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Short Communication

Development of a Method of Volumetric Activated Photodynamic Therapy Based on the Use of Raman Fluorescence Technology

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Introduction

The method of photodynamic therapy (PDT) is based on the ability of the photosensitizer to selectively accumulate in malignant, pathologically altered or virus-affected cells and/or microbes. After the introduction of the photosensitizer, the next step of the procedure is to use a laser radiation source to irradiate the area of tissue subject to pathological change. condition with the formation of reactive oxygen species in the irradiated BO, which in turn causes damage and necrosis of tissues that have most intensively accumulated this drug. Thus, only those cells and tissues that have been changed (dysplasia foci, tumor, etc.) are subjected to necrosis, and healthy cells do not react to radiation (because such a power and dose of radiation is selected that does not damage healthy tissue cells). Moreover, the effect of treatment (exposure) significantly depends on the accessibility of the irradiated object for laser exposure to it, the depth of its effective penetration into the pathological focus, the concentration of the photosensitizer in it and the volumetric density of the laser radiation power. That is, in most clinical applications, the effect of treatment due to, for example, the shallow depth of penetration of laser radiation into tissues, is insufficient. This leads to relapses of the disease and life-threatening complications. This also applies to the foci of inflammation. The situation is further aggravated by the impossibility of topical detection of the object, which completely excludes the effect of treatment. We set ourselves the task of developing a PDT method in which the photosensitizer can be activated outside the object. At the same time, penetrating into the BO, the drug should accumulate in the tissues of the organs and activate the PDT process there in its entirety (a pathological object of microbial or neoplastic nature). Based on this concept, a culture of microorganisms was used as a model object.

Implementation of the method

The stages of preparation of the photosensitizer (chlorophyll-containing preparation contains magnesium-porphyrin, similar in composition to ironporphyrin-hemoglobin) included

- Selection of a photosensitizer with the highest quantum yield of fluorescence during its resonance irradiation (the stage includes the selection of probing laser radiation)
- Selection of optimal radiation dose parameters in the diagnosis and treatment of diseases (this stage includes the selection of such radiation parameters and signal registration that do not distort the signal during recording and do not damage healthy cells).

Figure 1 shows the fluorescence spectra of seven different chlorophyll-containing preparations obtained using different purification methods (PX1, PX2, PX3, PX4, PX5, PX6, PX7). Preparations PX1, PX3, PX4, PX5, PX6 have a weak quantum yield of fluorescence compared to PX2 and PX7 preparations, so these five drugs are not suitable for diagnosis and treatment. Their accumulation in tissues is small and the fluorescence signal is difficult to diagnose and effectively excite with laser radiation for treatment. Thus, the initial stage of PDT is carried out – the selection of an adequate drug with a high quantum yield of luminescence.

The next two stages are the selection of the wavelength of the probing radiation and the search for the dose parameters of irradiation (time and power of laser radiation).

At the second stage , all excitation schemes of the drug were tested (based on the absorption spectrum of figure 2 and it was shown that the most effective wavelengths for excitation are the

