

CHARLES UNIVERSITY PRAGUE

**faculty of mathematics and physics**



**Luminescence Study of Excitation Energy Transfer  
between Porphyrins and Oxygen**

Doctoral thesis

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## List of abbreviations

[A] – concentration of a compound A

<sup>1</sup>carotene – ground state of carotene

<sup>3</sup>carotene – triplet state of carotene

DMSO – dimethylsulfoxid

EET – excitation energy transfer

HpD – hematoporphyrin derivatives

HSA – human serum albumin

ISC – intersystem crossing

$I_{PS}$  – intensity of photosensitizer phosphorescence

$I_{SO}$  – intensity of singlet oxygen phosphorescence

$k_{NO}$  – rate constant of non-radiative deactivation of singlet oxygen

$k_{NP}$  – rate constant of non-radiative deactivation of photosensitizer triplets

$k_{PO}$  – rate constant of phosphorescence of singlet oxygen

$k_{PP}$  – rate constant of phosphorescence of photosensitizer

$k_{TO}$  – rate constant of excitation energy transfer

$k_{TT}$  – rate constant triplet–triplet quenching of photosensitizer

M – mol per litre

<sup>1</sup>O<sub>2</sub> – first singlet excited state of oxygen

<sup>3</sup>O<sub>2</sub> – ground state of oxygen

PDT – photodynamic therapy

PpIX – protoporphyrin IX

PS – photosensitizer

<sup>3</sup>PS – triplet state of photosensitizer

$t_1$  – lifetime of photosensitizer triplets

$t_{SO}$  – lifetime of singlet oxygen

TPP – meso-tetraphenylporphyrin

<sup>1</sup>TPP<sub>ground</sub> – ground state of TPP

<sup>1</sup>TPP\* – first singlet excited state of TPP

<sup>3</sup>TPP – first triplet excited state of TPP

TPPS<sub>4</sub> – meso-tetra(4-sulfonatophenyl)porphyrin

$\Phi_T$  – quantum yield of photosensitizer triplet states

$\Phi_\Delta$  – quantum yield of singlet oxygen production

# 1 Motivation and the aim of this work

Photosensitized generation of singlet oxygen by molecules of photosensitizer is a crucial process in photodynamic therapy (PDT). This therapeutic method combines simultaneous presence of photosensitizer, oxygen, and light in the cancerous tissue. Its main advantage compared to chemotherapy or radiotherapy is the selective damage of the targeted tissue. Although more than 25 years have passed since PDT was first used on humans, there is still much to improve in this method and many chemists and biochemists carry out systematic research in PDT. As the first steps of the tumour destruction are purely physical processes (absorption of light, intersystem crossing, excitation energy transfer to oxygen, quenching of oxygen), physicists also contribute significantly to the progress of PDT. Optical spectroscopy proved to be an excellent tool to study the above mentioned phenomena.

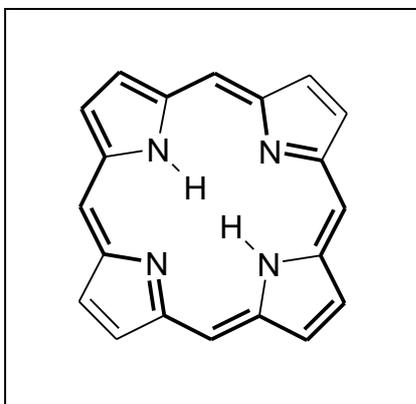
The aim of this work is to use optical spectroscopy to explain photophysical properties of several porphyrin photosensitizers in environments which simulate biological ones. Special attention was paid to the photogeneration of singlet oxygen in these environments. Furthermore, we focused on other processes competing in photosensitizer triplet state deactivation as well as on the deactivation of singlet oxygen. Basic experiments were carried out in well defined environment of a standard spectroscopic solvent (acetone). The first step towards mimicking *in vivo* conditions was done by the use of buffers with pH of human blood, cancer tissue, and skin. Subsequently, we observed processes of photosensitizer triplet state deactivation, which are often considered as negligible, in clinically used solvent – dimethylsulfoxide. Finally, the interaction of photosensitizer and singlet oxygen with the most abundant blood plasma protein (human serum albumin) was studied.

## 2 Introduction

### 2.1 Porphyrins

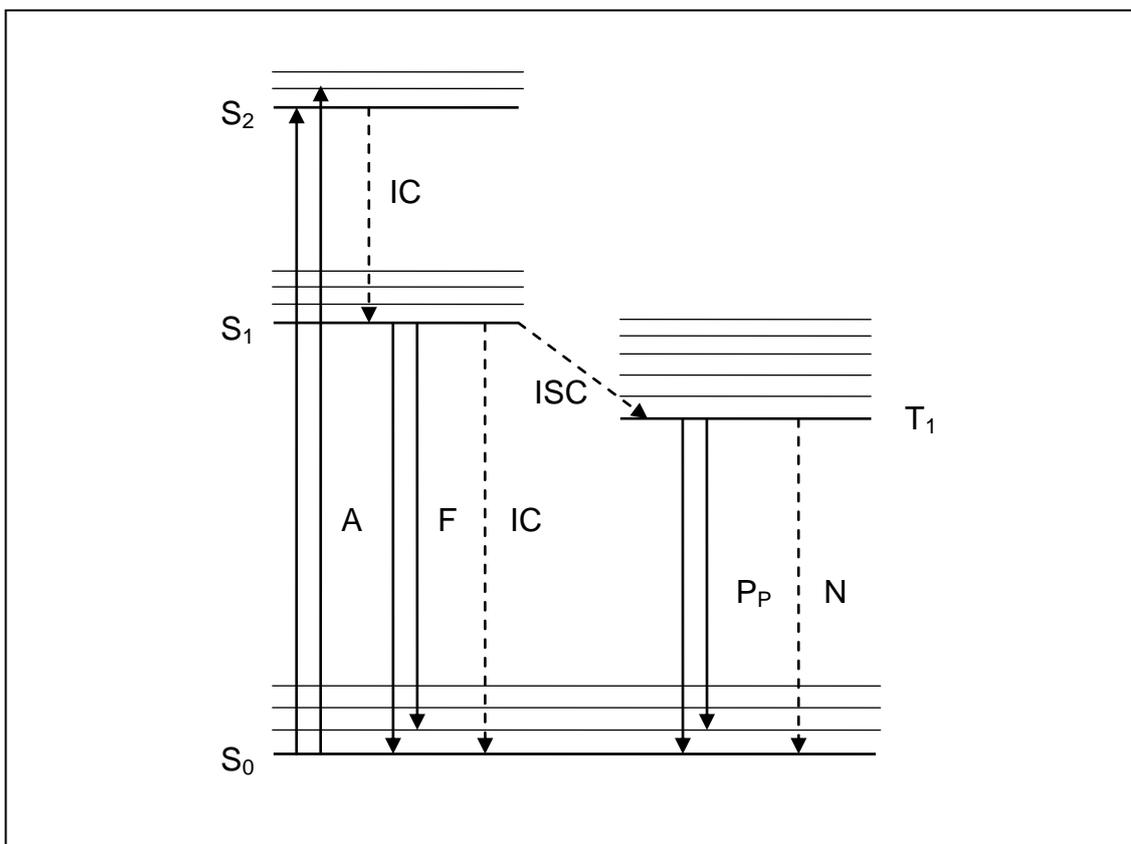
Porphyrins are organic dyes of particular biological importance. Their derivatives – chlorophylls play a crucial role in photosynthesis and haem is the part of haemoglobin which distributes oxygen in human body. There are also other porphyrins important in biology, e.g. prosthetic groups of cytochromes [1]. In the last years, porphyrins have proven to be useful in photodynamic therapy.

Porphyrins are colourful pigments due to the typical structure of their excited state energy levels. The absorption spectrum is characterized by bands between 500 and 650 nm and by so called Soret absorption band in blue part of the spectrum (around 400 nm). The ratio of absorbances of these bands determines their colour. For instance chlorophylls absorb similar amount of radiative energy in the red and blue region and thus they appear green. The key for understanding the energy levels of porphyrin molecules is the cyclic polyene (see Figure 1). The first proper explanation of the energy structure of porphyrins was proposed by Martin Gouterman in 1961. The details of his four orbital model are explained in [2].



**Figure 1.** All porphyrins contain above displayed porphin structure with cyclic polyene.

Figure 2 shows a typical Jablonski diagram of a porphyrin molecule together with absorption, radiative as well as non-radiative processes which occur after the excitation. All these processes are well known in optical spectroscopy and their details are described elsewhere [3,4]. Intersystem crossing (ISC), phosphorescence, and non-radiative deactivation of triplets have particular importance for our study so they are briefly described in the next paragraphs.



