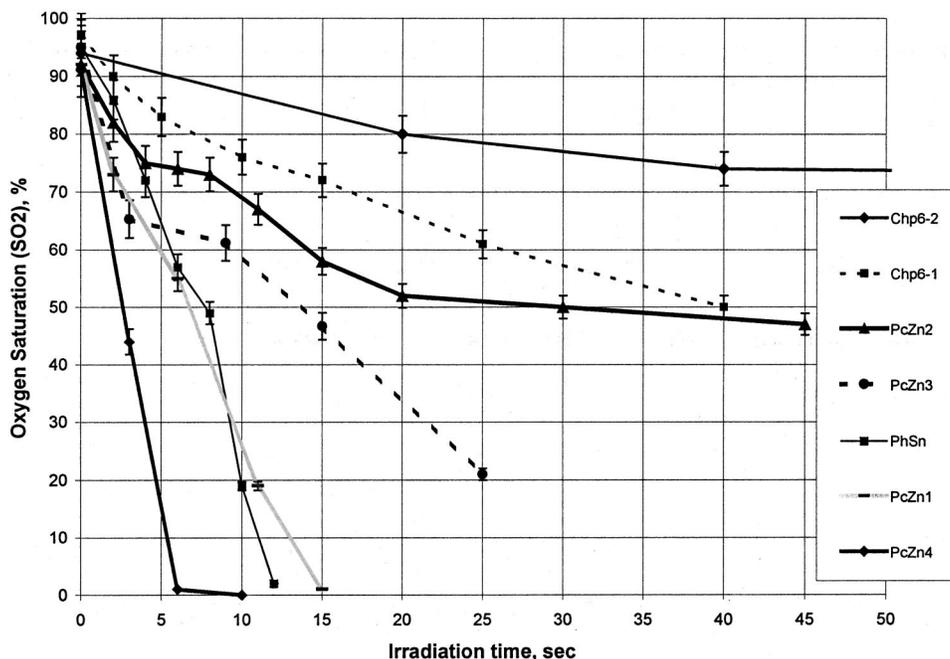
Fig. 1 Monitoring photodynamic deoxygenation *in vitro* for the whole blood via the glass sandwich method. $\lambda = 665-675$ nm, $P_m = 10$ mW/cm^2. Concentrations: chlorine $p6$ II (Chp6-2)—0.01 mg/ml; chlorine $p6$ I (Chp6-2)—0.01 mg/ml; zinc phthalocyanine II (PcZn2)—0.013 mg/ml; zinc phthalocyanine III (PcZn3)—0.012 mg/ml; photosence (PhSn)—0.01 mg/ml; zinc phthalocyanine I (PcZn1)—0.013 mg/ml; zinc phthalocyanine IV (PcZn4)—0.013 mg/ml. In this and subsequent graphs, the lines are a guide for the eye.

by the He–Ne irradiation ($P_m = 100-250$ mW/cm^2) was observed in the 640–900 nm spectral range. A fluorescence spectrum was acquired in 1 s.

Fluorescence spectra were measured in backscattering geometry and were dependent on both absorption and scattering properties of the tested object. To account for the changes in absorption and scattering of the sample, fluorescence spectra detected during the PDT session were normalized to the maximum amplitude of the excitation source spectrum.[23,24,28] In other words, fluorescence was calculated as the ratio of maxima of excitation source and fluorescence spectra (i.e., FL/EX).[28] The monitoring of photobleaching (diminution of fluorescence during irradiation) was performed as the ratio of fluorescence prior to irradiation (measured after the first second of switching on the excitation source) and fluorescence measured in the course of PDT irradiation. Evaluation of fluorescence recovery was carried out after switching off the He–Ne laser, in the same way as photobleaching monitoring. For excitation of fluorescence during its recovery measurements the He–Ne laser was periodically turned on for 2–3 s. The reproducibility in measured fluorescence for *in vitro* investigations was about 3%.

2.2.3 PDT Irradiation of Blood and of Tissue

The fluence rate was calculated as follows[19]:

$$P_m(\text{W/cm}^2) = P_s(\text{mW/cm}^2)(1 - Tt - Rd), \quad (3)$$

where P_s is the irradiance rate of the incident beam, and Tt and Rd are the total transmittance and the diffuse reflectance of the blood layer for the wavelength of interest.

We used a He–Ne laser (633 nm) coupled to a lightguide (diameter 200 μm) for both excitation of fluorescence and for

PDT irradiation during investigations of photobleaching. A confocal light emitting diode (LED) matrix (665–675 nm) (Biospec Lab., Moscow, Russia) was used for experiments with oxygen consumption evaluation. For more powerful irradiation for comparative studies of oxygen consumption, photohemolysis, and hemoglobin photodestruction, we used a diode laser (670 nm) (Biospec Lab., Moscow, Russia). A frequency-doubled solid state laser (672 nm) was used for *in vivo* PDT irradiation.