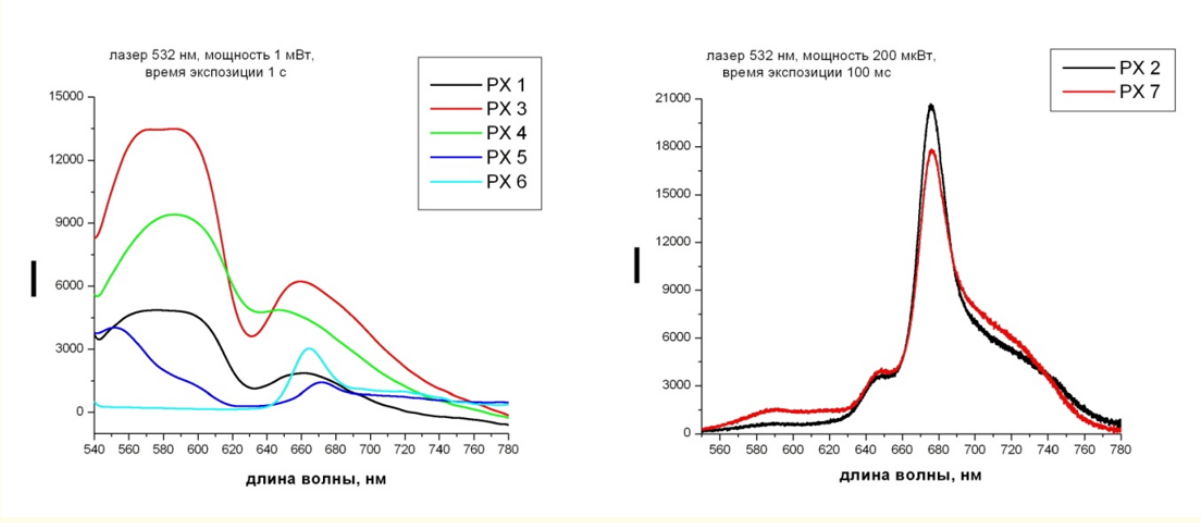

Figure 1: Fluorescence spectra of chlorophyll-containing products.

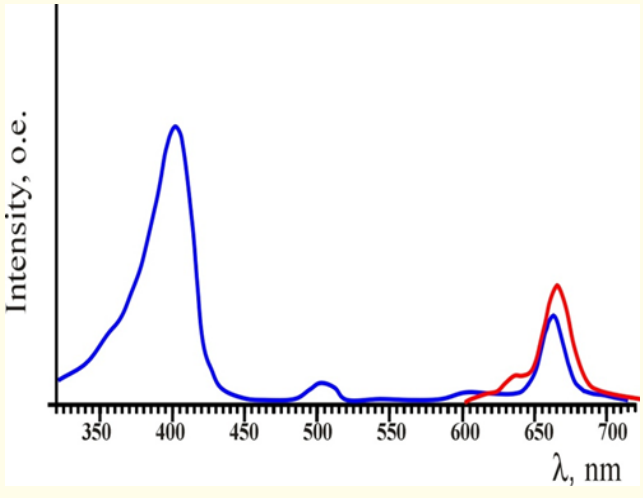

Figure 2: Absorption spectrum of radachlorophyll A solution in ethyl alcohol with a concentration of Radachlorophyll A® substance of 0.37 mg/ml. Red shows the fluorescence peak with a maximum at 668 nm, blue at 405 nm.

waves of 405 nm and 637 nm (which is fully consistent with the figure below). The wavelength of 405 nm is excellent for excitation of the drug in vitro, since it resonantly excites the drug and there is no need to meet the requirements for deep penetration of probing radiation (this would be important in the case of irradiation of the drug directly into the tissues).

Next, the optimal parameters of time and radiation power for the selected wavelength of 405 nm laser radiation were selected (Figure 3), at which the burn-in effect (decrease in the intensity of luminescence during exposure) of the pure PX preparation is no more than 7%. With these parameters, further measurement was carried out during PDT diagnostics (monitoring the PDT process and assessing its effectiveness).

The optimal signal registration parameters (power of 2.5 mW and exposure time of 50 ms) for the 405 nm laser were selected, at which the burn-in effect is 5%. Then, in the experiment, the judgment was tested that the effect of oxygen without the presence of laser irradiation does not lead to a change in the intensity of the luminescent glow of the PX preparation in a test tube.

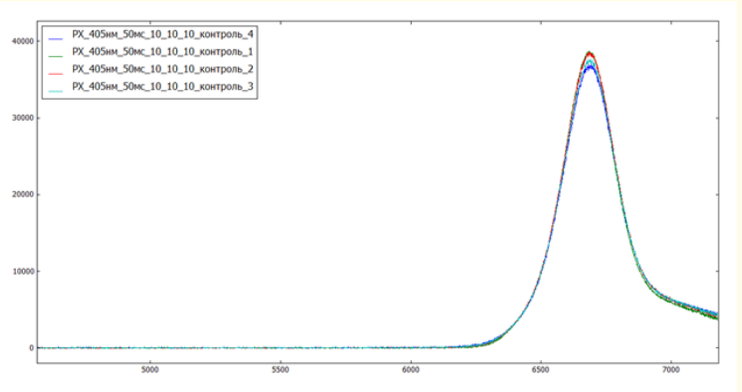

Figure 3: Control spectra of the RC under irradiation with a 405 nm laser.

