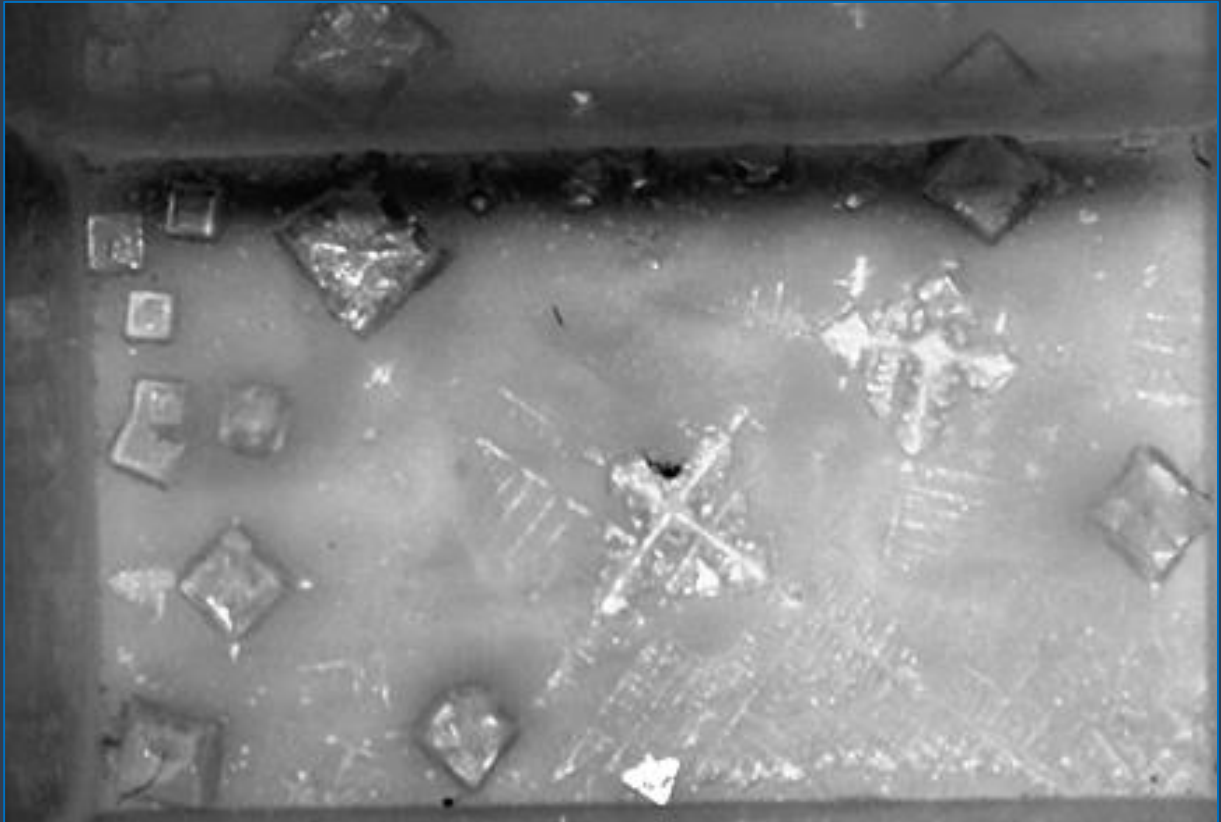


Special Issue

"Laser and optical technologies in biomedicine and ecology"

Journal of Biomedical Photonics & Engineering



Special Issue Editors

Aleksandra M. Mayorova, Valery P. Zakharov

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Special Issue

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Used Figure from this issue on the Cover

Alexander N. Malov, et al. On the mechanism of the gallstone nucleus formation and the impact of laser radiation on its growth, 020306, Fig. 3 (adopted).

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Foreword to the Special Issue on Laser and optical technologies in biomedicine and ecology

We are pleased to present the Special Issue of the Journal of Biomedical Photonics & Engineering, which focuses on laser and optical technologies for biomedical and ecological applications. This Special Issue consists of the selected papers of the Workshop “Biophotonics” of the XIV all-Russian Youth Samara conference-contest on optics and laser physics (Samara, November 8-12, 2016).

