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FLUORESCENCE DIAGNOSIS OF DAMAGE TO TUMOR TISSUES DURING PHOTODYNAMIC THERAPY WITH THE PHOTOSENSITIZER PHOTOLON®

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We have studied the feasibility of using an indotricarbocyanine dye as a marker for the efficacy of photodynamic therapy (PDT) of cancers with the photosensitizer Photolon®. We have established that on exposure to laser emission at λ = 667 nm with an exposure dose of 100 J/cm², we observe that the Photolon® concentration drops by about a factor of two in the exposed part of the tumor, while the concentration of the indotricarbocyanine dye does not change in any region of the tumor node. We have observed a correlation between the change in the shape of the fluorescence spectra of the indotricarbocyanine dye in vivo in the 780–880 nm resulting from a PDT session with the photosensitizer Photolon® and the extent of damage to the tumor tissues. Changes in the shape of the fluorescence spectrum of the dye are interpreted in terms of a model involving the appearance of absorption by different forms of hemoglobin, and changes in their ratio in the exposed part of the tumor due to consumption of molecular oxygen.

Keywords: *indotricarbocyanine dyes, Photolon®, photodynamic therapy, fluorescence diagnosis.*

Introduction. A promising method for treating malignant neoplasms is photodynamic therapy (PDT). In order to achieve a successful treatment outcome in PDT, a photosensitizer in the pathological tissues is exposed to laser light with optimal parameters. A distinguishing feature of PDT is that a rather long time passes between the phototherapy session and identification of the results of this exposure. Accordingly, the quality of treatment can be improved by the availability of techniques letting us monitor the light exposure parameters on the fly and directly during the PDT session in order to predict the efficiency of damage to tumor tissues. Thus in [1], it was established that as a result of PDT with a photosensitizer taken from the class of polymethine dyes, changes in its fluorescence spectrum during photochemotherapy correlate with the depth of damage (necrosis) in the tumor tissues. In this case, from the extent of change in the shape and position of the spectra for this photosensitizer, we can predict the depth and extent of necrosis in tumor tissues during the photochemotherapy session, and make changes in the treatment protocol depending on the individual response of the body. Changes in the fluorescence spectra of the polymethine dye *in vivo* identified as a result of PDT are connected with a change in the ratio of the different forms of hemoglobin in the damaged tumor tissue. Such a conclusion is drawn based on overlap of the fluorescence spectra of the dye with the absorption spectra of the indicated endogenous biomolecules and the change in their concentrations as a result of photochemical reactions [1–3].

To date, there have been no studies of the question of the feasibility of an analogous approach for analysis of the extent of damage during PDT of tumor tissues with another photosensitizer when using a polymethine dye as a fluorescent probe. In this paper, we present the results of a study of the spectral properties of indotricarbocyanine dye *in vivo*, with the aim of observing correlations between changes in the fluorescence spectral and the extent of damage to tumor tissues during PDT with the photosensitizer Photolon®.

Materials and Measurement Methods. As the photosensitizer for PDT, we used Photolon®, produced by Belmedpreparaty [4]; as the fluorescent probe, we used a symmetric indotricarbocyanine dye (polymethine dye, PD): 2-{7-[3-methyl-3-ethyl-1-trimethylenecarbo-(6-O-D-glucosyl)-2(1H)-indolenylidene]-4-chloro-3,5-(O-phenyleno)-1,3,5-

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hexatrien-1-yl}-3-methyl-3-ethyl-1-trimethylenecarbo-(6-O-D-glucosyl)indolenylium bromide, synthesized in the spectroscopy laboratory of the A. N. Sevchenko Research Institute of Applied Physical Problems, Belorussian State University [5].