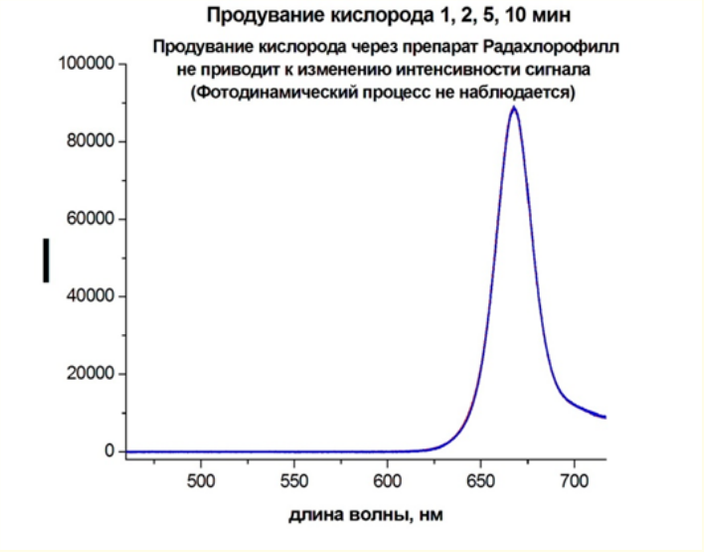

Figure 4: Fluorescence spectra of the PX preparation during oxygen supply.

The figure shows the fluorescence spectra of the PX preparation when oxygen is supplied for 1, 2, 5 and 10 minutes. All four spectra coincide (superimposed on each other). Thus, it can be concluded that the supply of oxygen through the PX without irradiation with laser light does not lead to a change in the intensity of the luminescence signal.

Further research concerned the physical and chemical basis of the action of activated PCs (irradiated with wave energy in the presence of oxygen donors) on cultures of bacterial cells or tissue cells.

As is known, Radachlorophyll A (RC) has the ability to absorb light in the visible area, resulting in its photoactivation. Classical PDT consists in the relaxation of the excited state of RC with the transfer of energy to molecular oxygen dissolved in tissues and then to the carbon of organic substrates. Radachlorophyll A is capable of destroying biological substrates after being excited by light with a wavelength of 350-670 nm. The most preferable excitation band for PDT is the longest wavelength absorption band of PX (662 nm), since with an increase in the wavelength, the penetration of light into biological tissues increases (up to 7 mm). However, in our case, in the case of excitation of the drug in a test tube, the best excitation is achieved with irradiation with a wavelength of 405 nm, because, as mentioned above, there is no requirement for light to penetrate deep into the tissue.

It is known that the formation of singlet oxygen (1O2) requires the presence of PS molecules, with the help of which the energy of photons is transferred to oxygen molecules. It is also known that the main pigments of photosynthesis, for example, chlorophyll derivatives, are effective PS for the formation of 1O2. Singlet oxygen (1O2) is electron-excited O2 molecules located at one of two singlet levels - lg+ and lg. Thus, 1O2 differs from other reactive oxygen species (O2 radicals, LEO, OH, or hydrogen peroxide H2O2) in that it requires only energy absorption without chemical modification of oxygen molecules.

The p-electron molecular orbitals of the porphin nucleus (the 18-electron aromatic system) stabilize the superoxide anions produced by the action of wave energy on the PS, leading to the chemical stability of the activated form of the PS for at least a few days (http://bd.patent.su/2345000-2345999/pat/servl/servletb4f1.html). Peroxides formed under the influence of light and oxygen from the above-mentioned groups, attached directly to the aromatic system of chlorophyll derivatives, stabilized by the presence of a porphin macrocycle and therefore long-lived, after introduction into the human body, accumulate according to the property inherent in chlorin E6 derivatives in the foci of tumor, infectious, parasitic, dermatological, immunological or allergic diseases, and can transfer the atom oxygen, hydroxyl radical, or electron both on each other and on the biomolecules of the membranes of pathologically altered cells or microorganisms. The presented literature data theoretically substantiate the proposed innovative concept of PDT.

Our studies have shown that the accumulation of chlorophyll-containing drugs in tissues and organs when taken per os significantly depends on the concentration of the PS used, while the effect of its intracellular (interstitial) activation practically does not depend on the dose of the drug. These data were obtained in experiments on microbial cell culture and mice (presented below).