**Figure 2.** Jablonski diagram of a molecule of photosensitizer. S<sub>0</sub> – ground energetic state, S<sub>1</sub>, S<sub>2</sub>, – singlet excited states, T<sub>1</sub> – first triplet excited state, A – absorption, IC – internal conversion, F – fluorescence, ISC – intersystem crossing, P<sub>p</sub> – phosphorescence of photosensitizer, N – non-radiative deactivation.

### 2.1.1 Intersystem crossing

Intersystem crossing from the first singlet excited state into the first triplet excited state involves the change of the total spin of the electrons in the molecule and therefore it is spin-forbidden. Nevertheless, the spin-orbital interaction, which causes mixing of singlet and triplet states, makes this process possible [4]. Quantum yield of triplet states  $\Phi_T$  is a quantity which defines the efficiency of ISC:

$$\Phi_T = \frac{\text{number of molecules undergoing ISC}}{\text{number of absorbed photons}} \quad (1)$$

### 2.1.2 Phosphorescence

Phosphorescence of porphyrins is a radiative transition from the first triplet excited state to the ground singlet state. Since it also involves the change of the total spin of

electrons, it is a very improbable process compared to fluorescence, which results in substantially lower intensity of phosphorescence compared to fluorescence.

### **2.1.3 Non-radiative deactivation of triplet states**

Non-radiative deactivation of triplet states is the intersystem crossing from a triplet state to the ground singlet state. It is as well a spin forbidden process, thus also very improbable.

### **2.1.4 Quenching of triplets by oxygen**

Contrary to phosphorescence and non-radiative deactivation, quenching of porphyrin triplets by other molecules can be much more efficient way of triplet porphyrin deactivation. Oxygen, which is a typical quencher of triplets [3,4], is usually present in dissolved form in liquid samples or in the cytoplasm of a cell. This phenomenon, which is called excitation energy transfer to oxygen (EET) or photosensitized generation of singlet oxygen, is extremely important for our study. Therefore chapter 2.3 describes the details of excitation energy transfer.

### **2.1.5 Repopulation of excited singlet state**

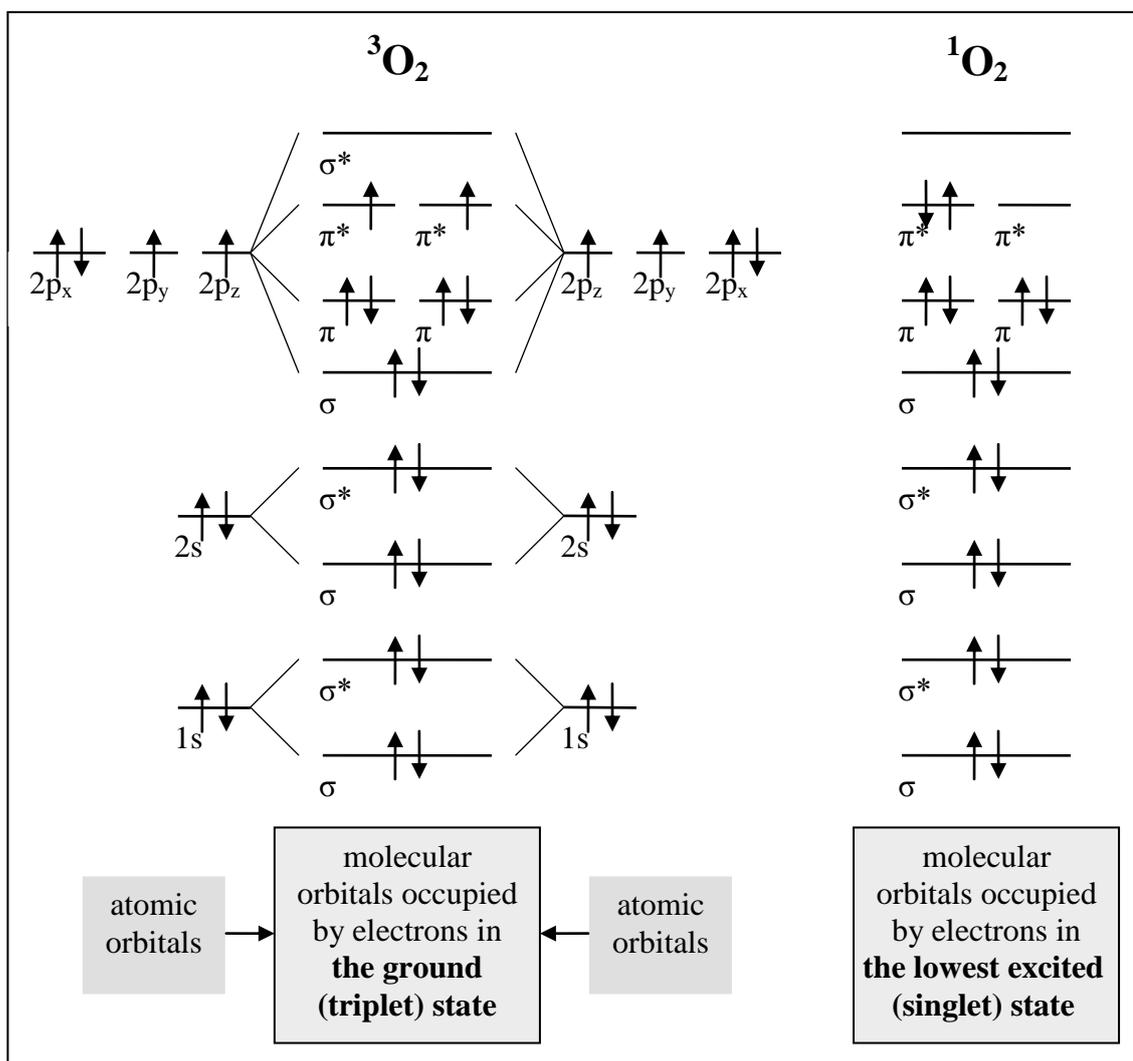
Molecules in the long-lived triplet state can return to the first singlet excited state and exhibit delayed fluorescence. Parker describes two ways of this repopulation [3]: E-type may occur if the energetic gap between the triplet and the first singlet excited states is relatively small. The first singlet excited state is repopulated by thermal activation in this case. P-type occurs after triplet–triplet quenching. Two molecules in the triplet state quench each other producing one molecule in the ground state and the second one in the first singlet excited state.

The first singlet excited state can be also repopulated by the reaction of photosensitizer triplet with singlet oxygen [39]. This reaction produces triplet oxygen and photosensitizer in the first excited state. This process is called singlet oxygen mediated mechanism.

## 2.2 Molecule of oxygen and its electrons

Michael Faraday was the first who noticed the exceptional property of oxygen: in contrast to other gasses, soap bubbles filled with oxygen were attracted to a magnet. Faraday himself called this phenomenon paramagnetism.

Paramagnetism of oxygen is caused by the fact that, unlike most of other molecules, the ground state of O<sub>2</sub> molecule is triplet [5,6]. Figure 3 explains this fact using simple MO-LCAO (Molecular Orbital-Linear Combination of Atomic Orbitals) method [7].



**Figure 3.** A scheme of energetic levels of a molecule of oxygen in its ground (triplet) and the first excited (singlet) states.

Each pair of atomic orbitals gives rise to bonding and anti-bonding molecular orbitals with different energies. Since 2p<sub>y</sub> and 2p<sub>z</sub> atomic orbitals have equivalent orientation in the molecule, they give rise to two bonding orbitals of equal energy as

well as two anti-bonding orbitals of equal energy. Figure 3 shows how electrons in the ground and the lowest excited states occupy molecular orbitals.

The total spin of the electrons in the ground state is equal to one, in other words oxygen ground state is triplet ( $^3\text{O}_2$ ). The electrons of the lowest excited state of oxygen have their total spin equal to zero. This excited state is called singlet oxygen ( $^1\text{O}_2$ ). Its energy lies 0.98 eV above the ground state which corresponds to singlet oxygen absorption wavelength of 1269 nm in the gaseous phase [5]. There are also other excited states of molecular oxygen – both singlet and triplet [5] – however none of them was a subject of our study.

### 2.2.1 Singlet oxygen generation

Singlet oxygen can be generated in a variety of chemical reactions (e.g.  $\text{ClO}^- + \text{H}_2\text{O}_2 \rightarrow \text{Cl}^- + \text{H}_2\text{O} + ^1\text{O}_2$ ), examples of which are described by Gilbert [8]. Another possibility of  $^1\text{O}_2$  production is its production in an electric discharge and particularly photosensitized generation which is explained in the chapter 2.3 and which is very important for PDT.