In vivo experiments were conducted on an experimental model for the tumor strain sarcoma M-1 on outbred white rats. There were six rats in each of the control and experimental groups. The tumors were exposed to light with an exposure dose of 50–100 J/cm^2 using a therapeutic diode laser LD680-2000 (BIOSPEK, Moscow), λ = 667 nm, power density 0.1 W/cm^2. The antitumor efficacy of photodynamic therapy was assessed based on data on the depth of necrosis, detected in the tumors by the vital staining method 24 hours after exposure. Tumor zones which died due to the direct cytotoxic effect or as a result of structural and functional disturbances of the microcirculation were not stained blue. In order to ensure conditions for which 100% incidence of complete tumor necrosis was achieved, the PDT session with Photolon® was conducted according to the protocol defined by the developers of this photosensitizer [6]. Note that all *in vivo* studies were conducted on equipment used previously for studying the antitumor activity of the photosensitizer Photolon® [6]. Photolon® was injected into the caudal vein of the animals in a concentration of 2.5 mg/kg; the indotricarbocyanine dye was injected immediately after the Photolon®. The fluorescence spectra of the dye PD *in vivo* with excitation by emission with λ = 683 nm were recorded using a spectrometric system (developed at the A. N. Sevchenko Research Institute for Applied Physical Problems) which was optimized for the 700–950 nm range [7]. In order to detect the fluorescence of the photosensitizer Photolon®, we used a Lesa-6 spectrometer with an He–Ne laser (λ = 632.8 nm) as the source of exciting radiation. The exciting radiation was delivered to the sample and the fluorescence was collected in the spectrometer system and the Lesa-6 spectrometer using optical fibers.

Results and Discussion. In order to evaluate the prospects for using PD as a marker for determining the efficiency of damage to pathological tissues during exposure to light with the photosensitizer Photolon®, we studied the fluorescence of PD in tumor tissue and healthy muscle tissue. The studies were conducted with excitation of fluorescence by emission at λ = 632.8 or 683 nm. Figure 1 shows the fluorescence spectra (λ_{ex} = 632.8 nm) of tumor tissues *in vivo* for the untreated animal (which was not injected with the dye), when Photolon® was injected into the animal, and also when Photolon® and PD were sequentially injected. The first fluorescence band with maximum at 670 nm corresponds to the emission of Photolon® [6, 8]; the second band in the 750 nm region is due to the presence of PD in the tissues. Note that when using exciting radiation with λ = 632.8 nm, emission by endogenous biomolecules in the tumor tissues (curve 1) make a significant contribution to the recorded spectrum. Emission in this spectral region is most likely due to the presence of endogenous porphyrins in the tissues [9], the molecules of which are rather efficiently excited by radiation at λ = 632.8 nm.

On going to excitation by the emission from a semiconductor laser with λ = 683 nm, no significant photoluminescence signal appears from the tumor or thigh muscle tissue for the untreated rat or for the animals treated with Photolon® (Fig. 2). The fluorescence spectra of the PD when 2.5 mg/kg Photolon® is injected into the animal or when Photolon® is not present coincide in shape and position. Consequently, the fluorescence of Photolon® is not excited by 683 nm radiation and does not make a contribution to the spectral characteristics of the PD.

The behavior of the change in spectral properties of the fluorescent probe PD during photochemotherapy with the photosensitizer Photolon® must be studied under conditions where the presence of the probe does not have an effect on the therapeutic properties of Photolon. In order to achieve such conditions, PD was injected in a dose of 1 mg/kg, which is 1/5 of the concentration at which PD is used as a photosensitizer for PDT of tumor tissues [5, 10]. In addition, we considered that for PDT using the photosensitizer PD during exposure to light at λ = 667 nm, the damage to the tumor tissues is less than for radiation sources at λ = 740 nm or 780 nm (by more than a factor of three) [10]. In order to establish the possible effect of PD in tumor tissues on the extent of damage to the tumor, we conducted studies with injection of only PD in a concentration of 1 mg/kg into the animal, i.e., in the absence of Photolon®. Exposure to light in this case was carried out according to the protocol for using Photolon, i.e., with radiation at λ = 667 nm for an exposure dose of 100 J/cm^2, 2 h after injection of PD. It was established that for such animals, no necrosis occurs in the tumor tissues.

