In Situ Luminescence Detection of Singlet Oxygen

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Abstract. Photodynamic therapy is a method for treatment of cancer and various chronic diseases. Singlet oxygen generated by triplet states of photosensitizers is well known for its high reactivity and plays a key role in the elimination of the affected tissue. A unique set-up for detection of weak infra-red luminescence of both the photosensitizers and the singlet oxygen with parallel time and spectral resolution was built at MFF UK. It is a suitable tool for examination of excitation energy transfer processes between photosensitizers and oxygen, as well as interactions of these molecules with their environment. Investigation of these processes is crucial for understanding the mechanisms of photodynamic therapy. The former arrangement of the set-up designed for detection of emission from solutions in spectroscopic cells was adapted to collect signal from solid samples using a bifurcated optical fibre with luminescence probe to be able to measure luminescence from tissues in vivo. In a first approach we successfully measured the phosphorescence from polymer films with defined concentrations of protoporphyrin IX. Moreover, we were able to measure the phosphorescence from thin layers of living cells treated by different photosensitizers.

Introduction

Photodynamic therapy (PDT) is a very promising method for treatment of various oncological and chronic diseases (psoriasis, age-related degeneration of macula, arthritis). The PDT is based on administration of a drug called photosensitizer (PS) into a patient body either intravenously or locally using an ointment. After some time (depending on the type of the PS and others factors), PS becomes specifically accumulated in the affected tissue due to morphological and metabolic differences in healthy and affected tissues. The PS itself is inefficacious and it must be activated using a light to excite it from its ground singlet state to an excited singlet state, which subsequently converts to a triplet state by inter-system crossing. Both the preferential accumulation of the PS in the target tissue and the local illumination make PDT very efficient in the confinement of the therapeutic action. The PS in excited triplet state has already influence on specific elimination of affected tissue, but moreover it's able to generate singlet oxygen \(1O_2\) by triplet-triplet energy transfer to oxygen in the triplet ground state. Singlet oxygen is well known for its high reactivity and plays a key role in the elimination of the affected tissues in PDT [1].

A unique spectroscopic set-up for detection of very weak infra-red luminescence of both the PS and the singlet oxygen with parallel time and spectral resolution was built at Faculty of Mathematics and Physics of the Charles University in Prague. It is a suitable tool for the examination of processes of excitation energy transfer between PS and oxygen, as well as interactions of these molecules with their environment. Investigation of these processes in living tissues is crucial for understanding the mechanisms of photodynamic therapy. The former arrangement of the set-up designed for efficient detection of luminescence from solutions in spectroscopic cells (in vitro) was recently adapted to be able to measure luminescence from solid samples. The new set-up was tested on polymer films containing PS and on living cells treated by PS.

Experimental

Scheme of the setup is presented in Figure 1. The excitation 6 ns laser pulses at 420 nm and energy 15 \(\mu\)J are provided by dye laser FL1000 (Lambda Physik) pumped by excimer laser ATLEX-500i (ATL Lasertechnik). The excimer laser is able to pulse at 500 Hz repetition rate, but because of thermal instability of the laser head, only 200 Hz repetition rate was used to ensure constant energy of laser pulses. Laser beam is then split in two beams. One is used to trigger the photon counter by fast
PIN photodiode and the second one is focused into the sole fibre of the excitation leg of the bifurcated lightguide Avantes FCR-7IR400-2-ME. The lightguide is ended by a luminescence probe where the excitation and detection fibres are mixed together. Signal from the six fibres of the lightguide used for collection of a luminescence signal are then displayed through a longpass filters that block scattered excitation light (TECHSPEC 450 nm) and fast fluorescence of the photosensitizer (2× Schott RG7) on high luminosity monochromator (H20IR Jobin-Yvon). The output of the monochromator is then projected on a photocathode of IR-sensitive photomultiplier (Hamamatsu R5509) cooled to -80 °C by liquid nitrogen. The photomultiplier is working in single photon counting mode and amplified pulses are detected by a counter (Becker-Hickl MSA 200) providing time resolution of 5 ns per channel.

Materials and methods

The polymer films were prepared from polymethyl-methacrylate (PMMA, Mw = 120.000, Aldrich). The polymer pellets were dissolved together with protoporphyrin IX (PpIX, ≥ 95%, Sigma) in tetrahydrofuran (≥ 99.8% for UV spectroscopy, Fluka). The solution was placed on a Petri dish and let the tetrahydrofuran evaporate. Polymer films with a uniform thickness of (0.45 ± 0.05) mm containing PpIX in various concentrations were obtained this way.

The 3T3 murine fibroblast cells were cultivated on 75 cm² culture flask in Dulbecco’s Modified Eagle Medium (Invitrogen) supplemented by 10 % of fetal bovine serum. Cells were incubated in humidified atmosphere with 5% of CO₂ at 37 °C until confluence. Then either the 5,10,15,20-tetrakis(4-sulfonatophenyl)porphine (TPPS4, Frontier Scientific) or 5,10,15,20-tetrakis(1-methyl-4-pyridinio)porphine (TMPyP, Aldrich) dissolved in a small amount of phosphate buffered saline (PBS, pH = 7.2) was added into the medium to reach the PS concentration of 100 μM in the medium. The cells were cultivated with PS for additional 20 hours. Finally, the cells were trypsinized and moved into 1×1 cm silica spectroscopic cell with optically polished bottom (Hellma) and centrifuged in the PBS to obtain a thin layer of cells on the bottom. The PBS was bubbled by oxygen 10 min before and during the whole experiment to provide enough oxygen for the living cells.