3 Results and Discussions

The analysis of the absorption spectra showed that there are no pronounced changes of the optical properties of blood samples without photosensitizers due to laser irradiation. The changes of the blood oxygen saturation deduced from absorption spectroscopy measurements due to photodynamic reactions in human blood *in vitro* are presented in Figures 1–3. In this and other graphs we use abbreviated names of photosensitizers defined in Table 1. Results with phtalocyanines and chlorines photosensitizers are shown in Figure 1. We note that oxygen consumption during light exposure for phtalocyanines is greater than for chlorines by a ratio of 2:20. This may be due to a considerable difference in extinction coefficient between these two photosensitizer types.[15,16,20] The fastest photodynamic deoxygenation was demonstrated by zinc phthalocyanine IV.

Results obtained from absorption spectra measurements for four other photosensitizers in whole blood under irradiation with $\lambda = 675$ nm are presented in Figure 2. It is clear that uroporphyrin demonstrated no photodynamic activity as measured by oxygen consumption. Moreover, this photosensitizer demonstrated the same null result with a much higher fluence
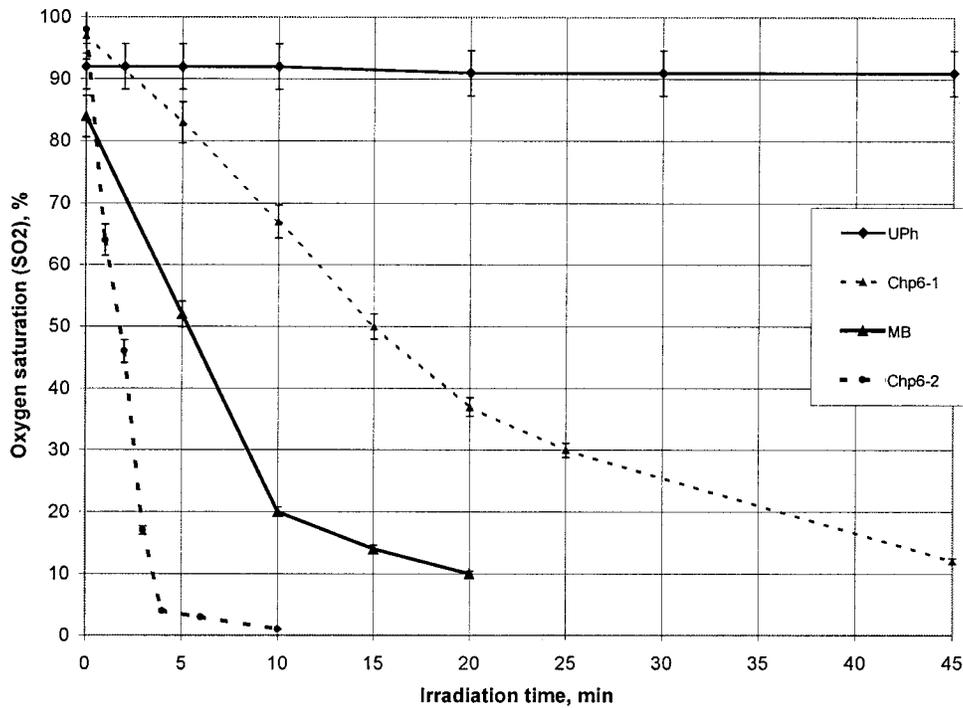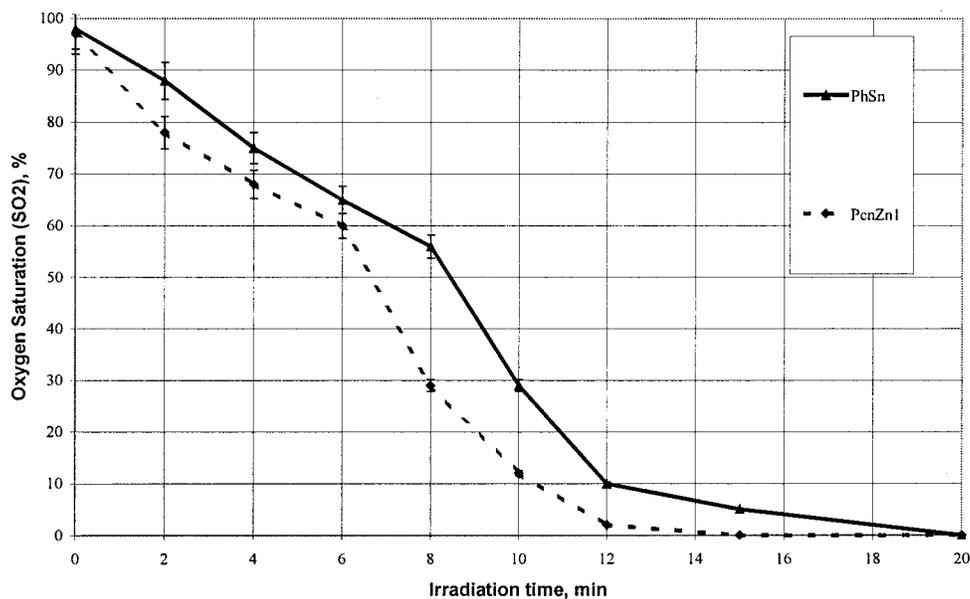
Fig. 2 Monitoring photodynamic deoxygenation *in vitro* for the whole blood via the glass sandwich method. $\lambda = 675$ nm, $P_m = 35$ mW/cm^2. Concentrations: uroporphyrin III (UPh)—0.023 mg/ml; chlorine $p6$ II (Chp6-2)—0.025 mg/ml; Methylene Blue—0.01 mg/ml; chlorine $p6$ I (Chp6-1)—0.025 mg/ml.

