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Delayed luminescence of erythrosine in biological tissue and photodynamic therapy dosimetry

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ABSTRACT

The features of delayed fluorescence (DF) and phosphorescence in erythrosine stained healthy and cancer-diseased mammary gland tissues of BYRB-line mice were investigated *in vitro*.
Thermoactivated delayed fluorescence (TDF), triplet-triplet annihilation (TTA) and originated from singlet-triplet annihilation (STA) delayed fluorescence are investigated as competing channels of radiative relaxation of the triplet states of erythrosine. The dominant role of diffusive-mobile molecular oxygen in deactivation of triplet-excited long-live states of the dye molecules in cells is determined.
The previously not described phenomenon of light quenching of DF under pulsed laser irradiation (light quenching of DF, LQDF) in stained tissue was revealed.
LQDF is increased if the energy of excited pulses is rise and their sequence period is decreased. The DF depletion disappears if time interval between pulses in the series is >5 s. This phenomenon is due two process competition: fast consumption of singlet oxygen by oxidation of cell organelles right after laser pulse excitation and slow diffusive recovery of oxygen concentration in the pause between the pulses.
Statistically valid distinction between DF characterization as well as LQDF erythrosine extent in healthy and pathological tissues was established.
The use of this phenomenon will greatly simplify the determination of radiation "dose" in photodynamic therapy (PDT) directly during the treatment session.

1. Introduction

The relaxation processes of long-lived excited fluorophores in tissues under the presence of diffusive-mobile oxygen are of significant practical interest due to the active PDT methods development and optical tissue diagnostics.

These processes in lifeless homogeneous media are studied quite explicitly both in theory and experimentally [1–5]. The results of these investigations are applied in PDT and optical diagnostics, however heterogeneity of cell contents and the differences of environment physical properties of a photo sensitizer localized in a cell limit significantly the capabilities of simple models usage.

The main efforts of PDT improvement are concentrated in several directions. A retrieval of novel effective photosensitizers (PS) of oxygen active forms including singlet oxygen $^1O_2(^1\Delta_g)$ which meets all the requirements of PDT applications is carried out [6].

The characteristics of photosensitizer DF or singlet oxygen phosphorescence (sometimes with 2D visualization) are used to determine the local oxygen concentrations on macro- or microscale [7–10].

The problem of dosimetry in PDT remains important because the cells of healthy tissues are also exposed to photodynamical action and thus the effective dose has to be controllably restrained during PDT session. Most of the studies [11–13] are devoted to the solution of this problem where some direct and indirect dosimetry methods are proposed. The direct methods are based on the registration of singlet oxygen phosphorescence [14–16] which is proportional to oxygen concentration in tissue. Unfortunately, they are insufficient because of low quantum yield of $^1\Delta_g(O_2)$ phosphorescence and low sensitivity of photodetectors in the near IR region.

Among the indirect dosimetry methods one distinguishes special molecular indicator probes [17–20] which photophysical properties change in the course of chemical reactions with oxygen. These changes can be registered in the convenient visible range and then the quantity of oxygen active forms produced can be evaluated. However, the use of such probes does not solve the problem entirely. The indicators remove

Abbreviations: DF, delayed fluorescence; PS, photosensitizer; LQDF, light quenching of DF; PDT, photodynamic therapy; STA, singlet-triplet annihilation; TDF, thermoactivated delayed fluorescence; TTA, triplet-triplet annihilation.

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some part of oxygen active forms out of PDT main process. Besides, the localization of these probes in the cell often does not coincide with PS location and thus the probe reaction does not quite correspond to photodynamical action produced.

To evaluate the quantity of singlet oxygen produced one can use calculating way [7,8,21–26] by numerical fitting of registered PS delayed fluorescence and phosphorescence curves that can be observed at the PDT session under pulsed laser excitation. While using this technique it is not necessary to register weak IR luminescence of $^1\Delta_g(O_2)$ or to use additional reagents with separate measurement of their characteristics. However, calculations are time-consuming and therefore it is difficult to use such a technique directly during a treatment. Besides, to evaluate the amount of $^1\Delta_g(O_2)$ produced it is necessary to know the PS concentration in the cell as well as other parameters that is not always possible. And finally, in model calculations [21–26] due to the difficulties of mathematical treatment, the only type of dye localization in the cell is proposed that does not always represent the facts.

For these reasons the abovementioned methods are not widely used in clinical practice and the search of novel effective dosimetry methods in PDT is still of great importance.

The set of experiments [21,26–28] on delayed erythrosine luminescence in mice normal and cancer tissues is being carried out since dosimetry method based only on the analysis of PS phosphorescence seems to be the most attractive. On the base of the phenomenon revealed the dosimetry technique in PDT can be created where the dose of the oxygen used in chemical reactions is evaluated by photosensitizer luminescence characteristics.

1.1. Deactivation Channels of Luminophore Electron Excitation Energy in Biotissues

Delayed luminescence characteristics of PS in tissues depend on photophysical and photochemical processes that are important for photodynamic therapy.