The XIV Youth Samara conference-contest on optics and laser physics was organized by Samara Branch of P.N. Lebedev Physical Institute of Russian Academy of Sciences and Samara National Research University with support of Russian Foundation for Basic Research (grant 16-32-10320), Administration of Samara Region and Samara SPIE Student Chapter. The conference program included lectures of leading scientists, reports of young PhDs and contest reports of students, graduate students and young researches on wide range of theoretical and experimental issues of coherent, physical and quantum optics, spectroscopy, the interaction of light with matter, nanophotonics and other contemporary topics of optics and laser physics. Many interesting reports on applications of laser and optical techniques in biomedicine and ecology were presented at the Workshop “Biophotonics”. The Special issue of JBPE will introduce you to some of them.

Laser speckle contrast imaging (LSCI) is a perspective noninvasive, contactless technique for visualization of capillary blood flow in a real-time without laser beam scanning. **Polina A. Timoshina** and co-authors used LSCI for study of the change in blood microcirculation of the pancreas in rats with alloxane-induced diabetes and under influence of optical clearing agent “Omnipaque TM -300”. The authors demonstrated that Omnipaque TM -300 caused changes in the blood flow in the pancreatic vessels only in diabetic mice and suggested that this fact allowed using this clearing agent for monitoring of certain complications caused by some diseases.

Alloxane-induced diabetes of rats was also studied by **Daria K. Tuchina** and co-authors. In particular, the authors carried out the comparative analysis of glycerol diffusion rate in skin and myocardium of rats in normal condition and under the conditions of model alloxan-induced diabetes. The changes in diffusion rate of glycerol clearing agent reflects the changes of tissue structure and can be used as a biomarker.

The Issue includes two papers of the researchers from Irkutsk Technical National University and Irkutsk State Medical University: **Alexander N. Malov** and co-authors. In [the first study](#) the experimental research of influence of laser radiation on the bacteria's growth dynamics in the nutrient medium as well as on the antibiotics activity is presented. The authors propose that the decrease in the colonies growth or the increase of the antibiotics efficiency under the laser radiation impact occurs due to the fact that the laser radiation breaks the molecules clusters of the organic solution or of the antibiotic accordingly. And in [the second paper](#) on the basis of published data and their own experimental results A. Malov and co-authors suggest possible mechanisms of the formation of gallstones and effect of laser radiation on their growth.

The paper of **Vladislav S. Krasnoukhov** and co-authors has an ecological focus. The authors studied the reaction between cyclopentadienyl and methyl radicals. The practical importance of the study is determined by the fact that this reaction is a potential source of polycyclic aromatic hydrocarbons having a large negative impact on the environment and human health. The authors found the reaction paths, clarified the energies of compounds and obtained the rate constants of some reactions.

The research by **Anastasiya N. Markina** and **Aleksandr A. Fedotov** is focused on the development of new approaches for diagnostic of arterial vessels. The authors suggest that the signal analysis of the pulse wave is noninvasive, relatively cheap and safe diagnostic technique and so it can be used as the most suitable method of screening diagnostics of atherosclerosis.

The paper of **Natalia S. Ksenofontova** and co-authors is devoted to investigation of optical immersion clearing of laboratory animal's skin *in vivo*. A comparative analysis of the changes in the optical depth of the skin detection at preliminary laser ablation of epidermis, the application of immersion agent and the combination of these two techniques is provided.

The principal possibility of the selection of lung cancer samples among the tumors of other localizations by means of Raman spectroscopy and autofluorescent analysis was studied by **Ludmila A. Shamina** and co-authors. Raman and autofluorescence spectra of blood and urine from patients with cancers of different localization were obtained and analyzed with PLS method. The authors demonstrated that simultaneous study of two human body fluids allows increasing informativeness of the analysis.

The study by **Anastasiya A. Lykina** and co-authors is directed on the development of non-resonant techniques of Raman spectroscopy for albumin analysis. The authors demonstrated that it is possible to increase the registration efficiency of Raman scattering spectra using special cuvette-reflectors of a certain shape. The quantitative characteristics of low albumin's concentrations with dependence on the shape and geometry of the cell were determined, which is very important for development of justified recommendations for biomedical applications.