rate of $P_m = 300$ mW/cm^2. Similarly, the photosensitizer photogem did not exhibit any measurable blood oxygen saturation decrease at $\lambda = 633$ nm and $P_m = 100$ mW/cm^2 (results not shown). It also appears that the rate of deoxygenation for chlorines and Methylene Blue are similar. For photosensitizers presented in Figure 2, we used a higher incident of irra-

diation because of the lower values of extinction coefficients compared with photosensitizers in Figure 1.

As was shown in Figure 1, we obtained a similar result for photodynamic oxygen consumption with aluminum phthalocyanine and zinc phthalocyanine I (curves 1 and 2) with the slide method. To examine these two phtalocyanines further,

Fig. 3 Monitoring photodynamic deoxygenation *in vitro* for the erythrocyte suspension via the gel slab method. $\lambda = 665-675$ nm, $P_m = 10$ mW/cm^2. Concentrations: photosence (PhSn)—0.01 mg/ml; zinc phthalocyanine I (PcZn1)—0.013 mg/ml.
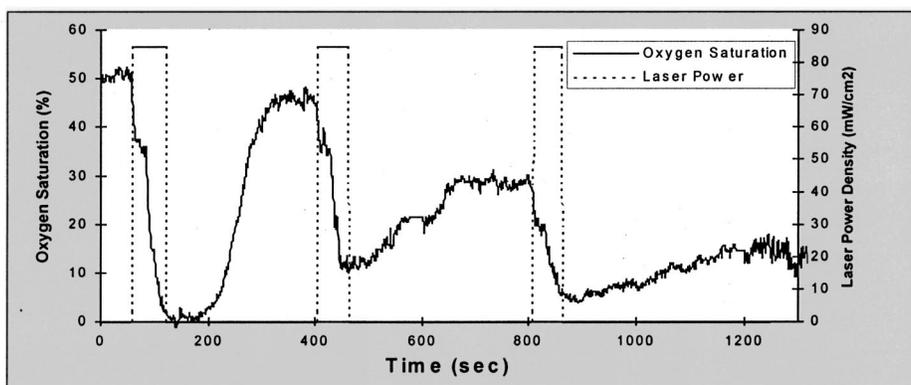
a)
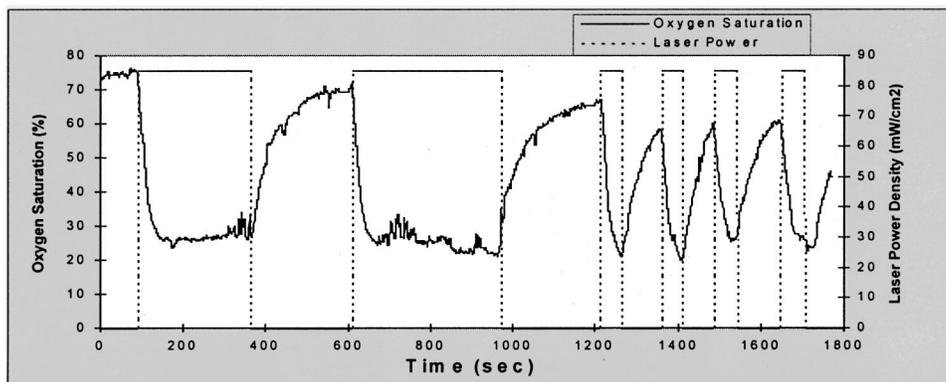
b)