Further, in the experiment, the optimal irradiation parameters were selected to obtain the highest amplitude contrast between the irradiated and non-irradiated sample. The temporal kinetic dependence of the effect of fluorescence restoration of the activated drug in the absence of probing radiation was studied. The time of fluorescence recovery was determined. Various options for oxygen delivery (peroxide, oxygen cylinder, etc.) were considered.

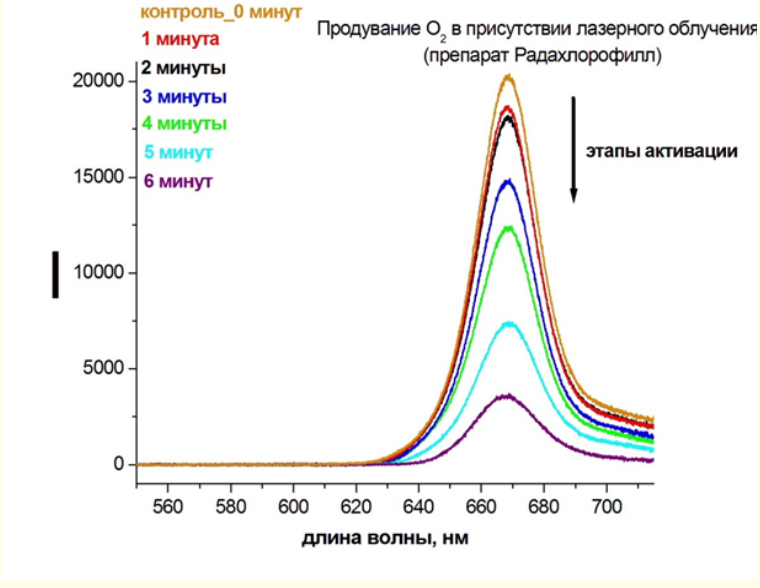

Figure 5: Activation of the drug by IR laser radiation (1.06 μm) for 6 minutes at a power of 6 mW (energy absorbed by the PX solution is 2.2 J). There is a 7-fold decrease in the luminescence peak.

Figure 5 shows the successive stages of drug activation when it is irradiated with light in the presence of oxygen. When 2 J of energy is transferred to the drug, its activation occurs.

We studied the effect of the accumulation of the PX drug in different concentrations in the organs and tissues of mice. The initial maximum concentration (0.7%) of the aqueous solution of Radachlorophyll "A" was administered orally to group 1 mice.

Here the indicators of integral fluorescence intensity of tissues averaged over group 1 are presented, normalized by the indicators of integral fluorescence intensity of the same tissues averaged over the control group of mice (not taking the drug).

Figure 6: Indicators of the integral intensity of fluorescence of tissues of group 1 mice.

The concentration of 0.07% aqueous solution of Radachlorophyll "A" was administered orally to group 2 mice.Here (Diagram 3) the indicators of integral fluorescence intensity of tissues averaged over group 2 are presented, normalized by the indicators of integral fluorescence intensity of the same tissues averaged over the control group of mice (not taking the drug).

The concentration of 0.007% aqueous solution of Radachlorophyll "C" was administered pyrally to group 3 mice.Here (Diagram 4) the indicators of integral fluorescence intensity of tissues averaged over group 3 are presented, normalized by the indicators of integral fluorescence intensity of the same tissues averaged over the control group of mice (not taking the drug).

Figure 7: Indicators of the integral intensity of fluorescence of tissues of group 2 mice.

Figure 8: Indicators of the integral intensity of fluorescence of tissues of group 3 mice.

Thus, on the basis of the presented study, the real possibility of accumulation of chlorophyll containing photosensitizer in various organs and tissues of CO was convincingly shown. This made it possible to develop a method of volumetric activated PDT. Two microorganisms Ps.aeruginosa and S.aureus (test objects) were taken as a basis for the experiment. As a basis for the experiment, the disk-diffusion method was taken, based on the diffusion of antibiotics from the carrier into a dense nutrient medium and inhibition of the growth of the studied culture in the zone where the concentration of the antibiotic exceeds the minimum inhibitory concentration.

To conduct the experiment, the device of the InSpectrum company was used (Figure 6).