Nikolay P. Kozlov and Rafael I. Fairushin used the Fourier spectroscopy technique for research of the oxidative degradation of polypropylene under the impact of ultraviolet radiation. The object of study is a polypropylene mesh, which is widely used in medicine. The presence of through-holes in the mesh allows passing only a part of the IR radiation onto the sample determining the features of processing spectra of such meshes.

Thus, the selected papers presented in this Special Issue, introduce you to the major current issues discussed at the workshop "Biophotonics" of the XIV All-Russian Youth Samara conference-contest on optics and laser physics.

Special issue Editors:

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Study of blood microcirculation of pancreas in rats with alloxan diabetes by Laser Speckle Contrast Imaging

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Abstract. The blood microcirculation of the pancreas in rats with diabetes was studied using Laser Speckle Contrast Imaging (LSCI). The impact on blood flow of x-ray contrast “OmnipaqueTM-300” ($n = 1.438$) and aqueous solution of “OmnipaqueTM-300” ($n = 1.407$) used as optical clearing agents (OCAs) was also investigated. The alloxan induced animal model of diabetes was exploited. The results obtained in the study of blood microcirculation disorders of pancreas in diabetes and under topical application of optical clearing agents show that disease development in animals causes changes in the microcirculatory system response to application of “OmnipaqueTM-300” solutions. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: speckle, blood flow, speckle contrast, diabet.

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1 Introduction

Many diseases, such as cardio-vascular diseases, atherosclerosis, diabetes, chronic venous insufficiency, cause functional and morphological changes of blood flow in single vessels and blood microcirculation within the microvasculature. In the case of diabetes, diabetic angiopathy (generalized defeat of blood vessels) arises. There are two types of diabetic angiopathy: microangiopathy arising as result of destruction of small vessels – capillaries, arterioles, and venules, and macroangiopathy – ischemic brain disease, peripheral vascular occlusion - arising as result of destruction of large vessels [1]. Diabetes is characterized by the elevation of blood glucose level, for the reason that the peptide hormone insulin is produced insufficiently in the beta cells of the pancreas (type I insulin-dependent), or the body cells not responding properly to the insulin produced (Type II, non insulin-dependent or “adult onset diabetes”) [2,3]. According to the data of World Health Organization, the incidence of diabetes in the world is about 347 million people. Diabetes is ranked as the third in the world after cardiovascular and oncological diseases. Hemodynamic changes in diabetes contribute to the emergence of hypoxemia in various organs, leading to retinopathy, neuropathy, nephropathy, coronary heart disease, and peripheral arterial disease. Thus, all these changes in blood flow and microcirculation within the microvasculature are primarily associated with endothelial dysfunction. The multiple functions of vascular endothelium include regulation of vessel integrity, vascular growth and remodeling, tissue growth and metabolism, immune responses, cell adhesion, angiogenesis, hemostasis and vascular permeability. Endothelial dysfunction is characterized by the following features: reduced endothelium-mediated vasorelaxation, hemodynamic deregulation, impaired fibrinolytic ability, enhanced turnover, overproduction of growth factors, increased expression of adhesion molecules and inflammatory genes, excessive generation of reactive oxygen species, increased oxidative stress, and enhanced permeability of the cell layer [4].

Currently, the most effective diagnostic methods for determining the physiological parameters of blood flow and microcirculation are the methods based on dynamic light scattering [5], as well as on principles of low-coherence optical coherence tomography (OCT), the so called Doppler OCT (DOCT) [6-8]. One of the prospective methods for the assessment of blood flow is the Laser Speckle Contrast Imaging (LSCI) [9-15]. When a diffuse object is illuminated with laser light, a random interference known as a speckle pattern is produced. If there is movement within the object, the intensity of speckles fluctuates correspondingly. These fluctuations can be used to provide information about the red blood cells (RBCs) movement in vessels. A simple way of accessing this information is to image the speckle pattern – the fluctuations cause a blurring of the speckle pattern, leading to a reduction of the local speckle contrast [9]. Thus, the LSI is a noninvasive,