### 2.2.2 Singlet oxygen deactivation

Certain ways of singlet oxygen deactivation are in principle the same as those of porphyrin triplets: phosphorescence and non-radiative deactivation. Moreover, singlet oxygen is quite easily deactivated by physical or chemical quenching. Physical quenching is a deactivation of an excited molecule by the interaction with another molecule causing no chemical changes. It is important e.g. in photosynthesis where one of the roles of molecules of carotene is to quench  $^1\text{O}_2$  and protect the cell from reactive singlet oxygen in this way.



Chemical reaction of  $^1\text{O}_2$  with an adjacent molecule is called chemical quenching of oxygen. Since singlet oxygen is extremely reactive, oxidation of vitally important substrates within the cell is the main biochemical action in PDT [9,10].

In biological environment, these are mainly proteins which exhibit the highest reaction rates for oxidation by singlet oxygen [11]. Singlet oxygen reacts with a

considerable degree of selectivity particularly with tryptophan, tyrosine, and histidine as well as with sulphur-containing amino acid residues (cystein, cystine, methionin). However, oxidation of other molecules (RNA, DNA, unsaturated lipids) also contributes significantly to the cell damage [11].

The reciprocal value of the lifetime of singlet oxygen in the absence of quenchers is given as a sum of non-radiative rate constant ( $k_{\text{NO}}$ ) and phosphorescence rate constant ( $k_{\text{PO}}$ ):

$$1/t_{\text{SO}} = k_{\text{NO}} + k_{\text{PO}} \quad (3)$$

Even in the absence of quenchers, the value of  $t_{\text{SO}}$  differs dramatically in various environments. For unperturbed molecule  $t_{\text{SO}} = 72$  minutes [5]. This value proves that  $^1\text{O}_2$  phosphorescence is one of the least probable processes in nature and its detection is extremely demanding in the sense of sensitivity of a detector. On the other hand the lifetimes of  $^1\text{O}_2$  in solvents (reviewed in [12]) rarely exceed 1 ms (e.g. in  $\text{CCl}_4$ ) and they usually reach just units or tens of microseconds. In water or in buffer of neutral pH,  $^1\text{O}_2$  lifetimes between 3.1 and 4.2  $\mu\text{s}$  are reported [12]. On the contrary, in most organic solvents the lifetimes of singlet oxygen are longer. For instance in acetone,  $t_{\text{SO}}$  is around 50  $\mu\text{s}$  [12]. Very short lifetime of singlet oxygen in water compared to acetone is caused by high concentration of OH groups quenching  $^1\text{O}_2$  [5]. Many other considerations explaining broad diversity of the lifetimes of  $^1\text{O}_2$  in various solvents are described in the Schweitzer's review article [5].

Due to the high concentration of quenchers within the cell, the lifetime of singlet oxygen *in vivo* is assumed to be extremely short, corresponding to extremely weak phosphorescence. The first detection of the emission of  $^1\text{O}_2$  from a tissue was achieved by Niedre *et al.* using infrared sensitive photomultiplier [13]. Even the photomultipliers do not allow to measure  $t_{\text{SO}}$  in a tissue directly. Its value was estimated: 100–250 ns [9], 30–180 ns [13]. However, Skovsen *et al.* has recently measured surprisingly long value of 3  $\mu\text{s}$  in a nerve cell nucleus [14].

### 2.3 Excitation energy transfer

Systematic study of excitation energy transfer from triplet photosensitizer ( $^3\text{PS}$ ) to oxygen is one of the main objectives of this work.

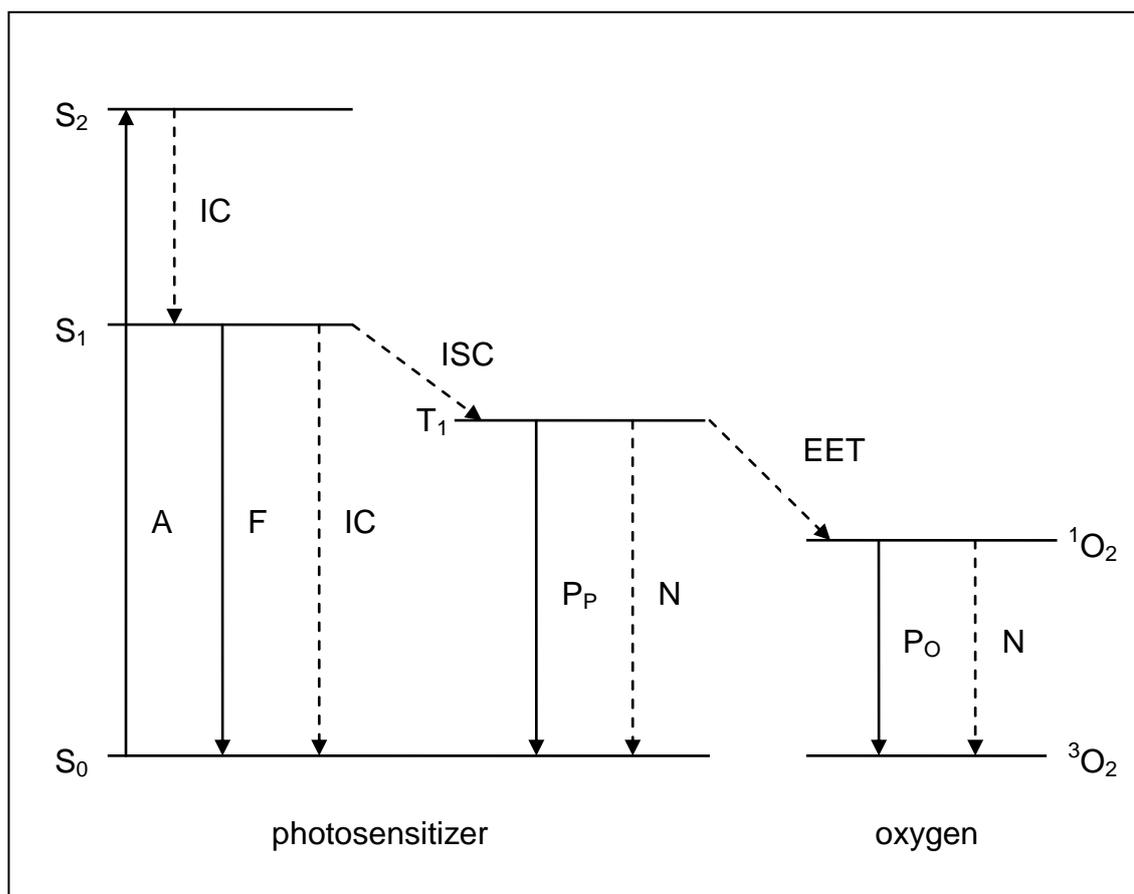


Since this process is allowed by Wigner's rule [6], it is a rather fast one compared to other ways of  $^3\text{PS}$  deactivation. Its rate constant  $k_{\text{TO}}$  depends on the solvent. Because it is a diffusion limited process,  $k_{\text{TO}}$  also depends on the solvent viscosity [15]. The overall efficiency of intersystem crossing and excitation energy transfer to oxygen is quantified by the quantum yield of singlet oxygen production  $\Phi_{\Delta}$

$$\Phi_{\Delta} = \frac{\text{number of photogenerated } ^1\text{O}_2 \text{ molecules}}{\text{number of absorbed photons}} \quad (5)$$

Singlet oxygen is the most important tumour destroying moiety in PDT thus high  $\Phi_{\Delta}$  is an inevitable prerequisite for a good PDT photosensitizer. Since many porphyrins dissolved in solvents exhibit very high  $\Phi_{\Delta}$  (often exceeding 0.5 [16,17]), they are in the focus of PDT research.

Figure 4 offers a nice summarization of EET and other processes described in the previous chapters.



**Figure 4.** Simplified Jablonski diagram of photosensitizer and  $\text{O}_2$  molecules depicting processes of excitation energy transfer and both radiative and non-radiative deactivation of  $^3\text{PS}$  and  $^1\text{O}_2$ .  $P_o$  – phosphorescence of oxygen.