Fig. 4 Monitoring of blood oxygen saturation during PDT *in vivo*. The influence of laser irradiation ($P_s = 85$ mW/cm^2, $\lambda = 670$ nm) on hemoglobin oxygen saturation in microvasculature of mice during photodynamic therapy with photosense, at the injection dose of 4 mg/kg, is shown. The time course of incident laser power is indicated by dotted line. (a) The first PDT session (photosensitizer accumulation time is 6 h); (b) The second PDT session (photosensitizer accumulation time is 28 h).

we used the gel method of the sample preparation. The outcome of this experiment is displayed in Figure 3. As seen, the faster photodynamic deoxygenation occurs in the blood sample photosensitized by zinc phtalocyanine I.

Selected independent checks of the *in vitro* reflectance results in Figures 1–3 were performed through the gas analyzer to determine PDT-induced oxygen consumption in blood with photosence and Methylene Blue. We observed the decrease of pO_2 in the samples with photosensitizers after light irradiation from 100 to 170 mm Hg down to 0–2 mm Hg. Both pH and pCO_2 were virtually stable. The attempts of the reoxygenation of the irradiated deoxygenated blood samples were successful. Gas analysis showed that the blood oxygen saturation level was restored to almost 97%. This implies that the use of photosence and Methylene Blue photosensitizers does not cause oxygen transport dysfunction of blood during PDT.

The results of oxygen consumption *in vivo* during PDT measured with fiberoptic spectrometer LESA-5 are shown in Figures 4(a) and 4(b). The irreversible vessel damage was observed for the first PDT session, when photosensitizer accumulation time was 6 h (a). Therefore, only incomplete recovery of oxygenation is seen. The blood oxygen saturation was restored reversibly after switching off the laser during the

second PDT session, when photosensitizer accumulation time was 28 h (b). This was enough time for elimination of the photosensitizer from blood and blood vessel tissue to prevent photodynamic reaction directly in the blood and damages to blood vessels.

Photohemolysis (destruction of RBCs due to development of photodynamic reaction[9]) is presented in Figure 5. This phenomenon was evident for chlorines and was not present for phthalocyanines. Photohemolysis was also detected for porphyrins at a rather high fluence rate of 500 mW/cm^2 ($\lambda = 633$ nm) (results not shown).

The differences in the photohemolysis level between photosensitizers could be in the ability of the photosensitizer molecule to bind with erythrocyte membranes. From Figure 5 we can suppose that chlorines $p6$ are most binding with erythrocyte membranes, based on their highest determined hemolysis. Therefore, we investigated hemoglobin photodestruction specifically in these photosensitizer systems. The results of the studies are presented in Figure 6.