Exposure to light was carried out according to the standard technique for using Photolon®, with accumulation of the maximum Photolon concentration in the tumor tissues [6]. The tumor nodes were exposed to the emission from a diode laser with λ = 667 nm, exposure dose 100 or 50 J/cm^2. We note that the pharmacokinetics of PD in tumor and normal muscle tissues with additional injection of Photolon or in its absence are identical, i.e., the presence of Photolon® does not have an effect on the processes of accumulation and clearance of PD from the tissues. In this case, over a time interval of 2–3 h after injection of the drugs, both for Photolon® and for PD we observe a maximum and approximately constant concentration in the tumor tissues (Fig. 3).

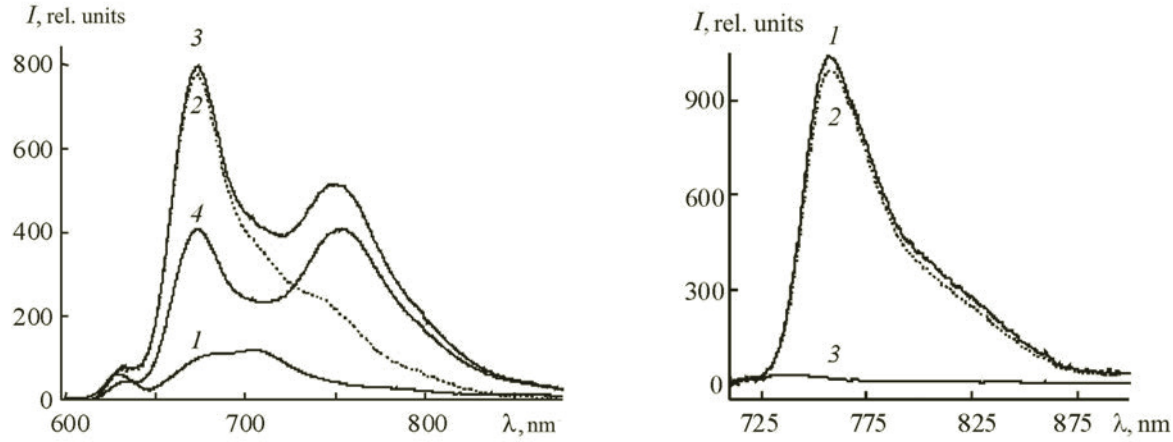

Fig. 1. Fluorescence spectra with excitation by the emission from an He–Ne laser at λ = 632.8 nm, from tumor tissues of an untreated rat *in vivo* (1), when Photolon® was injected into the animal in a dose of 2.5 mg/kg (2), when Photolon® 2.5 mg/kg and PD 1 mg/kg were injected before exposure to laser light began during PDT (3) and after exposure (4).

Fig. 2. Fluorescence spectra with excitation at λ = 683 nm from tumor tissue with injection of Photolon® 2.5 mg/kg and PD 1 mg/kg to the animal before (1) and after (2) exposure to laser light at λ = 667 nm with exposure dose 100 J/cm^2, and without injection of the photosensitizers (3).