### 2.3.1 Kinetics of phosphorescence of photosensitizer and singlet oxygen

Shortly after excitation pulse, when fluorescence becomes negligible, photosensitizer triplets are not generated by ISC anymore and their concentration [ $^3\text{PS}$ ] decays according to the following equation:

$$\frac{d[{}^3\text{PS}]}{dt} = - (k_{\text{PP}} + k_{\text{NP}} + k_{\text{TO}} [{}^3\text{O}_2]) [{}^3\text{PS}] \quad (6)$$

where  $k_{\text{PP}}$  and  $k_{\text{NP}}$  stand for the rate constant of radiative and non-radiative deactivation of photosensitizer respectively. Any decrease of  $^3\text{PS}$  population due to the repopulation of singlet state is assumed to be negligible in Equation 6. Since the concentration of oxygen in the samples containing  $\text{O}_2$  is usually substantially bigger than the concentration of PS, any decrease of [ $^3\text{O}_2$ ] due to the generation of  $^1\text{O}_2$  can be neglected. Therefore, [ $^3\text{O}_2$ ] in Equation 6 can be considered as a constant parameter and thus the intensity of photosensitizer phosphorescence  $I_{\text{PS}}(t)$  is proportional to the solution of Equation 6:

$$I_{\text{PS}} = I_{\text{PS}} e^{-t/t_1} \quad (7)$$

where

$$t_1 = 1 / (k_{\text{PP}} + k_{\text{NP}} + k_{\text{TO}} [{}^3\text{O}_2]) \quad (8)$$

represents the lifetime of triplet states of photosensitizer.

On the contrary, time evolution of singlet oxygen population is more complicated. In the absence of quenchers, singlet oxygen is generated by EET and deactivated non-radiatively and by phosphorescence:

$$\frac{d[{}^1\text{O}_2]}{dt} = + k_{\text{TO}} [{}^3\text{O}_2] [{}^3\text{PS}] - (k_{\text{PO}} + k_{\text{NO}}) [{}^1\text{O}_2] \quad (9)$$

Assuming [ $^3\text{O}_2$ ] as a constant parameter, system of Equations 6 and 9 provides time evolution of  $^1\text{O}_2$  phosphorescence:

$$I_{\text{SO}} = \frac{I_0}{1/t_1 - 1/t_{\text{SO}}} (e^{-t/t_{\text{SO}}} - e^{-t/t_1}) \quad (10)$$

where  $I_0$  is a constant. This formula describes typical kinetics of photogenerated singlet oxygen. It reflects the initial rise of the population (and emission) of  $^1\text{O}_2$  due to the EET. After reaching its maximum,  $^1\text{O}_2$  deactivation processes prevail and cause the decay of  $^1\text{O}_2$  population (and emission). Such kinetics have been observed in the solutions of meso-tetra(4-sulfonatophenyl)porphyrin (TPPS<sub>4</sub>) [18], tetrasulphonated aluminium phthalocyanine [13], and PDT photosensitizer Photofrin [19].

Equations 7 and 10 were used to analyse kinetics of phosphorescence measured during our experiments. This analysis enabled us to find the lifetimes and subsequently the quenching processes occurring in our samples.

## **2.4 Current knowledge of the interaction between photosensitizer, oxygen, and albumin**

The last part of this work concentrates on the study of the interaction of TPPS<sub>4</sub> as a photosensitizer and photogenerated singlet oxygen with human serum albumin (HSA). As HSA is the most abundant serum protein and photosensitizers are mostly applied intravenously, this study is an important step towards simulating *in vivo* conditions. HSA consists of 585 amino acid residues and its molecular weight is 66 kDa. It exhibits high affinity towards wide diversity of ligands that can be reversibly bound and thus distributed around human body [20,21]. Transient absorption measurements appeared to be a powerful tool to study the interaction of photosensitizers with HSA [22,23]. Since albumin induces considerable shifts of absorption and fluorescence bands of bound photosensitizer molecules, absorption and fluorescence are also a good way to study binding of photosensitizers to albumins [24-26]. Furthermore, surface-enhanced Raman spectroscopy was used to find the character of photosensitizer–albumin bond as well as the binding site of photosensitizer on the surface of the protein [27]. All these measurements revealed a dynamic equilibrium between photosensitizer bound to HSA and free one. According to Bartošová [25], HSA possesses one major binding site for TPPS<sub>4</sub> with a binding constant of  $3 \times 10^6 \text{ M}^{-1}$  and two to three sites of substantially lower affinity. The literature pays much smaller attention to the photogeneration of singlet oxygen and its interaction with albumin. Only the reaction constant of  $(5 \pm 3) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$  for oxidation of HSA by <sup>1</sup>O<sub>2</sub> was determined by Davila [28]. Since hardly anything is known about the production of <sup>1</sup>O<sub>2</sub> by TPPS<sub>4</sub> in the presence of HSA, we concentrated on this phenomenon. The direct measurements of time resolved phosphorescence of photosensitizer and singlet oxygen represent an excellent tool to do such observations. Furthermore, chemical quenching of <sup>1</sup>O<sub>2</sub> by HSA was studied.

## 2.5 Photodynamic therapy

Photodynamic therapy is a promising method of fighting cancer, macular degeneration and cutaneous lesions. It was first used on humans in 1978 [9]. The treatment of a patient begins with intravenous or topical administration of photosensitizer. Afterwards, the patient must stay in dim light conditions since the photosensitizer spreads through his whole body. Approximately 24 to 72 hours later, photosensitizer is retained predominantly in the cancerous tissue. The tumour is then locally irradiated by a lamp or laser. The usage of fibre optics and endoscopy is necessary to reach directly inaccessible parts of human body. The light is absorbed by the photosensitizer molecules which leads to singlet oxygen photogeneration via above described processes. Highly reactive photogenerated singlet oxygen then strikes vital functions of cancerous cells and destroys the tumour. Further details concerning general aspects of PDT can be found in the recent review by Brown [29].

To be more precise, there are two ways of tumour destruction by photosensitizer. These are called Type I and Type II reactions [30,31] and they act simultaneously within cells. The above mentioned action of singlet oxygen is denoted as Type II. Type I reactions are based on the direct reaction of photosensitizer triplet with a reducing substrate by the transfer of a hydrogen atom or electron to photosensitizer. Sensitizer radicals ( $PS^{\bullet-}$  and  $PS-H^{\bullet}$ ) may then react with  $O_2$  to produce reactive oxygen species (superoxide ion  $O_2^{\bullet-}$  and hydroxy radical  $HO^{\bullet}$ ) [31]. Superoxide ion and hydroxy radicals are toxic to living cells. Adding singlet oxygen quenchers to cells proved that Type II is more important for cell killing [10].

Brown and Brown mentions following photosensitizers approved for clinical use in PDT by 2004 [29]: **Porfimer sodium** (commercial name **Photofrin**) is the first and most widely used photosensitizer based on a mixture of hematoporphyrin derivatives, used for lung cancers, superficial gastric cancer, cervical cancer, and bladder cancer and tested for many other kinds of cancer. **Temoporfin** is a chlorin-based photosensitizer used for palliative treatment of head and neck cancer. **Mixture of aluminium sulphonated phthalocyanines** is approved as PDT photosensitizer only in Russia. **Verteporfin** (also called **Visudyne**) is chlorin-based photosensitizer widely used in ophthalmology for the treatment of the age related macular degeneration. **Protoporphyrin IX** is a metabolic precursor of haem and is used for the treatment of

cutaneous lesions. It is synthesized by cells after topical administration of 5-aminolevulinic acid (commercial name **Levulan**).

### 2.5.1 An ideal photosensitizer

Further improvement of PDT method has very well defined objectives. It is focused on the development of so-called ideal photosensitizer [9] and better delivery and quantification of applied light [29]. Some of the main properties of ideal photosensitizer are those which the first approved photosensitizer – Photofrin (Hematoporphyrin derivative) lacks [9,32]:

- 1) It should be chemically pure.
- 2) It should exhibit a high quantum yield of singlet oxygen production.

Porphyryns and porphyrin-based molecules generally satisfy this criterion well and thus they are in the focus of PDT research.

- 3) It should have a significant absorption at the long wavelength region (700–800 nm).

All currently approved photosensitizers have the redmost absorption band bellow 700 nm where the absorption of tissues limits PDT to the treatment of superficial tumours. The research aims at photosensitizers with absorption between 700 and 800 nm where lowest absorption of tissue occurs. The potential candidates are particularly chlorin and bacteriochlorin derivatives, expanded porphyrins, phthalocyanines, and naphthalocyanines [9].

- 4) Highly preferential tumour localization is required.