From a pure daily culture of Ps.aeruginosa and S.aureus, grown on a non-selective dense nutrient medium with distilled water, a 0.5 McFarland inoculum was prepared, which corresponds to a concentration of 1×10^8 CFU/ml. Then discs impregnated with Radachlorophyll, pre-activated laser radiation with a wavelength of 637, 532, 405 nm at a dose of 0.2-20 J/ml were placed. in a concentration of 0.7%,0.07%,0.007% and saturated with oxygen. Sterile disks, disks with a non-activated drug (in the same concentrations), disks with the drug after exposure to an oxygen-containing aqueous solution, disks with an oxygen-containing aqueous solution (hydrogen peroxide 3%) were used as controls.

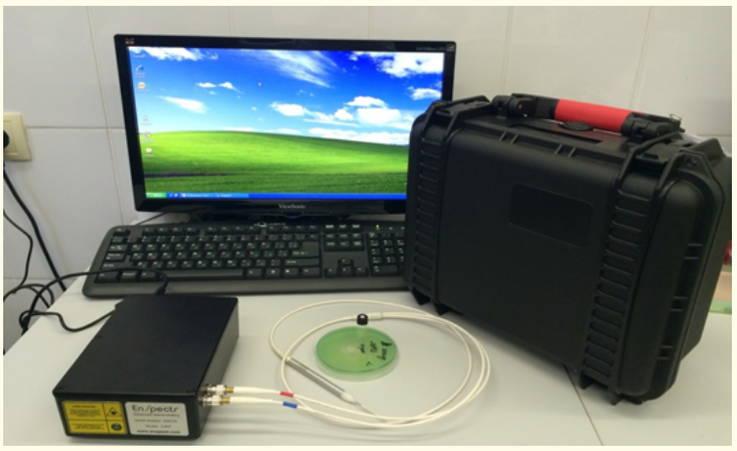

Figure 9: InSpectrum M device with a computer.

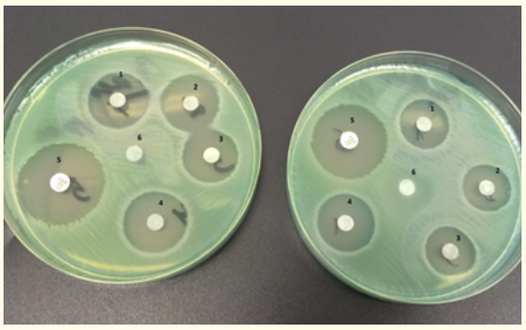

Figure 10: *Ps.aeruginosa* and radochlorophyll "C" (activated) immediately and after 40 min.

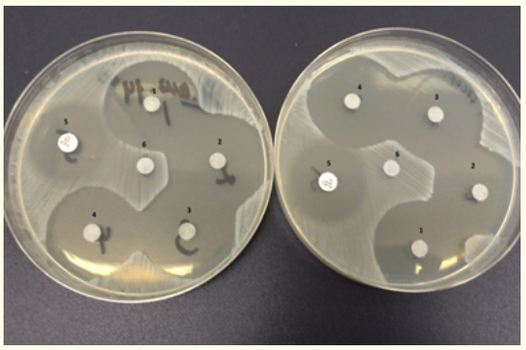

Figure 11: *S.aureus* and radochlorophyll "C" (activated) immediately and after 40 min.

	Growth retardation zone mm		
	Radoklorin 0.7%	**Radoklorin 0.07%**	**Radoklorin 0.007%**
Clean preparation	No growth retardation zone	No growth retardation zone	No growth retardation zone
	No growth retardation zone	No growth retardation zone	No growth retardation zone
+ H2O2	No growth retardation zone	No growth retardation zone	No growth retardation zone
	No growth retardation zone	No growth retardation zone	No growth retardation zone
+ H2O2 (laser radiation)	19	18	15
	21	20	18
+H2O2 (laser irradiation) h/w 40 min	24	20	21
	21	17	17
Control	H2O2 3%	Cefepime 30 mg/l	Clean Disc
	12	29	No growth retardation zone
	11	29	No growth retardation zone
	12	30	No growth retardation zone
	12	30	No growth retardation zone
	13	30	No growth retardation zone
	13	30	No growth retardation zone
	13	29	No growth retardation zone
	11.5	29	No growth retardation zone

Table 1

In the course of the second experiment, an inoculum was prepared from a pure daily culture of *Ps.aeruginosa*, grown on a non-selective dense nutrient medium, using distilled water, 0.5 according to McFarland, which corresponds to a concentration of 1×10^8 CFU/ml. Next, the spectra were measured on the InSpectrum M device and inoculated on a dense nutrient medium. The study was conducted in the following time intervals: immediately, after 30 minutes, 1 hour, 1 hour 30 minutes, 2 hours, 2 hours 30 minutes.