For the group of animals exposed to light at an exposure dose of 100 J/cm^2, we detected necrosis with depth of damage ~15 mm, typical for this photosensitizer. In this case, in the exposed part of the tumor, the intensity at the maximum for fluorescence of Photolon® with λ_{ex} = 632.8 nm drops by a factor of ~2, while for the surrounding muscle tissue or the unexposed part of the tumor, the signal level remains unchanged (Fig. 1). In the fluorescence spectra of Photolon®, we do not observe changes in the position or the shape. For such exposure to light, the fluorescence intensity for PD with excitation at λ = 683 nm in any region of the tumor node is practically constant (≤5%), i.e., the PD concentration does not change. Moreover, for the region of the tumor tissues exposed to light, in which necrosis was detected later, the shape of the fluorescence spectrum of PD changes in the 780–860 nm region. For comparison of the shape, we averaged 10 fluorescence spectra of the dye, normalized with respect to the maximum, recorded *in vivo* for each of the considered cases (Fig. 4a). The standard error for the intensity averaged over 10 normalized spectra is ≤ 0.01. The indicated distortion of the fluorescence spectrum of PD, normalized with respect to the maximum, correlates with the depth of necrosis in the tissues. Thus for the group of animals exposed to light with exposure dose 50 J/cm^2, the depth of necrosis fluctuates from 7 mm to 4 mm, the changes in the shape of the fluorescence spectrum of PD are not so significant. Figure 4b shows the difference fluorescence spectra for samples of tumor tissues in which necrosis of depth 4–15 mm were detected. As we see, distortion in the 780–860-nm region of the PD spectra are more significant for tissue samples with a greater extent of damage. At the same time, we do not observe changes in the fluorescence spectrum of PD in the unexposed part of the tumor or in the muscle tissue. During a time interval of 2–3 h after injection of the drugs, no changes are observed in the fluorescence spectra of PD in the tumor tissue or in the healthy muscle tissues of animals from the control group, who were not exposed to laser light. The reason for the changes in the shape of the fluorescence spectrum of the dye PD in the tissues *in vivo* may be the presence of endogenous biomolecules having appreciable absorption in the same spectral region [2, 11]. The fluorescence spectra of the studied PD overlap with the absorption bands of hemoglobin, melanin and water [12]. A change in the absorption spectra of water and melanin during exposure to light seems unlikely, while the ratio of the different forms of hemoglobin (oxyhemoglobin (HbO_2), deoxyhemoglobin (Hb), and methemoglobin (MetHb)) may change. We should consider that during the photochemotherapy session, due to a decrease in oxygen in the tumor tissue, the ratio of the concentrations of the different forms of hemoglobin changes: the HbO_2 fraction decreases [2, 3]. The absorption spectra for HbO_2, Hb, and

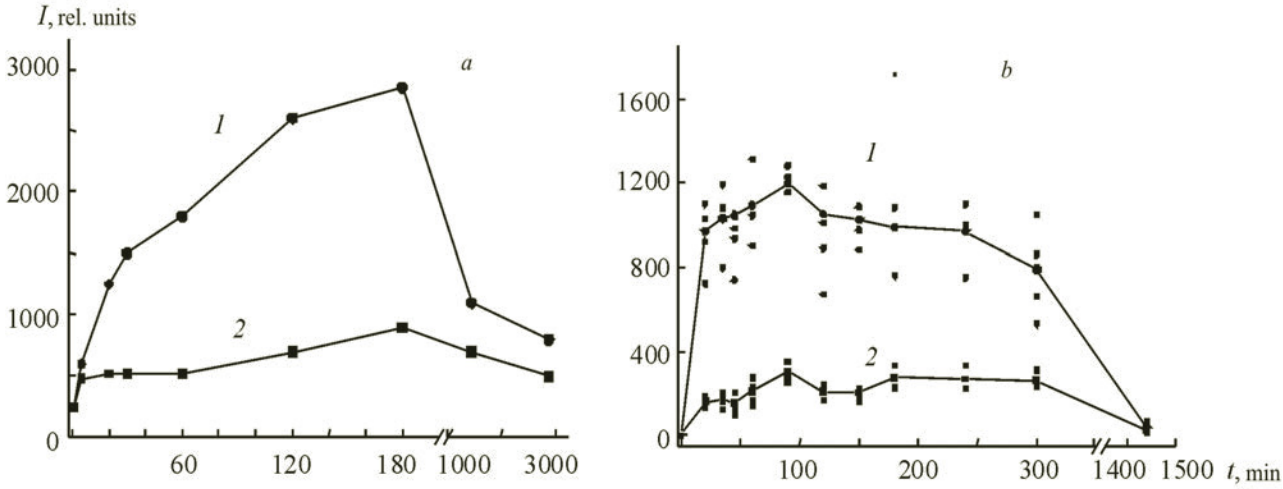

Fig. 3. Accumulation of Photolon® (a) and the indotricarbocyanine dye (b) in tumor tissue (sarcoma M1) (1) and normal tissue (thigh muscle) (2).

MetHb in the fluorescence band of PD (710–875 nm) have significant differences [12–14], and so a change in the ratio of the concentrations of the different forms of hemoglobin can affect the shape of the fluorescence band of the dye. Accordingly, in analogy to [1, 15], we performed a numerical calculation of the PD spectra, taking into account absorption of some of the fluorescence emission by different forms of hemoglobin.