We should note that the results of Figures 5 and 6 might have a similar underlying cause. It is possible that the ability of chlorine $p6$ I to bind with both erythrocytes and hemoglo-
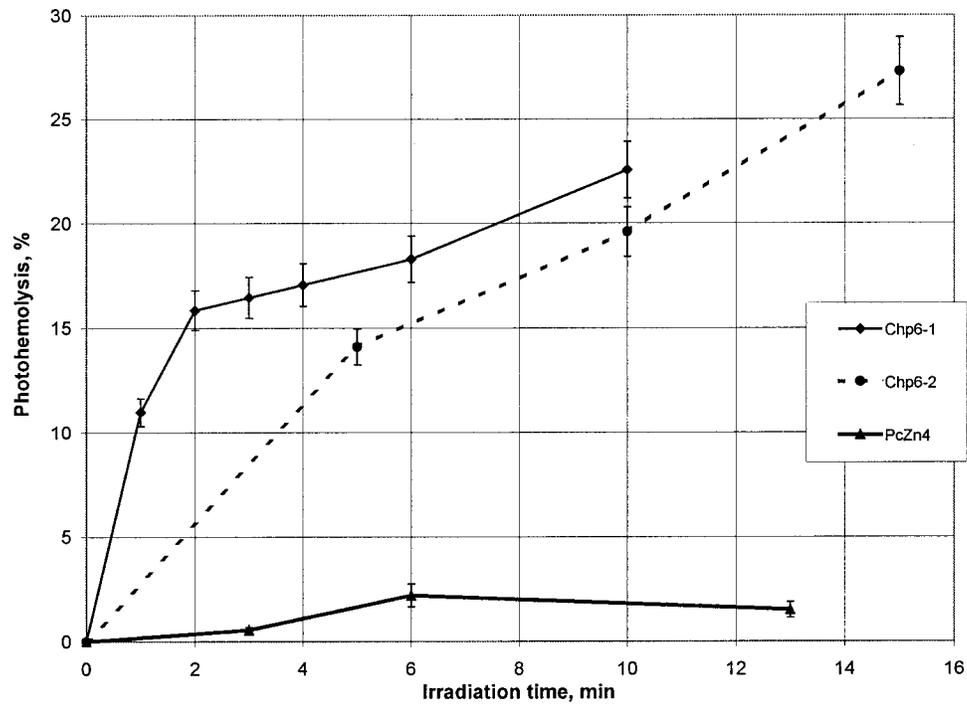
Fig. 5 Monitoring of the photohemolysis *in vitro* in whole blood via the glass slide method. Photohemolysis is measured in percents of the control (nonirradiated) sample, which is assumed to have 0% of hemolysis. $\lambda = 675$ nm, $P_m = 35$ mW/cm^2. Concentrations: chlorine $p6$ I (Chp6-1)—0.025 mg/ml; chlorine $p6$ II (Chp6-1)—0.025 mg/ml; zinc phthalocyanine IV (PcZn4)—0.013 mg/ml.
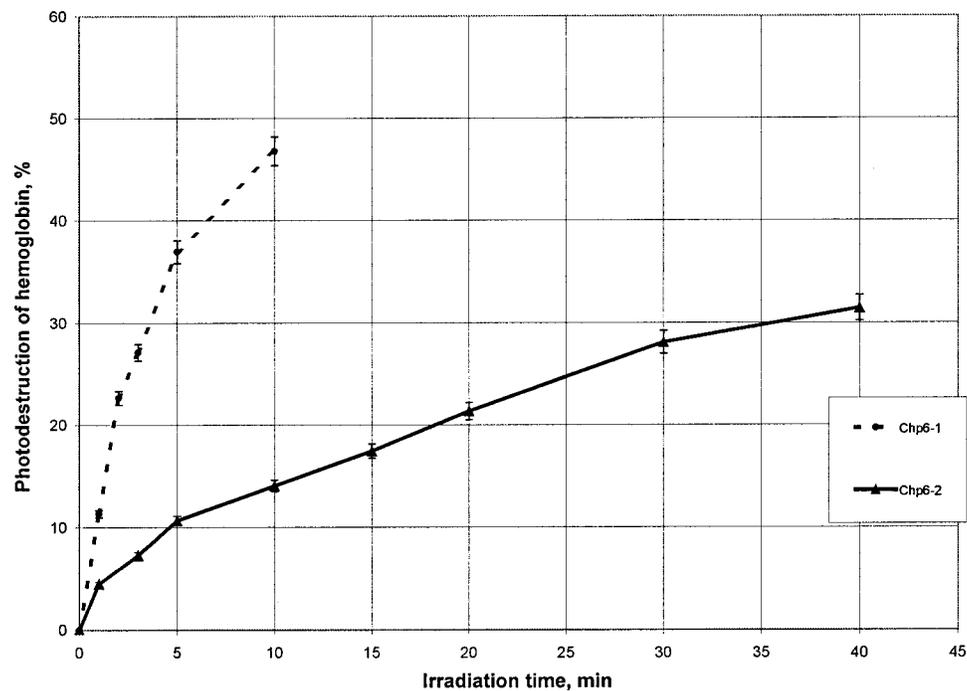
Fig. 6 Monitoring of hemoglobin destruction during irradiation *in vitro* in hemolyzed blood via the glass slide method. Photodestruction of hemoglobin is measured in percents of the control (nonirradiated) sample, which is assumed to have 0% of hemoglobin photodestruction. $\lambda = 675$ nm, $P_m = 35$ mW/cm^2. Concentrations: chlorine $p6$ I (Chp6-1)—0.025 mg/ml; chlorine $p6$ II (Chp6-2)—0.025 mg/ml.

Fluorescence, rel.un.

(a)

Fluorescence, rel.un.