However, determination of different competitive channels contribution in relaxation of long-lived excited states of endogenous or exogenous fluorophores *in vivo* is complicated because of the wide variety of possible PS locations and their excited states quenchers due to the natural heterogeneity of the medium [29].

Electronic excited states relaxation for diffusion-mobile fluorophores in the presence of molecular oxygen in homogeneous media has been studied thoroughly [1–5]. The intersystem crossing in fluorophore molecules and bimolecular energy exchange processes in donor-acceptor pairs are taken into account.

The considerable amount of long-lived triplet states is accumulated during 15–20 ns excite pulse in molecules with a great intersystem crossing yield and S_1-singlet state lifetime about 1 ns including xanthene dye erythrosine used in our research. Triplet states of luminophore molecules are quenched efficiently by oxygen resulting in $^1\Delta_g(O_2)$ formation.

Long-lived luminescence of dyes in tissues registered experimentally in spectral range corresponding to radiative $S_1 \rightarrow S_0$ transition consists of:

- thermoactivated delayed fluorescence (TDF) caused by reverse $T_1 \rightarrow S_1$ intersystem crossing$_1$ in molecules;
- annihilation component the source of which is contact pairs of triplet-excited diffusion-mobile luminophore molecules (triplet-triplet annihilation, TTA);
- delayed fluorescence originated from annihilation T_1-state luminophore molecule with singlet oxygen $T_1 + {}^1O_2({}^1\Delta_g) \rightarrow S_1 + {}^3O_2({}^3\Sigma_g^-)$ (singlet-triplet annihilation, STA).

The other type of long-lived molecular luminescence is the phosphorescence. This luminescence is caused by radiation transitions between the states of different multiplicity: for triplet dye state it is transition to the general singlet state, for $^1\Delta_g(O_2)$ is transition to the basic triplet state.

Taking the abovementioned into consideration, DF kinetics can be described by following equations system:

$$I_{PP}(t) = A_1 n_T(t), \tag{1}$$

$$I_{DF}(t) = B_1 n_T(t) + B_2 n_T^2(t) + B_3 n_T(t) n_\Delta(t), \tag{2}$$

$$I_\Delta(t) = C_1 n_\Delta(t), \tag{3}$$

where $I_{DF}(t)$ is the dye molecules DF intensity, $I_{PP}(t)$ the dye phosphorescence intensity; $I_\Delta(t)$ the singlet oxygen $^1\Delta_g(O_2)$ phosphorescence intensity. A_1, B_1–B_3, C_1 - are time independent coefficients; $n_T(t)$ and $n_\Delta(t)$ the triplet states of dye molecules and the singlet oxygen concentrations.

In the presence of oxygen triplet state relaxation can be accompanied by O_2^- superoxide formation. At the first stage of such reactions the excited dye molecule captures electron from environment that leads to the formation of fluorophore radical and surrounding molecule radical. At the second stage dye molecule returns the captured electron to triplet oxygen and transfers to the basic singlet state. Thus oxygen converts to superoxide and can take part in many restoration reactions resulting in the formation of strong oxidants able to easily diffuse in the cells.

2. Experimental Setup and Samples

The investigations were conducted *in vitro*. The tissue fragments were obtained surgically from mammary, liver and lung tissues of diseased and healthy animals. Tissue staining was accomplished by 1 mM dye aqua solution and subsequent excess dye washing by physiological solution. The BYRB genetic line mice were used as the experimental animals. The females of this line are known for the same spontaneous mammary tumors.

As the PS we used erythrosine - vital xanthene dye that is widely applied in histology. The use of erythrosine as a PS is known in oral cavity tumor PDT [30–31]. Though erythrosine is useful only for PDT surface tissue layers due to its peculiarities, it can serve as a convenient model photosensitizer because of good solubility in water, ability for penetration inside the cells, high triplet state quantum yield and it can be easily excited by *YAG-Nd* laser.

Stationary luminescence spectra were registered by excitation of the dye with xenon lamp that illuminates the sample through exciting monochromator.

The second harmonics (532 nm) of pulsed *YAG:Nd*$^{3+}$ LQ-129 laser was used for the delayed luminescence excitation. Gated photomultiplier (FEU-84-3) was used as a photodetector.

The sample was placed into a hermetical chamber with temperature and oxygen content regulation.

3. Results and Discussion

3.1. Stationary Erythrosine Luminescence Spectra in Tissues

Fig. 1 shows normalized erythrosine fluorescence spectra in tumorous and healthy mice mammary tissues and in polyvinyl alcohol.

The position of erythrosine spectra maximum in biotissues differs from the one in polyvinyl alcohol. Dye spectrum in healthy tissue (*Normal* curve, Fig. 1) is slightly displaced to the blue side. The shift is not too large and, possibly, is not concerned with features of dye-environment interaction.

In contrast to the healthy tissue, the 7 nm red shift of the dye luminescence spectrum is observed in tumor (*Tumor* curve, Fig. 1).

As one can see the influence of environment on the energetic spectrum of erythrosine in tumorous tissues is much larger than in normal