In the spectrometric system used, fluorescence of the dye molecules in the tissues is excited and detected from a depth of ~1.5 cm from the surface [16]. Therefore in the general case, the expression for the fluorescence spectrum of PD *in vivo*, determined by emission by the dye molecules from a depth in the biological tissues down to l = 1.5 cm, taking into account absorption by the different forms of hemoglobin and according to the Bouguer–Lambert–Beer law, has the form:

$$I(\lambda) = I_0(\lambda)\int_0^{1.5} 10^{l(C_{HbO_2}\varepsilon_{HbO_2}(\lambda)+C_{Hb}\varepsilon_{Hb}(\lambda)+C_{MetHb}\varepsilon_{MetHb}(\lambda))}dl\,, \quad (1)$$

where $I(\lambda)$ is the calculated fluorescence spectrum of PD *in vivo*, taking into account the effect of absorption by oxyhemoglobin, deoxyhemoglobin, and methemoglobin on the fluorescence spectrum $I_0(\lambda)$, emitted by the dye molecules from a depth l; C_{HbO_2}, C_{Hb}, C_{MetHb}, and $\varepsilon_{HbO_2}(\lambda)$, $\varepsilon_{Hb}(\lambda)$, $\varepsilon_{MetHb}(\lambda)$ are the concentrations and molar absorption coefficients for oxyhemoglobin, deoxyhemoglobin, and methemoglobin, respectively [12–14].

According to available data [10], the scattering coefficient for scattering by biological tissues is practically constant in the spectral range corresponding to PD fluorescence (700–900 nm). Consequently, this parameter cannot have an effect on the shape of the fluorescence spectrum of PD. Accordingly, scattering was neglected in this model. In order to calculate the hemoglobin concentration in the tumor tissue of the rat, we took into account the total blood volume, weight and size of the animal. The hemoglobin concentration in the blood of the animal is 110–120 g/L [17]. Due to averaging of the signal over the volume of the samples using light beams of diameter ~5 mm on the surface, in the model we assumed that the blood was uniformly distributed over the volume under consideration. In this case, we took into account a correction for the weight and volume of bone tissue, which does not contain a large amount of blood.

Due to the change in the ratio of the different forms of hemoglobin after the photochemotherapy session was completed, we can write an expression for the recorded fluorescence spectrum of PD based on (1):

$$I'(\lambda) = I_0(\lambda)\int_0^{1.5} 10^{l(C'_{HbO_2}\varepsilon_{HbO_2}(\lambda)+C'_{Hb}\varepsilon_{Hb}(\lambda)+C'_{MetHb}\varepsilon_{MetHb}(\lambda))}dl\,, \quad (2)$$

where $I'(\lambda)$ is the calculated fluorescence spectrum of PD *in vivo*; the C'_x are the concentrations of the different forms of hemoglobin after exposure to light.