(b)
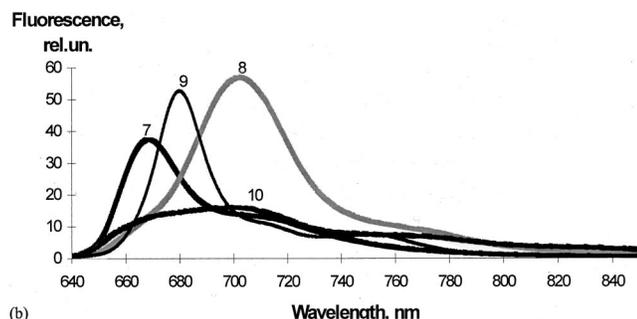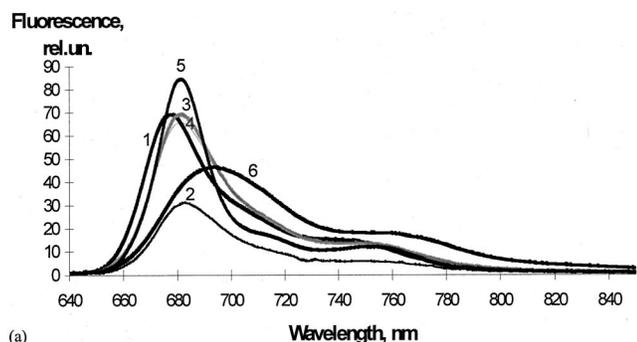
Fig. 7 Fluorescence spectra of studied photosensitizers in PBS solution. Excitation $\lambda = 633$ nm. (1) Zinc phthalocyanine I—0.007 mg/ml; (2) zinc phthalocyanine II—0.013 mg/ml; (3) zinc phthalocyanine III—0.012 mg/ml; (4) zinc phthalocyanine IV—0.013 mg/ml; (5) aluminum phthalocyanine (photosence)—0.001 mg/ml; (6) Methylene Blue—0.01 mg/ml; (7) chlorine $p6$ I—0.01 mg/ml; (8) chlorine $p6$ II—0.01 mg/ml; (9) uroporphyrin III—0.023 mg/ml; (10) hematoporphyrin derivative (photogem)—0.5 mg/ml.

bin is higher than that of chlorine $p6$ II. Interestingly, we also observed a substantial increase in viscosity of whole blood photosensitized with chlorine $p6$ I in the cuvette after laser irradiation ($\lambda = 675$ nm, $P_m = 150$ mW/cm^2). This effect did not occur with any other photosensitizer studied. This phenomenon is perhaps related to protein denaturation in the chlorine $p6$ I system, due to its expected highest ability among investigated photosensitizers for binding with erythrocyte membrane and hemoglobin.

The results of the fluorescence measurements of the photosensitizers in PBS solution are presented in Figure 7. Concentrations of photosensitizers have been selected to ensure similar fluorescence intensity for each system, allowing the

presentation of all curves in one scale. Essentially, this necessitated concentration adjustment of Uroporphirin III and Photogem. Fluorescence was very stable during the measurements. No photobleaching effect for any of the photosensitizers in PBS solution has been observed.

The largest fluorescence amplitude is seen for photosence (curve 5). The lowest amplitude is for zinc phthalocyanine II (curve 2), more than two times lower than for other photosensitizers from the Zn phthalocyanine group. It may be due to more pronounced aggregation of such photosensitizer molecules. Despite a very low level of oxygen consumption for Uroporphyrin during PDT (probably indicative of its real photodynamic activity), note the high level of fluorescence intensity (curve 9). One can assume that uroporphyrin III is a useful dye for diagnostic purposes. From Figure 7, we can divide all investigated photosensitizers into two groups based on the wavelength range of their fluorescence maxima. In the first group are zinc phthalocyanine I, II, III, IV, photosence, chlorine $p6$ I, and uroporphyrin III (curves 1, 2, 3, 4, 5, 7, 9), with fluorescence maxima at 670–685 nm. The second group includes chlorine $p6$ I, Methylene Blue, and photogem with peak fluorescence in the range 690–710 nm. These inferences obtained from fluorescence spectra of Figure 7 account for the dynamic range issues of the fiber optic system for fluorescence measurement in biological liquids and tissues.

Fluorescence spectra presented in Figure 8 were measured for chlorine $p6$ I in whole blood before (curve 3) and after (curves 2,4) sedimentation. This allows for observation of photosensitizer distribution between blood plasma and erythrocytes compartments. We supposed that fluorescence amplitude indicates photosensitizer concentration. The majority appears localized in blood plasma; hence, the concentration of chlorine $p6$ I in whole blood and plasma must be similar. However the fluorescence amplitude in blood plasma is considerably higher than in whole blood because of strong attenuation of exciting and fluorescent light by erythrocytes scattering and hemoglobin absorption.[19] We thus conclude that determination of relative photosensitizer concentration from fluorescence measurements can be quantified only in samples with similar types of optical properties. For example, fluorescence spectra from an absorbing only solution cannot be directly correlated with spectra from a turbid tissue. This imposes certain limitations of fiberoptic fluorescence spectroscopy usage in real biological tissues both *in vivo* and *in vitro*. It is seen that the spectral shape and peak positions of