Results and Discussion

We tested the ability of the adapted set-up to measure singlet oxygen from the surface of solid samples on polymer films containing a clinically used PS PpIX. The phosphorescence of the PpIX and generated singlet oxygen in PMMA films with 100 μM of the PS with time and spectral resolution is presented in Figure 2. Fast photobleaching of the PpIX during the experiment was observed. This was also confirmed by fluorescence measurements (data not shown). Therefore, the excitation beam spot on the film was moved to a fresh place of the specimen before accumulation of the luminescence data at each wavelength to avoid artifacts caused by the photodegradation of PpIX. The luminescence
kinetics was independent of the excitation spot position across the foil area. It was checked by series of independent measurements of the phosphorescence kinetic from distinct places at the same wavelength (data not shown). Two distinct phosphorescence bands of PpIX were present in PMMA. The PpIX phosphorescence spectrum is dominated by the band located around 1038 nm. It is accompanied by a weaker band at 958 nm. The maximum of singlet oxygen phosphorescence was found around 1278 nm. The differences in kinetics of singlet oxygen luminescence in films of different concentrations of PpIX in PMMA are presented in Figure 3. Corresponding time-constants are summarized in Table 1. The presented data document sufficient sensitivity and time resolution of the modified set-up for detection of PS and singlet oxygen phosphorescence from solid samples.

3T3 fibroblast cells treated by two different photosensitizers were used as a model of a tissue treated by PDT to prove the ability of the set-up to measure singlet oxygen from a tissues of living laboratory animals. Water soluble negatively charged TPPS₄ [3] and its positively charged counterpart TMPyP [4] were used. Their opposite charges determine different localization of each PS in the cells. The differences in subcellular localization of the two photosensitizers are documented in

Table 1. Rise- ($τ_1$) and decay-times ($τ_2$) of singlet oxygen phosphorescence generated by various concentrations of PpIX in PMMA.

<table>
<thead>
<tr>
<th>PpIX concentration [µM]</th>
<th>$τ_1$ [µs]</th>
<th>$τ_2$ [µs]</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>15.6 ± 0.2</td>
<td>64.2 ± 0.8</td>
</tr>
<tr>
<td>20</td>
<td>15.1 ± 0.3</td>
<td>58.1 ± 1.1</td>
</tr>
<tr>
<td>5</td>
<td>13.5 ± 0.8</td>
<td>54.7 ± 2.5</td>
</tr>
</tbody>
</table>

Figure 2. Luminescence of 100 µM PpIX and singlet oxygen in PMMA with time and spectral resolution.

Figure 3. Kinetics of singlet oxygen phosphorescence generated by different concentrations of PpIX in PMMA at 1278 nm.
Figure 4. Both microphotographs showing red fluorescence (around 650 nm) of the cells treated by the PS were obtained using a fluorescence microscope. The preferential accumulation of TMPyP in the cell nuclei is in contrast with TPPS4, which is located in lysosomes. This is in agreement with data obtained on human skin fibroblasts by Jimenez-Banzo [2]. To demonstrate the ability of our set-up to detect weak phosphorescence of singlet oxygen from the cells \textit{in vivo} with sufficient time resolution to distinguish both time constants, kinetics of the singlet oxygen luminescence at 1278 nm were recorded. The kinetics are shown together with their corresponding fits in Figure 5. Both rise- and decay-times were resolved with substantially better precision than data published up to now. Our data are presented in Table 2 where the previously published data [2] are also provided for the sake of comparison. The rise times correspond well to the previously published results [2]. On the contrary, substantially shorter decay times compared to [2] can be possibly attributed to faster quenching due to the higher concentration of oxygen dissolved in the PBS during our measurements.

\begin{table}[h]
\centering
\begin{tabular}{lcc}
\hline
 & $\tau_1$ & $\tau_2$ & $\tau_1$ [2] & $\tau_2$ [2] \\
 & [\mu s] & [\mu s] & [\mu s] & [\mu s] \\
\hline
TPPS4 & 0.99 ± 0.02 & 4.5 ± 0.2 & 1.5 ± 1.0 & 20 ± 1 \\
TMPyP & 1.11 ± 0.09 & 5.8 ± 0.1 & 1.7 ± 1.0 & 15 ± 2 \\
\hline
\end{tabular}
\caption{Rise- ($\tau_1$) and decay-times ($\tau_2$) of singlet oxygen phosphorescence in fibroblast cells generated by TPPS4 or TMPyP. Comparison with published results [2].}
\end{table}

Figure 4. Fluorescence pictures of fibroblast cells incubated with 100 $\mu$M TMPyP (left) and TPPS4 (right) for 20 hours.

Figure 5. Kinetics of singlet oxygen phosphorescence generated by TPPS4 and TMPyP in living cells at 1278 nm.
**Conclusion**

The unique set-up for measurements of singlet oxygen and photosensitizer phosphorescence with time and spectral resolution was adapted for in vivo measurements. Suitability of the new set-up for detection of infrared luminescence from solid samples was confirmed by measurements of the singlet oxygen production behavior in polymer films containing PpIX in different concentrations and in murine fibroblast cells containing PS. Our results confirm previously published lifetimes [2] of singlet oxygen using TPPS₄ and TMPyP PS in human fibroblasts suspensions. Moreover, the lifetimes were estimated with much higher precision in our study. The results are promising for planned investigations of time constants of singlet oxygen phosphorescence in skin of living laboratory rats undergoing a PDT treatment.

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**References**