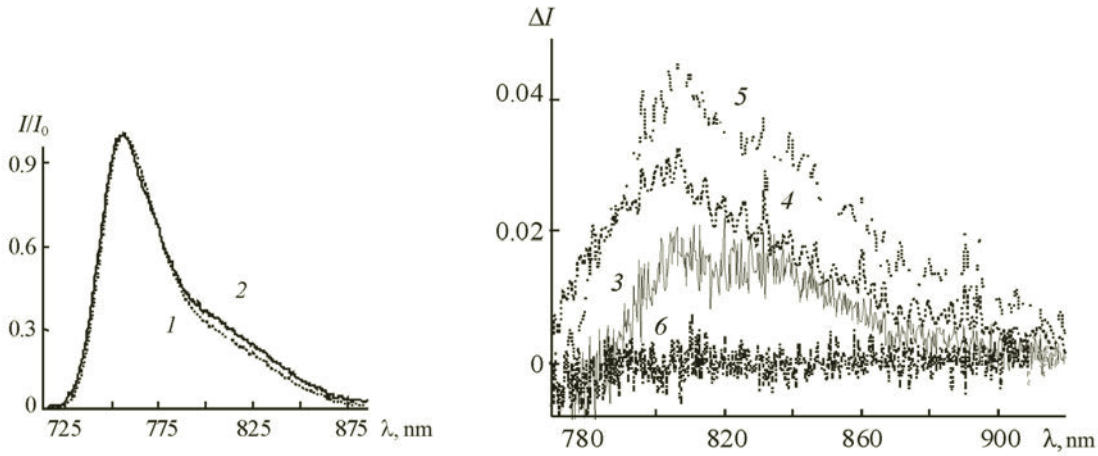

Fig. 4. Fluorescence spectra (λ_{ex} = 683 nm) of PD *in vivo* before (1) and after (2) exposure of Photolon® in tumor tissues to light for necrosis of depth 15 mm, the difference fluorescence spectra of PD for necrosis of depth 4 mm (3), 7 mm (4), and 15 mm (5), and in the unexposed part of the tumor tissue (6).

By varying the ratio of the concentrations of Hb, HbO_2, and MetHb, according to formula (2) we calculated the fluorescence spectrum of PD *in vivo* after exposure to light, taking into account absorption by the blood components. In this case, the parameters characterizing the changes in the concentrations of the different forms of hemoglobin as a result of exposure to light, $\Delta C = C - C'_x$, and specifically ΔC_{MetHb}, ΔC_{Hb}, ΔC_{HbO_2}, were determined by minimizing the function $\xi(\Delta C_{MetHb}, \Delta C_{Hb}, \Delta C_{HbO_2})$:

$$\xi(\Delta C_{HbO_2}, \Delta C_{Hb}, \Delta C_{MetHb}) = \sum_{\lambda} (I'(\lambda) - I_{exp}(\lambda))^2 . \quad (3)$$

The function $\xi(\Delta C_{MetHb}, \Delta C_{Hb}, \Delta C_{HbO_2})$ is the square of the difference between the experimental fluorescence spectrum of PD *in vivo* after exposure to light and the spectrum calculated according to formula (2).

The best match between the experimental and calculated fluorescence spectra of the dye in the exposed part of the tumor is observed as the Hb concentration increases from 14%–15% to 20% and the HbO_2 concentration symbatically decreases from 85% to 79%. The MetHb concentration in this case remains practically unchanged, and does not exceed 1%.

In contrast to the data obtained, when using PD as both a photosensitizer and a probe we observe a local increase up to 50% in the MetHb concentration in the exposed part of the tumor tissue relative to the total hemoglobin concentration in the blood, where the HbO_2 fraction is ~30% and the Hb fraction is ~20% [1]. Such significant differences in the change in the concentration of the different forms of hemoglobin for the same extent of damage to the tumor tissues during PDT with the photosensitizer Photolon® and the photosensitizer PD (depth of necrosis ~15 mm) indicate that the damage mechanism is different for these photosensitizers.

Conclusions. There is a regular change in shape of the fluorescence spectra of indotricarbocyanine dye during photodynamic therapy with the photosensitizer Photolon®, and it correlates with the depth of necrosis in the tumor tissues. The indicated differences in the fluorescence spectra of the dye are interpreted in terms of a model describing the effect on their shape from a change in the concentrations of the different forms of hemoglobin in the tissues. Indotricarbocyanine dye can be used as a marker for on-the-fly determination of the efficacy of photodynamic therapy.

REFERENCES

1. M. P. Samtsov, E. S. Voropay, L. S. Liashenka, D. G. Melnikau, K. N. Kapleusky, and A. P. Lugovskij, *Zh. Prikl. Spektrosk.*, **78**, No. 1, 121–127 (2011) [M. P. Samtsov, E. S. Voropay, L. S. Liashenka, D. G. Melnikau, K. N. Kapleusky, and A. P. Lugovskij, *J. Appl. Spectrosc.*, **78**, 110–116 (2011) (English translation)].