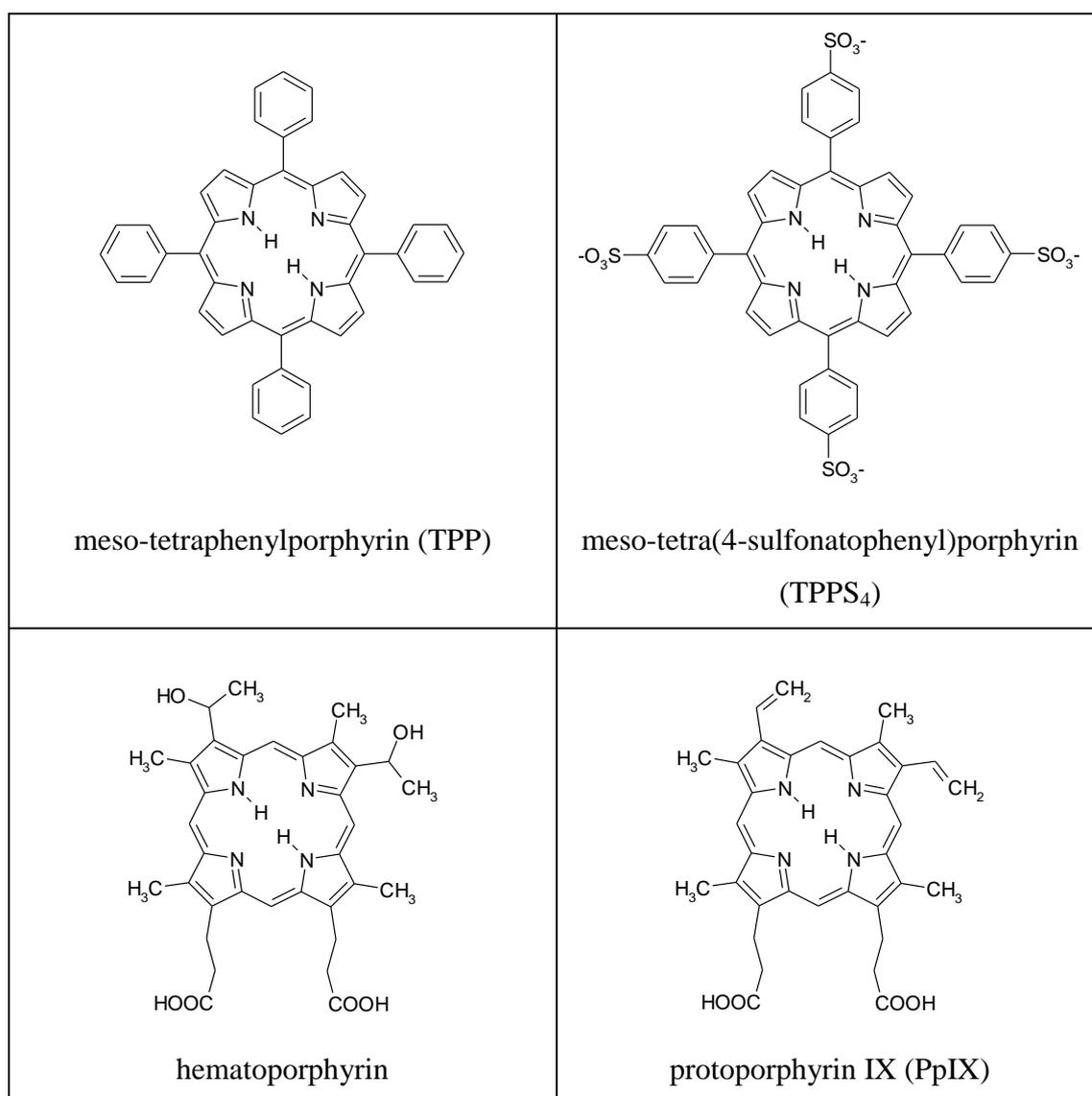
For currently used photosensitizers, the concentration in the tumour is 2 to 6 times higher compared to normal tissue.

- 5) Minimal dark toxicity and delayed phototoxicity is also desirable.
- 6) It ought to be stable and easy to dissolve in the injectable solvents.

Extensive research is also carried out in the use of PDT as a trigger of cell apoptosis [10]. Apoptosis is a well regulated process of cell death which the cell undergoes in a response to intensive stress which does not kill the cell immediately. Some photosensitizers (e.g. hypericin) are able to start cell apoptosis after the irradiation and kill the tumour cells by apoptotic way. Apoptotic cell death is not accompanied by strong inflammatory response as in the case of cell necrosis which follows the irradiation of tumour with currently used photosensitizers.

### 3 Materials

This work presents the investigation of photophysical properties of four photosensitizers (Figure 5): meso-tetraphenylporphyrin (TPP), protoporphyrin IX (PpIX), hematoporphyrin derivatives (HpD) and meso-tetra(4-sulfonatophenyl)porphyrin (TPPS<sub>4</sub>). All photosensitizers were purchased from Frontier Scientific Porphyrin products, Logan, USA and were used without any further purification.



**Figure 5.** Structures of the studied photosensitizers.

The measurements were carried out in solutions whose concentrations ranged from 1  $\mu\text{M}$  to 200  $\mu\text{M}$ . Acetone and DMSO were used as a solvent for hydrophobic

photosensitizers. Phosphate buffers of pH 7.4; 6.5; and 5.5, corresponding to pH of blood, cancer tissue, and skin respectively, were used to dissolve hydrophilic TPPS<sub>4</sub>.

Concentration of oxygen was changed in certain experiments by up to 1 hour purging the samples either by oxygen to reach oxygen saturated value of 1400  $\mu\text{M}$  in buffer [33] and 2200  $\mu\text{M}$  in DMSO [34] or by nitrogen to remove oxygen from the samples completely. The air-saturated concentration of oxygen is 280  $\mu\text{M}$  in buffer [33], 2400  $\mu\text{M}$  in acetone [35] and 460  $\mu\text{M}$  in DMSO [34].

Human serum albumin was purchased from Sigma and dissolved in phosphate buffer of pH 7.4. Its concentrations ranged from 1 to 50  $\mu\text{M}$  while TPPS<sub>4</sub> concentration was 10  $\mu\text{M}$  for all albumin experiments.

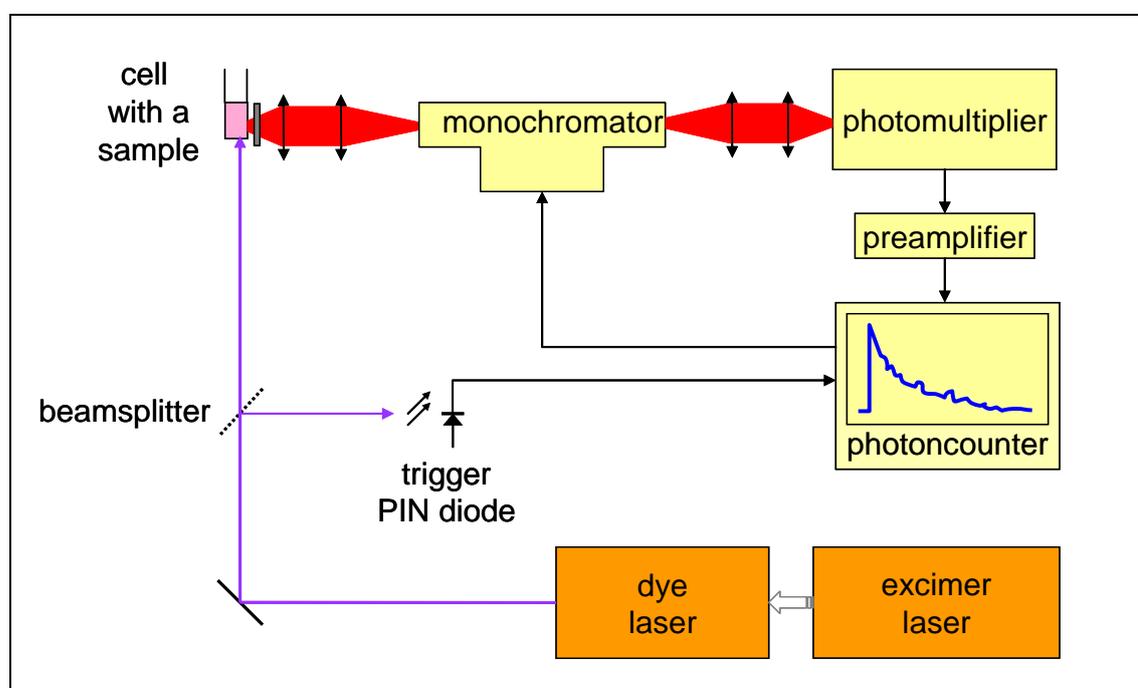
All experiments were carried out at 20°C.

