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Optical diagnostics of biological tissue cells during their cultivation in polymers

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Abstract. **The specific features of long-term luminescence of exogenous molecular probes in cells of pathogenic and normal biological tissues, cultivated on a solid nutrient medium, have been investigated by laser kinetic fluorimetry. It is proposed to use the Hyamatrix biopolymer as a nutrient medium. This polymer is formed on the basis of native, chemically unmodified hyaluronic acid and contains amino acids, microelements, vitamins, and other components. The possibility of using the obtained results to develop an alternative method of fluorescent diagnostics of biological tissues is discussed.**

Keywords: *laser diagnostics of biological tissues, cell cultivation, molecular probes, long-term luminescence.*

1. Introduction

Optical methods of study are widely used in various fields of biology and medicine, in particular, to develop methods of diagnostics of pathologies in biological tissues [1]. In optical diagnostics the state of biological tissues or cells extracted from them is generally estimated from the scattered light intensity [2] or from the fluorescence spectra of endogenous or exogenous fluorophores [3 – 6].

The main problems of such studies are insufficiently high accuracy and reproducibility of photometric measurements. These drawbacks can be eliminated by choosing the measured parameter to be not the desired signal intensity but the lifetime of excited states of fluorophores, which is estimated from the kinetics of photoprocesses with their participation. Living cells must be at hand to develop diagnostic methods. The necessary vitality of cells can be provided by growing them on special nutrient media.

In this paper, we demonstrated the possibility of cultivating somatic cells (extracted from normal and pathogenic tissues of animals) in the Hyamatrix nutrient medium and investigated the kinetics of long-term luminescence of exogenous fluorophores in these cells.

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2. Experimental

Hyamatrix biomaterial is a polymer plate prepared by photochemical cross-linking of macromolecules in a hydrogel based on native, chemically unmodified hyaluronic acid [7, 8]. Amino acids, microelements, vitamins, and other components were added to the hydrogel of hyaluronic acid.

Cells for cultivation were extracted from the mammary gland of healthy female mice of the BYRB line and individuals with spontaneous malignant tumours. Fragments of milky gland tissue were placed in a collagenase solution with a concentration of 0.5 mg mL^{-1} in a phosphate buffer (pH = 7.4) and incubated at a temperature of 37 °C [9].

After the end of enzymatic dissociation the cell suspension was deposited on a biomaterial plate with a thickness of 0.25 mm and density of 340 kg m^{-3}. The biomaterial with cells was placed in a Petri dish and incubated under standard conditions [10, 11].

Cuts of the biomaterial with cells were prepared for photographing according to the standard technique for histological preparations. The absorption spectra of the polymer plate were measured on a Solar SM-2203 spectrophotometer and the IR absorption spectra and frustrated total internal reflection spectra were recorded on a Varian 3100 FT-IR Fourier spectrophotometer.

The biomaterial structure was investigated using a CMM-2000 scanning atomic-force microscope (AFM). The AFM images in air were obtained using triangular cantilevers (rigidity 0.01 N m^{-1}) with a pyramidal tip having a radius of curvature $r \sim 15 - 25$ nm.

The oxygen permeability of the nutrient medium was investigated by laser flash-photolysis and estimated from the quenching of the delayed fluorescence (DF) and phosphorescence of the molecular probes introduced into the biomaterial by molecular oxygen. The probes were organic dyes of the xanthene series: erythrosine, rose Bengal, and eosin. This class of dyes was chosen because of their appropriate spectral-luminescence characteristics: high quantum yield to the triplet state, intense DF and phosphorescence [12], good solubility in water, and ability of penetrating into cells [13].

The setup for studying the DF and phosphorescence decay kinetics of dye molecules was based on a solid-state pulsed Nd : YAG laser (Fig. 1). Molecules were excited by the second-harmonic radiation ($\lambda_{exc} = 532$ nm). The pulse width was 10 ns, and the pulse energy varied from 10 to 50 mJ. The probe DF was recorded by an MDR-41 monochromator and an FEU-84 photoelectron multiplier.

The samples for study were placed in a special evacuated chamber. The air pressure (oxygen concentration) above the surface of coloured biopolymer changed from 0.01 to 760 Torr. The rate of decrease in air pressure in the chamber was chosen to gradually reduce the oxygen concentration in the cells but prevent the intracellular fluid from pronounced evaporation or freezing. Collection, accumulation, and primary processing of the experimental data were performed on an automatic system including a personal computer and CAMAC crate with a transient response leading edge of 200 ns.