Fluorescence, rel.un
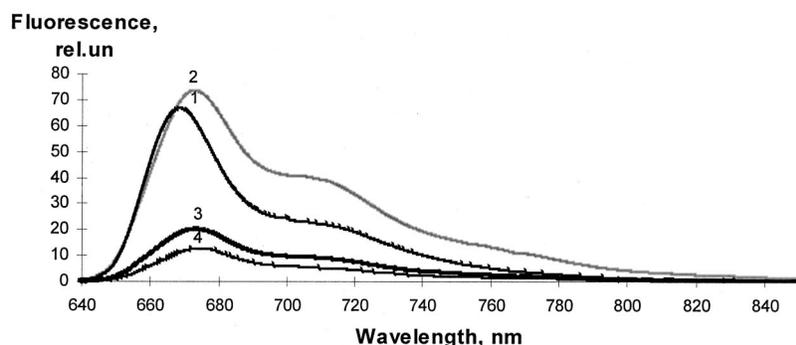
Fig. 8 Fluorescence spectra of chlorine $p6$ I in (1) PBS buffer; (2) plasma; (3) whole blood; (4) erythrocytes. Excitation $\lambda = 633$ nm.
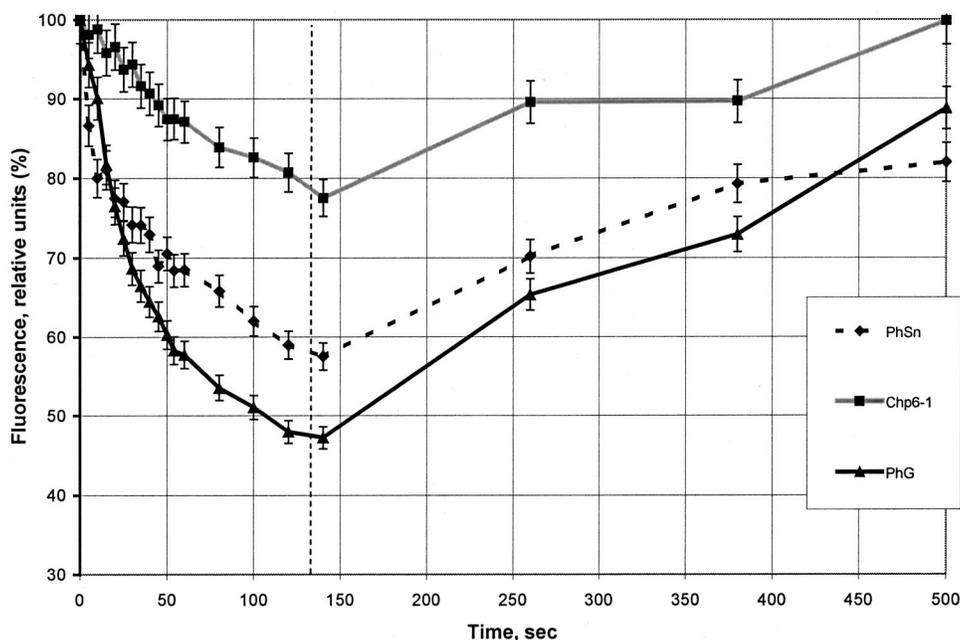
Fig. 9 Photobleaching of photosensitizers in precipitated erythrocytes. Photobleaching is measured in percent relative to a sample measured in the first 1–3 s of excitation, which is assumed to have 100% of its early fluorescence level. Dashed vertical line indicates the time at which the excitation laser was turned off. Excitation $\lambda = 633$ nm. Photosence (PhSn)—0.001 mg/ml; chlorine $p6$ I (Chp6-1)—0.01 mg/ml; photogem (PhG)—0.05 mg/ml.

photosensitizers in whole blood and erythrocytes are the same. Photosensitizer spectra in the whole blood and blood components, compared with PBS buffer spectra, exhibit a fluorescence peak shift by ~ 7 nm, which can be due to binding of the photosensitizers with the blood components (protein binding–plasma proteins or erythrocytes).

The main problem of the experimental blood measurements was the change of the fluorescence signal during excitation. There was a diminution of fluorescence of blood with dissolved photosensitizer during irradiation by the He–Ne laser. This so-called photobleaching phenomenon is well known.[7] However, we did not observe this in PBS solution and blood plasma. Evidently, the presence of membrane structures was necessary for photosence, chlorine $p6$ I, and photogem photobleaching. We therefore used erythrocytes in our experiments to study this phenomenon. The most expressed photobleaching effect took place on precipitated RBCs. Figure 9 summarized the results of photobleaching monitoring in precipitated erythrocytes.