Table and figure 12,13 show that activation of Radachlorophyll A showed suppression of the growth zone of the microorganism after 24 hours, while the non-activated drug with *Ps. aeruginosa* did not delay the growth zone, and *S. aureus* showed minimal suppression of the growth zone. When an oxygen-containing aqueous solution was added to the non-activated chlorophyll (radachlorophyll "A") preparation, no increase in growth zone retention was observed in *Ps. aeruginosa*, and in *S. aureus* there was even a decrease in growth zone retention.

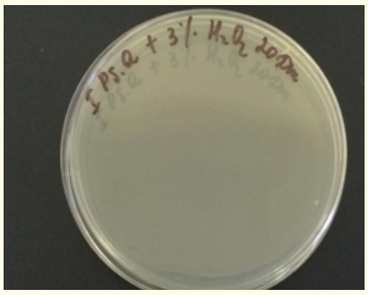

Figure 12: *Ps.aeruginosa* and radochlorophyll "C" with the addition of 3%H_2O_2, activated 20 J-growth no.

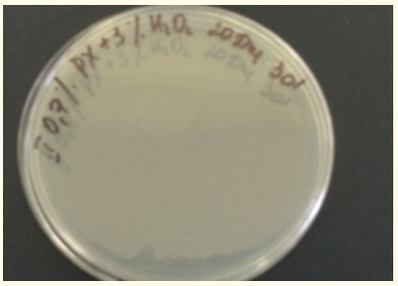

Figure 13: *Ps.aeruginosa* radochlorophyll "C" with the addition of 3%H_2O_2, activated by 20 J. After 30 minutes, there is no growth

As a result of the study, it can be assumed that when an oxygen-containing drug is added to Radachlorophyll "A" and activated by laser radiation with wavelengths of 405 nm, 532 nm and 637 nm with doses of 0.2-20 J/ml, an excited photosensitizer interacts with an oxygen molecule, resulting in the formation of singlet oxygen, which is cytotoxic for living cells, due to its property as a strong oxidizer of biomolecules. Singlent oxygen is also formed in phagocytes during respiratory explosion reactions. There is evidence of high bactericidal efficacy of reactive oxygen species (singlent oxygen, superoxide radical, hydrogen peroxide, hydroxyl radical) against most microbes. It can be assumed that the interaction of activated Radachlorophyll A with a microbial cell is similar to the oxygen-dependent subsystem of the microbicidal system of phagocytes, which is not addictive.

It was shown that the observed effect of the action of the activated drug on microbial cells does not significantly depend on its concentration (no significant differences were revealed), and that this effect is comparable in effect to the action of antibiotics. It was also shown that the quantitative effect of microbial cell death does not depend on the dose of irradiation with the light of a drug activated by an oxygen-containing substrate, while the accumulation in tissues depends significantly on the dose of the drug.

Conclusion

The ability of the photosensitizer to accumulate in altered tissues, microbial cells with the effect of lethal photosensitization of bacteria can be used in the treatment of antibiotic-resistant strains of pathogenic microorganisms. The developed technique was first used in women with chronic endometritis and infertility.

It can be assumed that for the prevention of oncological diseases in patients of "high-risk" groups, as well as in borderline tumors and the initial stages of malignant transformation, we plan to use the developed technology of volumetric activated PDT, when a small quantum yield of the PK drug suppresses the growth and development of initially changing cells (when they absorb activated

forms of the drug). clinical capabilities to combat diseases and processes of microbial and neoplastic nature and, most importantly, both in terms of their prevention and objective control of PDT (at the stage of drug activation and assessment of the effectiveness of its clinical use) based on RFD technologies.