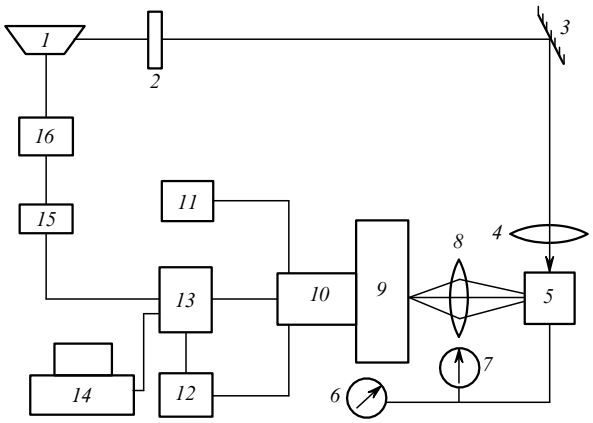

Figure 1. Schematic of the experimental setup: (*1*) solid-state Nd : YAG laser; (*2*) SZS-22 light filter; (*3*) rotational mirror; (*4*, *8*) focusing lenses; (*5*) evacuated chamber with samples; (*6*) vacuum pump; (*7*) manometer; (*9*) MDR-41 monochromator; (*10*) FEU-84; (*11*) power supply of FEU-84; (*12*, *15*) pulse generators, (*13*) CAMAC crate; (*14*) computer; and (*16*) laser power supply and control.

3. Results and discussion

Figure 2 shows the structure of Hyamatrix biopolymer. The AFM-image scan size was 1.7×1.7 μm. The bright segment in Fig. 2a shows the area where the dependence of the relief height h on the coordinate x was investigated (Fig. 2b). The difference in the heights corresponding to the beginning and end of the segment is 35.02 nm, and the difference in the ordinates of the intersection points of vertical markers with the dependence obtained is 2.235 nm (markers indicate the void boundaries). The coordinates of the first and second markers are, respectively, 354.9 and 435.8 nm; i.e., the void size is 80.88 nm. On the whole, the size of the void between filaments changes from 50 to 200 nm throughout the sample.

This spatial organisation of the material endows the nutrient medium with peculiar properties: plasticity, enhanced adhesion, drain ability, transparency, etc. It also significantly improves the biocompatibility due to the metabolisation of the material during cell cultivation. Possible changes in the character of intermolecular interactions under UV irradiation were investigated by IR spectroscopy. Figure 3 shows the IR absorption spectra of the Hyamatrix nutrient medium and the polymer film obtained by coating the substrate with the initial hydrogel. After UV irradiation the intensity ratio of the absorption bands changes only slightly, and a new peak arises at a wavelength of 870 cm^{-1}. The peak is most likely to be due

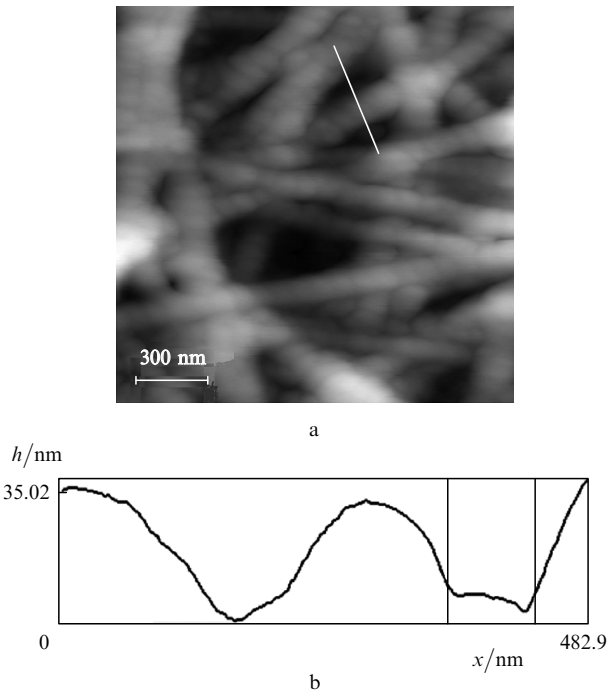

Figure 2. (a) AFM image and (b) surface relief of Hyamatrix biomaterial along the segment shown in panel (a).