2. O. Abdallah, W. Stork, K. Miller-Glaser, and A. Bolz, *Biomed. Technik*, **48** (2003).
3. S. A. Kutsenko, *Principles of Toxicology* [in Russian], Foliant, St. Petersburg (2004).
4. P. T. Petrov, V. M. Tsarenkov, A. L. Meshcheryakova, O. N. Al'bitskaya, V. I. Tyurin, M. V. Sarzhevskaya, M. A. Kaplan, N. D. Kochubeeva, and I. N. Zhuravskin, *Agent for Photodynamic Therapy of Malignant Neoplasms: Photolon*, Belarus Patent No. 5651 (2003).
5. Y. P. Istomin, E. N. Alexandrova, V. N. Chalov, E. A. Zhavrid, E. S. Voropay, M. P. Samtsov, A. P. Lugovskiy, A. A. Lugovskiy, and I. S. Mikhalovsky, *Experim. Oncol.*, **26**, No. 3, 226–231 (2004).
6. P. T. Petrov, G. A. Isakov, S. V. Shlyakhtin, L. N. Marchenko, and Yu. P. Istomin, *Vestn. Farm.*, **37**, No. 3, 69–81 (2010).
7. E. S. Voropay, M. P. Samtsov, I. M. Gulis, D. V. Glushkov, K. N. Kaplevsky, A. E. Rad'ko, and K. A. Shevchenko, in: E. S. Voropay, Ed., *Spectral Instruments for Analytical Applications. Promising Developments* [in Russian], BGU, Minsk (2005), pp. 5–9.
8. H. A. Isakau, M. V. Parkhats, V. N. Knyukshto, B. M. Dzhagarov, E. P. Petrov, and P. T. Petrov, *J. Photochem. Photobiol. B: Biol.*, **92**, 165–174 (2008).
9. Nirmala Ramanujam, Fluorescence spectroscopy *in vivo*, in: R. A. Meyers, Ed., *Encyclopedia of Analytical Chemistry*, Wiley, Chichester (2000), pp. 20–56.
10. M. P. Samtsov, E. S. Voropay, K. N. Kaplevsky, D. G. Melnikau, L. S. Lyashenko, and Yu. P. Istomin, *Zh. Prikl. Spektrosk.*, **76**, No. 4, 576–582 (2009) [M. P. Samtsov, E. S. Voropay, K. N. Kaplevsky, D. G. Melnikau, L. S. Lyashenko, and Yu. P. Istomin, *J. Appl. Spectrosc.*, **76**, 547–553 (2009) (English translation)].
11. P. Taroni, A. Pifferi, A. Torricelli, D. Comelli, and R. Cubeddu, *Photochem. Photobiol.*, **2**, 124–129 (2003).
12. S. Matcher, C. Elwell, C. Cooper, M. Cope, and D. Delpy, *Analyt. Biochem.*, **227**, No. 1, 54–68 (1995).
13. S. Matcher, M. Cope, and D. Delpy, *Phys. Med. Biol.*, **39**, 177–196 (1994).
14. W. Zijlstra, A. Buursma, and O. van Assendelft, *Visible and Near Infrared Absorption Spectra of Human and Animal Haemoglobin*, VSP, Utrecht (2000).
15. M. P. Samtsov, E. S. Voropai, D. G. Mel'nikov, L. S. Lyashenko, A. A. Lugovskii, and Yu. P. Istomin, *Zh. Prikl. Spektrosk.*, **77**, No. 3, 438–444 (2010) [M. P. Samtsov, E. S. Voropai, D. G. Mel'nikov, L. S. Lyashenko, A. A. Lugovskii, and Yu. P. Istomin, *J. Appl. Spectrosc.*, **77**, 406–412 (2010) (English translation)].
16. E. S. Voropai, M. P. Samtsov, V. N. Chalov, and E. A. Zhavrid, *Zh. Prikl. Spektrosk.*, **68**, No. 3, 359–362 (2001). [E. S. Voropai, M. P. Samtsov, V. N. Chalov, and E. A. Zhavrid, *J. Appl. Spectrosc.*, **68**, 468–472 (2001) (English translation].
17. W. Hsing-Wen, E. M. Putt, M. J. Emanuele, D. B. Shin, E. Glatstein, and A. G. Yodh, *Cancer Res.*, **64**, No. 20, 7553–7561 (2004).