## 4 Methods

Absorption spectroscopy (Perkin Elmer Lambda 12, Avantes Avaspec-1024) and fluorescence spectroscopy (Avantes S2000) were used mainly for spectral characterization and checking the quality of the samples. Besides these routinely used techniques, three unique experimental set-ups were used for time resolved measurements:

### 4.1 Experimental set-up for phosphorescence measurements

Experimental set-up for time and spectral resolved phosphorescence measurements of photosensitizers and singlet oxygen is displayed in Figure 6.



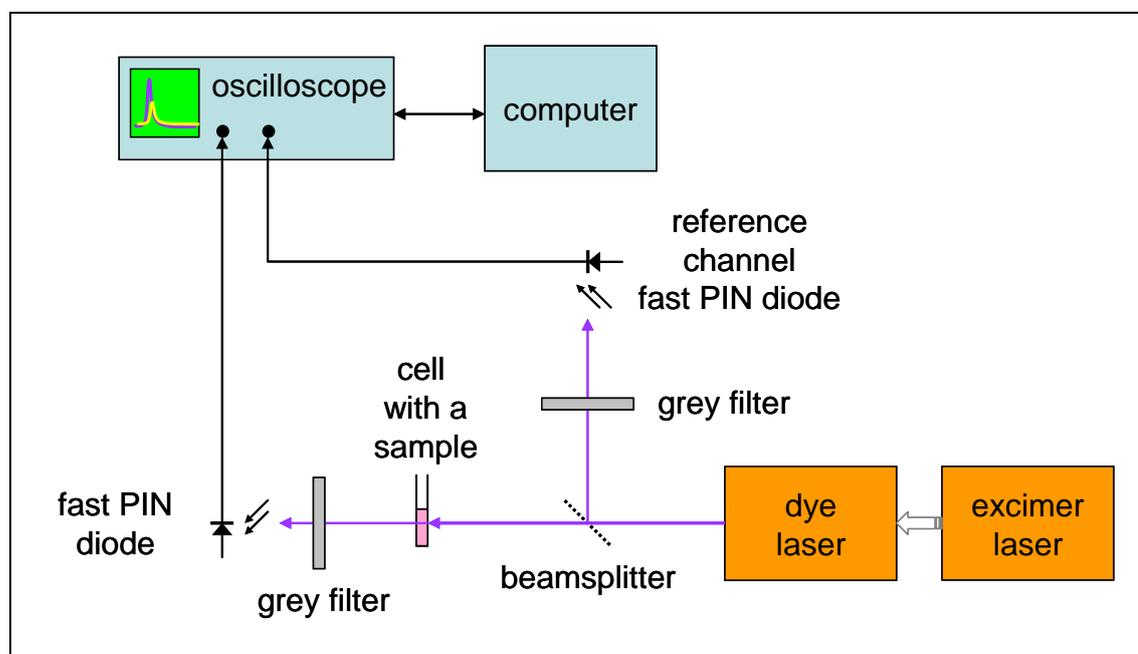
**Figure 6.** Experimental set-up for phosphorescence measurements

The samples were excited by excimer laser (Lambda Physics LPX 105ICC) pumped dye laser (Lambda Physics FL 3001). Stilbene 3 dye in methanol provided excitation pulses at the wavelength of 420 nm, DCM dye in DMSO provided 645 nm excitation. Dye laser beam was focused to the sample through optically polished bottom of a spectroscopic cell. Excitation energy was kept typically around 20  $\mu\text{J}$  per pulse. It ranged from 30 to 0.05  $\mu\text{J}$  per pulse in excitation intensity dependence measurements. Duration of the laser pulses was 20 ns with repetition frequency 40 Hz. IR phosphorescence was spectrally resolved by monochromator Jobin Yvon H20 IR

together with two long-pass filters Schott RG 7. Spectral width of monochromator slit was 16 nm. High IR sensitivity and the time resolution of 5 ns was achieved by cooled IR sensitive Hamamatsu R5509 photomultiplier together with Becker-Hickl HF AC-26 dB preamplifier and Becker-Hickl MSA 200 photoncounter. The photon counter was triggered by the signal from a fast PIN photodiode, on which part of excitation light was reflected by a beamsplitter. Time and spectral resolved emission of the samples was typically measured between 750 and 1342 nm with 16 nm step to observe photosensitizer and singlet oxygen phosphorescence together. The detail of singlet oxygen phosphorescence was measured in the region from 1242 to 1306 nm with 4 nm step. Phosphorescence data were corrected with respect to spectral sensitivity.

## 4.2 Absorption measurements with a subnanosecond time resolution

High luminous flux of excitation light combined with high  $\Phi_T$  and relatively long lifetime of photosensitizer triplet states cause temporal photo bleaching of the samples due to the decrease of concentration of photosensitizer in the ground state. It leads to substantial deviations from Lambert-Beer law. Therefore, the energy absorbed by the samples has to be measured directly. This was done in a two-channel way using fast PIN photodiodes together with 500 MHz oscilloscope (see Figure 7).

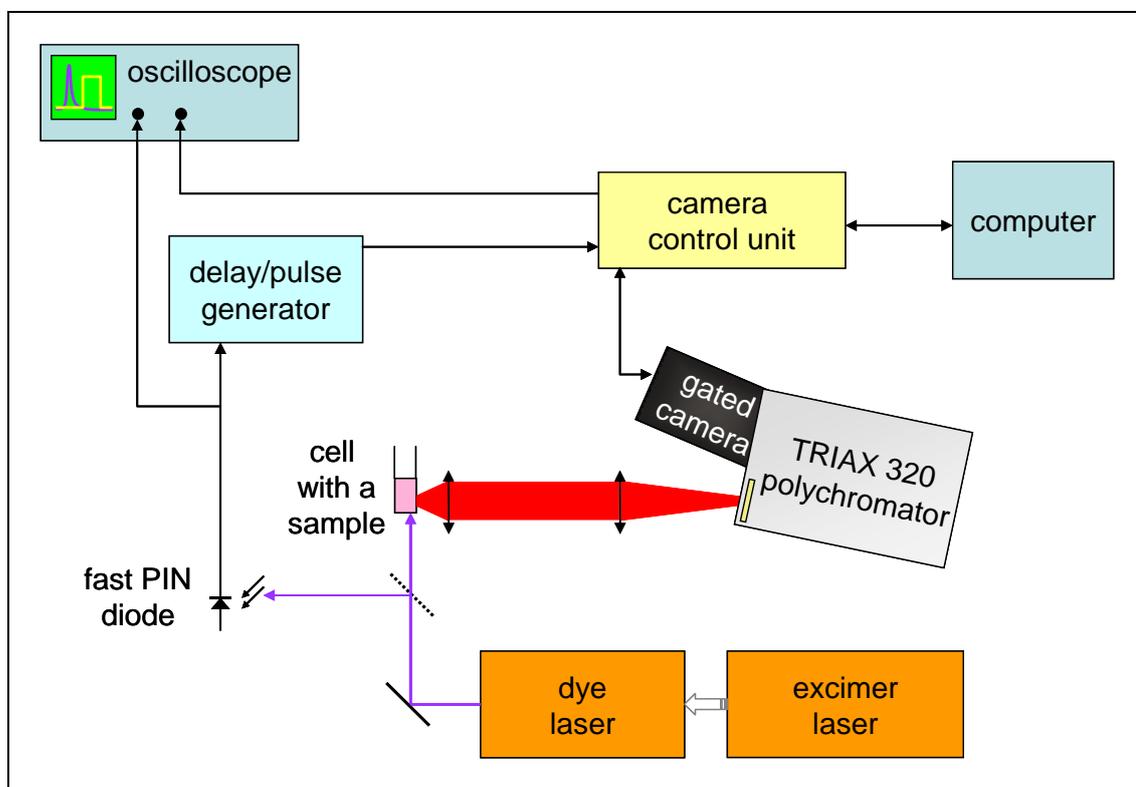


**Figure 7.** Experimental set-up for absorption measurements with a subnanosecond time resolution

The energy absorbed by the samples was obtained from the ratio of signals in the probing and reference channels integrated over the duration of excitation pulse.