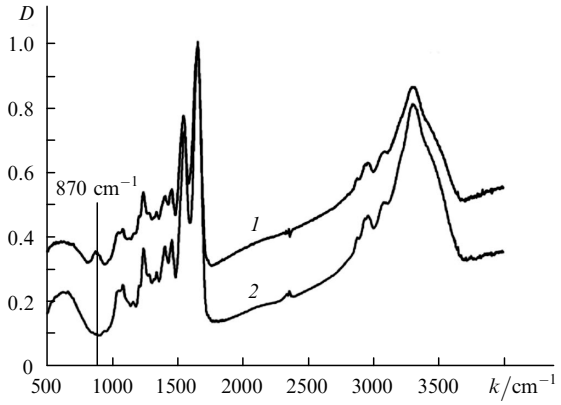

Figure 3. Normalised IR absorption spectra of (*1*) Hyamatrix biopolymer and (*2*) the polymer obtained from the initial hydrogel without UV irradiation.

to the C–C vibrations, symmetric or asymmetric C–O–C vibrations, or bending vibrations of C–H groups. However, on the whole, the used conditions of UV irradiation did not lead to significant degradation of the medium, and most macromolecules were not destroyed.

The absorption spectra of the biomaterial contain two wide structureless peaks in the UV region. The short-wavelength absorption ($\lambda < 230$ nm) is mainly due to the hyaluronic acid molecules, while the long-wavelength absorption peak ($\lambda = 280$ nm) is caused by the total absorption of the amino acids entering the composition of the nutrient medium.

To study the oxygen permeability of this medium and compare the oxygen content in the cells extracted from different tissues, we measured experimentally the decay kinetics of the long-term luminescence of organic dyes after pulsed photoexcitation. Delayed fluorescence is selective

with respect to oxygen, which is an effective quencher of the triplet states of probe molecules [8–10]. The character of DF decay allows one to draw unambiguous conclusions about the presence or absence of oxygen in the medium.

The DF kinetic curve of erythrosine in a polymer film without cells is an exponential with a decay rate constant $K = 1.8 \times 10^4$ s^{-1}. Xanthene dyes are characterised by small singlet–triplet splitting and exhibit thermally activated DF (TADF) [11, 12]. In the absence of quenchers this monomolecular process for erythrosine has a decay time of about 700 μs. The decrease in the TADF lifetime is due to presence of quenchers of the triplet states of molecules in the medium. The change in the air pressure above the surface of a coloured polymer film without cells from 150 Torr to atmospheric barely affects the DF kinetics of the dyes present in the film. This fact indicates penetration of erythrosine into the regions of nutrient medium that are difficult to access for oxygen.

The cultivated cells are located mainly in the thin layer on the biopolymer surface. An analysis of the transverse cuts of biomaterial with cells stained by hematoxylin–eosin indicates that actually a cell monolayer is formed in the surface layer.

The character of the kinetics of long-term luminescence of dyes in the cells deviates significantly from exponential (Fig. 4). The cells located in the surface layer of nutrient medium contain oxygen, which freely diffuses in the intracellular fluid. In this case, singlet oxygen $^1\Delta_g(O_2)$ is formed during sensitised occupation in the reaction

$$T + {}^3\Sigma_g^-(O_2) \rightarrow S_0 + {}^1\Delta_g(O_2)$$

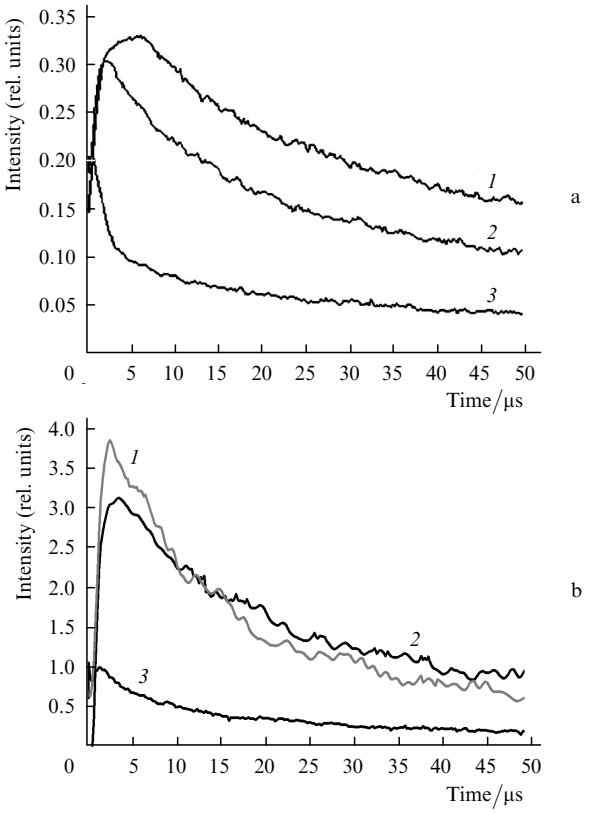

Figure 4. DF kinetics of erythrosine (*1*, *2*) in the cells of (*1*) normal and (*2*) tumour tissues and (*3*) in the biomaterial (a) at an air pressure of 150 Torr above the sample surface and (b) at an atmospheric pressure.