contactless method that allows for visualization of capillary blood flow in a real-time without laser beam scanning. It is particularly attractive as an intra operative tool for measuring blood flow in ophthalmology, dermatology, neurosurgery and other fields of medicine, as it provides full-field relative flow speed maps with the use of only a coherent light source and a camera, both easily adaptable to conventional microscopes. Laser speckle contrast imaging (LSCI) techniques are based on the registration of spatial and temporal statistics of the speckle pattern, calculation of contrast of time-averaged dynamic speckles in dependence on the camera exposure time [9-16]. LSCI can be realized as Spatial (SLSCI) or Temporal (TLSCI) modifications. When SLSCI is used, contrast is calculated as the ratio of the standard deviation of the pixel intensity fluctuations to its mean value within selected area ($N \times N$ pixels). The TLSCI is based on registration of temporal statistics and calculation of the image speckle contrast using a sequence of raw speckle images acquired along a few time points instead of a spatial window, i.e. contrast is calculated as the ratio of the standard deviation of the intensity fluctuations for a particular pixel at different times and mean intensity for this pixel [15, 16]. The most useful modification appeared to combine these two techniques into one, called the Spatio-Temporal Laser Speckle Contrast Imaging (STLSCI). The contrast of the speckle image is calculated as the ratio of standard deviation of the intensity fluctuations for many pixels within a chosen area at different times and the pixel mean intensity averaged for all pixels of the area [17]. These techniques and some other LSI modifications, are often used in biomedical applications.

The applicability of the method is mainly determined by the imbedding depth of the blood vessels under study, being more accurate for superficial vessels. However the method of optical clearing, which reduces the efficiency of scattering from static scatterers, allows one to extend the applicability of the method to larger imbedding depths of the vessels. Combination of LSCI with immersion optical clearing demonstrates a high efficiency for evaluation of microhemodynamics of different tissues. For example, authors of Ref. [18] have quantified blood flow in arteries and veins in mouse skin due to a high quality of the speckle images. Also, LSCI was used in conjunction with confocal microscopy to monitor light-evoked changes in blood flow in retinal vessels [19]. This dual imaging technique permitted authors to stimulate retinal photoreceptors and measure vessel diameter with confocal microscopy while simultaneously monitoring blood flow with LSI. And the authors concluded that use of LSI to image retinal blood flow holds promise in elucidating the mechanisms mediating functional hyperemia in the retina and in characterizing changes in blood flow that occur during retinal pathology. Estimation of the impact of clearing agents on blood flow is of great importance for further application of this combined technology for more in depth imaging of blood hemodynamics. For

example, Cheng et al. used LSCI to evaluate quantitatively OCA-induced changes of blood flow [20]. They found that after removing the rabbit skull and a small area of dura mater, the application of glycerol on the dura mater around the exposed cortex decreases the cerebral blood flow by 20-30% immediately. Zhu et al. [21] applied the LSCI to investigate both the short-term and long-term effects of glycerol and glucose on blood vessels in chick chorioallantoic membrane (CAM).

In this study, we present results of blood flow measurements within the microvasculature of the pancreas in rats with diabetes and under influence of optical clearing agents by using Laser Speckle Contrast Imaging (LSCI). The influence of “Omnipaque™-300” (radiopaque agent, active substance of iohexol) and of aqueous solution of “Omnipaque™-300” was investigated.

2 Methods and Materials

2.1 Laser speckle contrast imaging system

The local estimation of the contrast K for the fixed exposure time done within the areas with given number of speckles makes it possible to image tissue regions with essentially different velocity of scatterers [9, 10]:

$$K_k = \sigma_{I_k} / \bar{I}_k \quad (1)$$

where k is the number of frames in a sequence of speckle-modulated images, $\bar{I}_k$ and σ_{I_k} are the averaged over the analyzed frame scattered light intensity and the *rms* (root-mean-square) value of the fluctuation component of the pixel's brightness, respectively:

$$\bar{I}_k = (1/MN) \sum_{m=1}^M \sum_{n=1}^N I_k(m, n), \quad (2)$$

$$\sigma_{I_k} = \sqrt{(1/MN) \sum_{m=1}^M \sum_{n=1}^N \{I_k(m, n) - \bar{I}_k\}^2}, \quad (3)$$

where M and N are the number of pixels in rows and columns of the analyzed area of the frame, respectively; $I_k(m, n)$ is the brightness of the (m, n) -pixel of the k -frame.