### 4.3 Experimental set-up for delayed fluorescence measurements

Time resolved delayed fluorescence was measured using a polychromator and a gated intensified CCD camera. The set-up for these measurements is shown in Figure 8. Measurements were carried out with the same excitation laser as in phosphorescence measurements. The wavelength of 420 nm was used for excitation with 15 Hz repetition rate. Sample emission was collected perpendicularly to the excitation on the entrance slit of Jobin-Yvon Triax 320 polychromator. OG4 cut-off filter was employed to absorb scattered laser light. Gated intensified CCD camera (Roper Scientific PI-MAX 512RB) attached to the polychromator was used to detect delayed fluorescence spectra with up to 40 ns time resolution. To trigger the measurements, part of the excitation pulses was reflected by a beamsplitter to a fast PIN diode. Time resolution was achieved by built in programmable delay generator of a camera control unit. PIN diode signal and camera control unit output signal were monitored by oscilloscope to obtain the exact value of the delay between excitation and gate opening.



**Figure 8.** Experimental set-up for delayed fluorescence measurements

## 5 Summary of results and their discussion

### 5.1 General spectroscopic features of studied photosensitizers and singlet oxygen

All photosensitizers studied in this work exhibit typical spectral properties of porphyrins. The examples of spectral behaviour of our samples are shown in the figures of Enclosures 1 and 3. The absorption is dominated by a very intensive Soret band around 410 nm accompanied by much weaker Q bands covering spectral region from 500 to 650 nm. Fluorescence exhibits two bands at around 650 nm and 700 nm with Stokes shift of several nanometres. Broad phosphorescence band of photosensitizers has maximum between 800 and 1100 nm depending on the photosensitizer and the solvent. Relatively narrow phosphorescence of singlet oxygen appears at 1275 nm which is slightly bigger value than 1269 nm measured for gaseous oxygen [5].

### 5.2 Excitation energy transfer from photosensitizers to oxygen in solvents

Photophysical properties of photosensitizers and singlet oxygen were thoroughly explored for various concentrations of TPP, HpD and PpIX in acetone (Enclosures 1 and 3). Acetone was used as a standard spectroscopic solvent with well defined properties. To advance towards *in vivo* conditions, TPPS<sub>4</sub> was studied in buffers of pH of human blood, cancer tissue and skin (Enclosure 2). This basic set of experiments was carried out to find the lifetimes of triplet photosensitizer and singlet oxygen.

Oxygen proved to be a strong quencher of photosensitizer triplet states resulting in submicrosecond lifetimes of phosphorescence of acetone dissolved photosensitizers. In the case of TPPS<sub>4</sub>, which was dissolved in air saturated buffer, the lifetime of <sup>3</sup>TPPS<sub>4</sub> is longer ( $1.8 \pm 0.2$ )  $\mu\text{s}$  due to substantially lower concentration of oxygen in buffer compared to acetone. The increase of oxygen concentration in buffer to oxygen saturated value causes shortening of <sup>3</sup>TPPS<sub>4</sub> lifetime to ( $0.37 \pm 0.07$ )  $\mu\text{s}$  while removing oxygen from the samples by purging them by nitrogen results in <sup>3</sup>TPPS<sub>4</sub> lifetime exceeding 100  $\mu\text{s}$ .

Singlet oxygen kinetics in all above mentioned samples follow Equation 10. The lifetimes of singlet oxygen extrapolated to zero concentration of photosensitizer (53  $\mu\text{s}$

in air saturated acetone, 3.7  $\mu\text{s}$  in air saturated buffer) correspond to those shown in the literature [12].

The lifetime of singlet oxygen in acetone decreases with increasing TPP and HpD concentrations in accordance with observations by Krasnovsky [36]. This fact was explained as the result of quenching of  $^1\text{O}_2$  by photosensitizer, probably via the formation of charge transfer complex containing TPP and singlet oxygen [36]. However, we have proven in Enclosure 6 that quenching of singlet oxygen by singlet oxygen may also contribute to the decrease of singlet oxygen lifetime (see chapter 5.4).

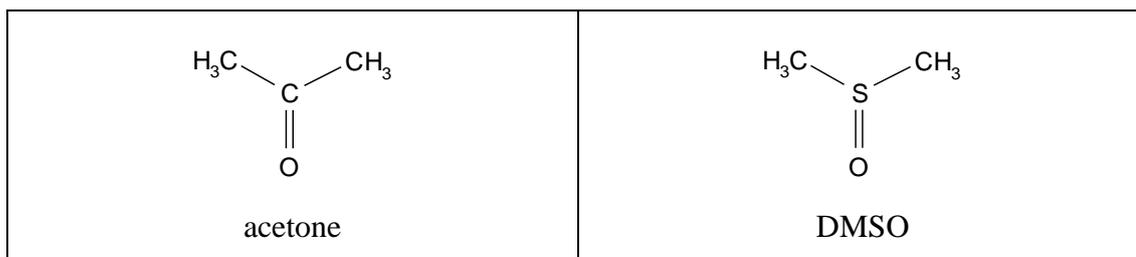
The rise-times of singlet oxygen kinetics in TPP and HpD samples differ slightly from the lifetimes of  $^3\text{TPP}$  and  $^3\text{HpD}$ . Nevertheless, both lifetimes remain constant within our experimental accuracy in the studied concentration range (1 to 200  $\mu\text{M}$ ).

Absorption measurements of TPPS<sub>4</sub> in buffer and PpIX in acetone at various concentrations revealed the presence of aggregates at concentrations exceeding 30  $\mu\text{M}$ . This fact is reflected in the concentration induced changes of the lifetimes of  $^3\text{TPPS}_4$  and  $^3\text{PpIX}$  which are shown in the Enclosures 2 and 3. On the contrary, the rise-time of singlet oxygen kinetics remains constant. The explanation of this fact is based on different behaviour of monomers and aggregates. In TPPS<sub>4</sub> solutions for instance, triplet molecules in aggregates do not transfer excitation energy to oxygen and thus their triplet state lifetime is longer. With increasing TPPS<sub>4</sub> concentration the aggregate/monomer ratio increases too and so does the effective  $^3\text{TPPS}_4$  lifetime. Since singlet oxygen is generated only by monomers, its rise-time is independent of the total TPPS<sub>4</sub> concentration.

Changing the pH value from 5.5 to 7.4 did not induce any changes of lifetimes, rate constants, or aggregation in TPPS<sub>4</sub> solutions.

### **5.3 Repopulation of the first excited singlet state and delayed fluorescence of TPP in DMSO and in acetone**

DMSO is a solvent clinically used as a penetration enhancer to carry drugs into tissues [37]. Its chemical structure is very similar to acetone (see Figure 9). However, Enclosures 4 and 5 show dramatic differences in photosensitized generation of singlet oxygen in these solvents. TPP was chosen as a photosensitizer for these measurements.



**Figure 9.** Chemical structures of acetone and DMSO differ in one atom.

On the contrary to the short lifetimes of phosphorescence of photosensitizers in acetone and in buffer, relatively long TPP phosphorescence was obtained when dimethylsulfoxide was used as a solvent (Enclosure 4). Moreover, phosphorescence kinetics do not exhibit monoexponential decays in DMSO. This fact implies that another phenomenon, which is not included in the Equation 6, must be taken into consideration. It is triplet–triplet quenching:



This process occurs with the rate constant  $k_{\text{TT}}$ . It represents another way of  ${}^3\text{TPP}$  deactivation, bringing non-linearity into the Equation 6 and resulting in non-exponential decay of phosphorescence.

$$\frac{d[{}^3\text{PS}(\text{I})]}{dt} = -\left(k_{\text{PP}} + k_{\text{NP}} + k_{\text{TO}} \cdot [{}^3\text{O}_2] + k_{\text{TT}} \cdot [{}^3\text{PS}(\text{I})]\right) [{}^3\text{PS}(\text{I})] \quad (12)$$

Triplet–triplet quenching is inevitably accompanied by delayed fluorescence which was measured and analysed in the Enclosure 5. Both TPP in acetone and in DMSO exhibit delayed fluorescence however that of acetone is substantially weaker.

A question arises why TPP in acetone and DMSO behave in such a different ways. The explanation is based on the six times higher viscosity of DMSO compared to that of acetone [38] as well as on the five times lower concentration of oxygen in DMSO compared to acetone [34,35]. Phosphorescence kinetics in acetone are therefore dominated by rapid excitation energy transfer to fast diffusing oxygen causing other  ${}^3\text{TPP}$  deactivation processes almost negligible. In DMSO, however, slower diffusion and lower concentration of oxygen result in relatively bigger role of triplet–triplet quenching accompanied by much smaller quantum yield of singlet oxygen. Detailed discussion of  ${}^3\text{TPP}$  decays in DMSO including their mathematical description can be found in the Enclosure 4.