At the initial instant the concentration of $^1\Delta_g(O_2)$ excited molecules is zero, and only TADF is recorded. Then the DF signal increases for some time, making the kinetic curve typically humpbacked. As was shown in [14–17], this is due to the annihilation of the remaining (unquenched) triplet excited dye molecules with migrating $^1\Delta_g(O_2)$ molecules, as a result of which singlet states S_1 of fluorophores are formed and additionally contribute to DF:

$$T + {}^1\Delta_g(O_2) \rightarrow S_1 + {}^3\Sigma_g^-(O_2).$$

Thus, the recorded DF kinetics is a superposition of two signals of different nature; each of them contains information about the state of the medium.

A change in the oxygen concentration in cells changes both the intensity and lifetime of fluorophore DF; this is related to the activation of the annihilation process $T \rightarrow {}^1\Delta_g(O_2)$. The experimental curves clearly indicate that the DF kinetics in cells changes with an increase in the air pressure above the sample surface. The increase in the signal intensity and the decrease in its accumulation time are indicative of the increase in the oxygen concentration in the cells [16, 17]. Note that effective singlet–triplet annihilation and DF enhancement are observed only in living cells.

Under normal conditions the dominant process in cytoplasma cells, which determines the character of the endogenous fluorophore DF, is quenching of their triplet states by molecular oxygen with subsequent annihilation: $T \rightarrow {}^1\Delta_g(O_2)$.

Taking this circumstance into account and assuming that the migration of oxygen molecules in the cells is fairly efficient, one can use the formal kinetic approach to describe the processes observed. Within this approximation the total DF intensity of molecules is determined by the expression [16, 17]

$$I_{DF}(t) = \varphi_{fl} k_2 p_S N_T(t) N_\Delta(t) + \varphi_{fl} k_1 N_T(t), \qquad (1)$$

where φ_{fl} is the quantum fluorescence yield; p_S is the probability of forming the S_1 states of molecules in the annihilation reaction $T \rightarrow {}^1\Delta_g$; k_1 is the singlet–triplet intersystem crossing rate; k_2 is the triplet–singlet annihilation rate; N_T is the concentration of molecules in the triplet state; and N_Δ is the concentration of singlet oxygen.

The first and second terms in (1) determine, respectively, the annihilation and thermally activated components of the luminescence. The short-time kinetics $I_{DF}(t)$ is mainly determined by the factor $N_\Delta(t)$ in the annihilation component, because the contribution of the second term in (1) is small.

As can be seen in Fig. 4a, at an air pressure of 150 Torr above the sample surface, the DF enhancement time in the cells extracted from a normal tissue (7 μs) is twice longer than that for the cells from pathogenic tissues (3.5 μs). At an atmospheric pressure (Fig. 4b) the DF lifetimes for molecules in the cells of normal and pathogenic tissues are 35± 3 μs and 20 ± 2 μs, respectively.

In contrast to DF, the phosphorescence intensity of the molecules monotonically changes with time. Figure 5 shows the phosphorescence decay kinetics of erythrosine in the cells extracted from normal and tumour tissues at an atmospheric pressure. Detection was performed at a wavelength of 680 nm. It can clearly be seen that the

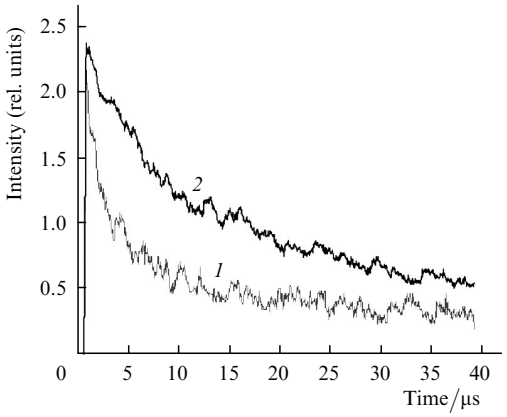

Figure 5. Phosphorescence decay kinetics of erythrosine in the cells extracted from (*1*) normal and (*2*) tumour tissues.

phosphorescence kinetics is different in cells of different type. In pathogenic cells the phosphorescence is quenched less, and the lifetime of the triplet states of dye molecules is longer.

The curves in Fig. 5 are approximated by the function

$$I_{\text{phs}}(t) = A_1 \exp\left(-\frac{t}{\tau_1}\right) + A_2 \exp\left(-\frac{t}{\tau_2}\right). \qquad (2)$$

The values of the parameters τ_1 and τ_2 are, respectively, 2.8 ± 0.3 μs and 55 ± 5 μs for curve (*1*) and 5.2 ± 0.5 μs and 41 ± 4 μs for curve (*2*).