We hypothesize that the observed photosensitizer fluorescence change during light excitation is caused by a combination of several different processes. In the following discussion, the minus sign indicates a process leading to a fluorescence decrease, while the plus sign denotes fluorescence-increasing process. The first is a formation of reversible complex between photosensitizer and oxygen during light irradiation. This process causes attenuation of fluorescence without absorption spectra changes ($-A$ process). The second is oxygen consumption during photodynamic reaction due to singlet oxygen generation ($+B$ process).[2,4] The decrease in oxygen concentration can lead to extraction of oxygen from complexes appearing during the $-A$ process. The third is direct bleaching of the photosensitizer because of

its destruction by light, a ''true photobleaching'' phenomenon ($-C$ process).[5] This leads to diminution of oxygen consumption during PDT for both $+B$[17] and $-A$ reactions. In addition to the above, other factors can also contribute. Let us introduce the space factor (S factor) describing the growth of probability of $-A$, $+B$, $-C$ processes in the presence of membrane suitable for photosensitized substrates. No changes of fluorescence in the absence of membrane structures were observed. Perhaps the S factor should be critical for the $-A$ process in particular. For *in vivo* investigations, we also have to consider the TO factor, indicative of tissue oxygen supply and blood microcirculation conditions. The importance of each process and factor could be very different even for photosensitizers with similar chemical composition. As an example, consider oxygen consumption and photobleaching for phtalocyanines or chlorines $p6$ (Figures 1 and 9). The fluorescence recovery processes for chlorine $p6$ I, hematoporphyrin derivate (photogem) and aluminum phthalocyanine (photosence) (curves in Figure 9) suggest that the absence of light irradiation can lead to regression and elimination of all of $-A$, $+B$, $-C$. The destruction of reversible complexes formed due to the $-A$ process can cause the recovery growth of the photosensitizer fluorescence in biological objects.

The presented hypotheses require further investigation to furnish decisive evidence concerning supposed relationships between processes of oxygen consumption and photobleaching during photodynamic reactions.

4 Summary

Given the complicity and interdependence of processes occurring in human blood during PDT, the purpose of the present study is not to reveal the whole picture. However, some useful

findings have emerged. Oxygen consumption can be measured both *in vitro* and *in vivo* by means of fiberoptic absorption spectroscopy in real time. If the level of deoxygenation is proportional to photodynamic activity, then comparative screening of various perspective photosensitizing compounds can be carried out according to their oxygen consumption rate during PDT. Zinc phthalocyanine IV was determined to provide the maximal rate of the photodynamic deoxygenation of blood. This photosensitizer is a mixture of approximately equal parts of tetrasulfonated (46%) and trisulfonated (44%) forms, with a minor content (10%) of the disulfonated form.

Photodynamic reaction directly in blood could express itself as a hemolysis (photohemolysis). In general, photohemolysis can develop quickly (immediately or within 1–2 h after light irradiation) or slowly (during 1–2 days after irradiation).[9,18] In our work, we tried to evaluate the level of fast photohemolysis, which was previously assumed to be very low.[1] However, a high level of photohemolysis (22%–27%) was achieved in the whole blood with the trisodium salt of chlorine $p6$ and trisodium salt 3-divinyl-3 formyl chlorine $p6$. For these photosensitizers, we also determined that maximal level of hemoglobin photodestruction under irradiation ($\lambda = 675$ nm) with the trisodium salt of chlorine $p6$ is 30%–45%.

Photosensitizer concentration monitoring prior to and during light irradiation may be furnished by photosensitizer fluorescent property changes due to interaction with blood and blood components.[29] Solutions of photosensitizer trisodium salt of chlorine $p6$ I in the whole blood and blood components, compared with the solutions in PBS buffer, exhibits the peak fluorescence shift to lower wavelength by 7 nm. This can be due to binding of the photosensitizers with the blood components, most likely plasma proteins.

We have proposed several mechanisms of deoxygenation and photobleaching during photodynamic reactions. The results of comparative studies of oxygen consumption and photobleaching correlate well for zinc phthalocyanine IV. On the other hand, the correlation is poor for chlorine $p6$ I. Therefore, comparative investigations of photosensitizer deoxygenation and photobleaching reactions make sense only when measurements of blood oxygen saturation and fluorescence within irradiation volume are conducted simultaneously. Understanding the mechanisms responsible for fluorescence recovery for chlorine $p6$ I, hematoporphyrin derivative (photogem) and aluminum phthalocyanine (photosence) in blood and in precipitated RBCs after interruption of light irradiation requires additional investigations. Nevertheless, this phenomenon could be used for development of an efficient irradiation procedure during PDT, such as intermittent irradiation synchronized to the photosensitizer fluorescence recovery intervals.

Acknowledgments

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