The kinetics of  ${}^3\text{TPP}$  in acetone do not show deviations from monoexponential decay. Weak delayed fluorescence in acetone also exhibits monoexponential decay with

the lifetime very similar to the lifetime of phosphorescence. It happens because the repopulation of excited singlet states in acetone is also caused by singlet oxygen reaction with triplet TPP:



This process was described by Levin [39] and results in so called singlet oxygen-mediated delayed fluorescence. Thus besides the intensive excitation energy transfer in acetone, we observe two weak ways of  ${}^3\text{TPP}$  deactivation both giving rise to delayed fluorescence.

The rate of triplet–triplet deactivation decreases as the concentration of  ${}^3\text{TPP}$  decreases. Contrary to that, the rate of singlet oxygen mediated deactivation increases with increasing concentration of singlet oxygen. Considering both these processes, the overall rate of repopulation of excited singlet state of TPP remains constant and therefore delayed fluorescence of TPP in acetone exhibits monoexponential decay with the same lifetime as phosphorescence. Detailed mathematical analysis of triplet–triplet and singlet oxygen mediated deactivation of  ${}^3\text{TPP}$  in acetone can be found in Enclosure 5. Since the quantum yield of singlet oxygen in DMSO is much smaller compared to acetone, the contribution of singlet oxygen mediated delayed fluorescence is negligible in this solvent.

Compared to acetone, singlet oxygen phosphorescence follows more complicated rise and decay kinetics in DMSO. It is because Equation 10 was derived assuming monoexponential decay of photosensitizer triplets which is not the case of TPP in DMSO. In spite of this, singlet oxygen lifetimes in DMSO were calculated (Enclosure 4):  $(1.8 \pm 0.5) \mu\text{s}$  under air-saturated conditions and  $(1.2 \pm 0.1) \mu\text{s}$  under oxygen-saturated conditions. The difference of these lifetimes is explained in the next chapter.

## 5.4 Singlet oxygen quenching by oxygen

Most authors who published their measurements of the lifetimes of singlet oxygen in various solvents (reviewed by Wilkinson [12]) determined precise values but did not discuss following facts: The lifetime of singlet oxygen is shorter when the concentration of oxygen in the solvent is increased by purging the samples by oxygen. This fact was observed both in buffer (Enclosure 2) and in DMSO (Enclosure 4). No

such experiment was carried out in acetone since any purging of acetone dissolved samples causes fast evaporation of the solvent. This observation led us to hypothesis that singlet oxygen is quenched by oxygen in our samples. To prove our hypothesis, a series of experiments was carried out and published in the Enclosure 6.

Kinetics of singlet oxygen photogenerated by TPPS<sub>4</sub> in buffer (pH 7.4) were measured at various concentrations of oxygen between zero and oxygen saturated value (Enclosure 6). The lifetime of singlet oxygen increases with decreasing concentration of oxygen which is a demonstration of quenching of singlet oxygen by oxygen. The lifetimes of <sup>1</sup>O<sub>2</sub> in buffer, which were published before, are between 3.1 and 4.2 μs [12] but these were typically measured at relatively high concentrations of oxygen (air or oxygen saturated buffers). Our measurements show that the lifetime of singlet oxygen in water extrapolated to zero oxygen concentration can be as high as (6.5 ± 0.4) μs.

We can not decide from the above mentioned experiment whether singlet oxygen is quenched by <sup>1</sup>O<sub>2</sub> or <sup>3</sup>O<sub>2</sub>. Therefore, the emission of singlet oxygen photogenerated by TPP in acetone was measured at various energies of 645 nm excitation pulses. The increase of singlet oxygen depopulation rate constant dependence on absorbed energy proves that singlet oxygen is quenched predominantly by singlet oxygen:



This process can be accompanied by the emission of one 635 nm photon and it is called dimol emission in this case [40]. This emission is overlaid by delayed fluorescence in our samples and we did not observe it.

## **5.5 The interaction of TPPS<sub>4</sub> and singlet oxygen with human serum albumin**

Deep understanding of mechanisms of interaction between photosensitizers, proteins, and oxygen is crucial for further progress in the PDT. That is why in the Enclosure 7 we used our previously acquired knowledge of photosensitizers dissolved in the solvents to investigate samples containing TPPS<sub>4</sub> and human serum albumin in buffer (pH 7.4 – the same as pH of human blood).

In accordance with other authors [25,26,41], two major groups of TPPS<sub>4</sub> molecules were distinguished in the presence of HSA. TPPS<sub>4</sub> phosphorescence of air

saturated samples follows biexponential decay. The lifetime of one component is equal to the lifetime of TPPS<sub>4</sub> in buffer therefore it can be attributed to free TPPS<sub>4</sub>. The other one, exhibiting the lifetime of tens of microseconds, originates from TPPS<sub>4</sub> bound to HSA. Relatively long lifetime of <sup>3</sup>TPPS<sub>4</sub> is due to the shielding of bound TPPS<sub>4</sub> by HSA against quenching by oxygen.

Time evolution of singlet oxygen emission consists of linear combination of two Equations 10. Their lifetimes  $t_1$  correspond to the lifetimes of free and bound TPPS<sub>4</sub> phosphorescence. It implies that singlet oxygen is photogenerated by free as well as by bound TPPS<sub>4</sub> and the above mentioned shielding is only partial. The lifetime of singlet oxygen is the same for both ways of its origin due to the relatively high diffusion constant. Singlet oxygen is able to cross distances exceeding the size of HSA as well as the space between HSA molecules in our samples.

Further interesting pieces of knowledge were gained when the samples were purged by nitrogen. Only one group of <sup>3</sup>TPPS<sub>4</sub> was detected. Its lifetime is in hundreds of microseconds and rises with increasing HSA concentration. This phenomenon is explained by HSA preventing depopulation of the bound triplet TPPS<sub>4</sub> molecules by water, analogically to what has been published on phthalocyanine–HSA solutions [22]. The exchange between free and bound forms is substantially faster than their respective triplet lifetimes and therefore only single lifetime of <sup>3</sup>TPPS<sub>4</sub> increasing with HSA concentration is observed.

All the details of our TPPS<sub>4</sub>–HSA–oxygen interaction study are summarized in Enclosure 7.

## 6 Conclusions

Optical emission spectroscopy was used to study photophysical properties of meso-tetraphenylporphyrin, protoporphyrin IX, hematoporphyrin derivatives, and meso-tetra(4-sulfonatophenyl)porphyrin in various environments. Firstly, acetone was used as a solvent to verify basic model of fast excitation energy transfer from photosensitizer to oxygen. Oxygen proved to be a very strong quencher of photosensitizer triplet states.

Further experiments were aimed at the observation of excitation energy transfer in conditions simulating biological ones. Only monomers of water soluble meso-tetra(4-sulfonatophenyl)porphyrin photosensitizer transfer energy to oxygen in buffers (pH of skin, tumour tissue, blood).

A comparison of photosensitizing properties of meso-tetraphenylporphyrin in acetone and in clinically used solvent dimethylsulfoxide revealed two processes contributing to the deactivation of photosensitizer triplets: triplet-triplet quenching and quenching of triplets by singlet oxygen. Both these processes result in the repopulation of the first excited singlet state of photosensitizer leading to delayed fluorescence. Triplet-triplet quenching dominates the depopulation of photosensitizer triplets in dimethylsulfoxide. In acetone, however, triplet-triplet quenching and quenching of triplets by singlet oxygen are only minor processes compared to excitation energy transfer to oxygen.

Concerning singlet oxygen, its rise and decay kinetics were measured and analysed revealing the lifetimes of singlet oxygen in all above mentioned solvents. Quenching of singlet oxygen by singlet oxygen as well as quenching of singlet oxygen by molecules of photosensitizers were observed.

The study of photosensitizer and singlet oxygen interaction with human serum albumin confirmed the presence of two groups of photosensitizer molecules: free ones and those bound to albumin. Although bound photosensitizer is partially shielded by albumin against quenching by oxygen, both these groups generate singlet oxygen. The exchange between free and bound form of the photosensitizer occurs with time constant substantially smaller than hundreds of microseconds.

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- 6) R. Dědic, **M. Kořínek**, A. Molnár, A. Svoboda, J. Hála, Singlet oxygen quenching by oxygen in tetraphenyl-porphyrin solutions, *Journal of Luminescence*, accepted for publication
- 7) **M. Kořínek**, R. Dědic, A. Molnár, and J. Hála, The influence of human serum albumin on the photogeneration of singlet oxygen by meso-tetra(4-sulphonatophenyl)porphyrin. Infrared emission study., *Journal of Fluorescence*, accepted for publication