The DF and phosphorescence kinetics was measured for two days during cell cultivation. No changes in the kinetics were revealed during this time.

It was suggested in [18] that hypoxia occurs in pathogenic tissues. The results of our experiments confirm this suggestion.

4. Conclusions

To study the regularities of the long-term luminescence of exogenous fluorophores, one needs cells that can retain vitality for a long time. The multicomponent biomaterial Hyamatrix can be used as a nutrient medium for cells. This medium not only provides the necessary cell vitality under *in vitro* conditions but also maximally approaches the experimental conditions to *in vivo* conditions, because its optical characteristics barely differ from those of living biological tissues.

The reliably detected differences in the kinetics of long-term luminescence of exogenous fluorophores in the cells of normal and pathogenic biological tissues can be used to develop an alternative method of laser fluorescent diagnostics based on measuring the lifetime of the fluorophore triplet states. We assume that the high sensitivity of this method will make it possible to detect pathological changes in cells at a very early stage. Any compounds exhibiting intense DF or phosphorescence and satisfying the requirements imposed on these preparations according to biological criteria can be used as exogenous fluorophores.

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References

1. Nenasheva T.A., Mashanov G.I. *Biofiz*., **51**, 454 (2006).
2. Tuchin V.V. *Usp. Fiz. Nauk*, **167**, 517 (1997).
3. Giraev K.M., Ashurbekov N.A., Medzhidov R.T. *Opt. Spektrosk*., **95**, 874 (2003) [*Opt. Spectrosc*., **95**, 819 (1995)].
4. Tchernyi V.V., Rogatkin D.A., Bychenkov O.A., Polyakov P.Y. *Proc. SPIE Int. Soc. Opt. Eng*., **5693**, 336 (2005).
5. Fu Sh., Chia T.Ch., Kwek L.Ch., Diong Ch.H., Tang Ch.L., Choen F.S., Krishnan S.M. *Proc. SPIE Int. Soc. Opt. Eng*., **5139**, 199 (2003).
6. Borisova E., Troyanova P., Pavlova P., Avramov L. *Kvantovaya Elektron*. **38**, 597 (2008) [*Quantum Electron*., **38**, 597 (2008)].
7. Rakhmatullin R.R., Pozdnyakov O.A. RF Patent No. 2367476; *Byull. Izobret*., No. 26, 12 (2009).
8. Rakhmatullin P.P., Barysheva E.S., Rakhmatullina L.R., Mitrofanova I.R. *Vestn. Orenburgskogo Gos. Univ*., **6**, 88 (2010).
9. Letuta S.N., Maryakhina B.C., Rakhmatullin P.P., Zabirov R.A. RF Patent No. 24009664; Priority of 29.04.2009.
10. Lezhnev E.I., Pankratov V.P., Koshevoi Yu.V. *Upravlyaemoe kul'tivirovanie kletok* (Controlled Cell Cultivation) (Moscow: Nauka, 1974) p. 270.
11. Conkie D., Erba E., Freshney R., Griffiths B., Hay R., Lasnitzki I., Maurer H., Morasca L., Wilson E. *Animal Cell Culture* (New York: Wiley, 1989).
12. Rohatgi-Mukherjee K.K., Mukhopadhyay A.K. *Indian J. Pure Appl. Phys*., **14**, 481 (1976).
13. Dobretsov G.E. *Fluorestsentnye zondy v issledovanii kletok and membran i lipoproteinov* (Fluorescent Probes in Study of Cells, Membranes, and Lipoproteins) (Moscow: Nauka, 1989) p. 277.
14. Kenner R.D., Khan A.U. *J. Chem. Phys*., **64**, 1877 (1976).
15. Bryukhanov V.V., Ketsle G.A., Laurinas B.Ch., Levshin L.V. *Opt. Spektrosk*., **60**, 205 (1986).
16. Kucherenko M.G., Ketsle G.A., Mel'nik M.P., Letuta S.N. *Izv. Ross. Akad. Nauk, Ser. Fiz*., **57**, 175 (1993).
17. Kucherenko M.G., Ketsle G.A., Mel'nik M.P., Letuta S.N. *Opt. Spektrosk*., **78**, 649 (1995) [*Opt. Spectrosc*., **78**, 621 (1995)].
18. Gorenkov R.V., Karpov V.N., Rogatkin D.A., Shumskii V.I. *Biofiz*. **52**, 711 (2007).