To perform the measurements and calculate contrast, we developed a software in the LabVIEW 8.5 environment (National Instruments, USA) that allows for real time recording of the intensity distribution of the speckle field with the rate of 100 frames per second. The monitoring of blood microcirculation in rats was carried out using a homemade experimental setup (Fig. 1). The observation area of the system was 1.5×1.5 mm². Within this area, the spatial distribution of the speckle contrast was calculated from the measurements of intensity fluctuations by using Eqs. (1) – (3). The local contrast was quantified as the mean contrast within the small area of 7×7 pixels. Operator has a possibility to control field of view (FOV) of the optical system to provide measurements within a single vessel with directional blood flow. In the setup, He–Ne laser GN-5P

(Russia) was used as a light source ($\lambda = 632.8$ nm). The monochrome CMOS camera (Basler A802f, number of pixels in the matrix 656×491 , pixel size 9.9×9.9 μm, 8 bit per pixel) with the fixed exposure time T of 10 ms, combined with the LOMO objective ($10\times$, St. Petersburg, Russia) was used as a detector. Figure 2 shows the spatial distribution of the measured local contrast (a) in coherent light, and its normalized distribution (b).

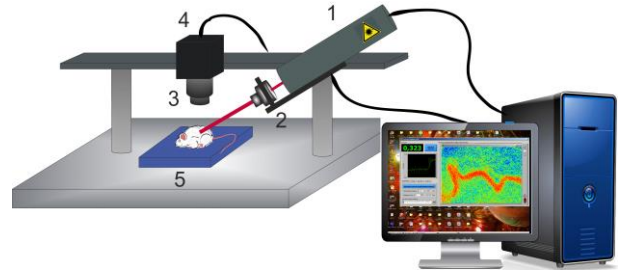


Fig. 1 1-He-Ne laser (632.8 nm); 2-objective (LOMO 20 $\times$); 3-microscope tube lens with objective (LOMO 10 $\times$); 4-CMOS-camera Basler A602f (656×491 pxls, pxl size 9.9×9.9 μm, 8 bits/pxl); 5- rat under study.

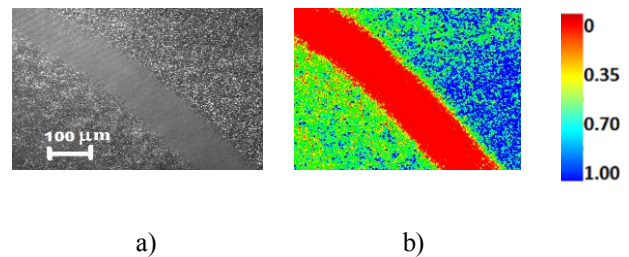


Fig. 2 The images of a vessel (diameter 280 μm): speckle image in laser light (632.8 nm) (a) and corresponding speckle contrast image as a calculated distribution of speckle contrast (1-0) (b).

The problem of quantitative velocity measurements is associated with understanding the inter-connection between the contrast of speckles and the velocity of scattering centers (or velocity distribution) [22]. As there is no an adequate theoretical description of this inter-connection, calibration should be used for the quantitative evaluation of the blood flow. This comparative study was conducted using a specially designed tissue phantom with capillaries (Fig. 3) [23]. The phantom had the following structure: the basic material was the epoxy resin with the inserted TiO₂ (3 mg/ml, diameter 200 nm) and SiO₂ (150 mg/ml, diameter 1000 nm) nanoparticles serving as scattering centers and Indian Ink (0.2 μl/ml) as an absorber; it was designed as a multi-layer plate of $27 \times 21 \times 3.2$ mm; thickness of the top layer (solid) was 150 μm; the second layer (porous) consisted of 29 capillaries with diameter of 180 μm; thickness of the third layer (solid) was 1 mm; the fourth layer (porous) consisted of 29 capillaries with diameter of 180 μm; thickness of the fifth layer (solid) was 150 μm; the sixth layer (porous) consisted of 58 capillaries with diameter of 180 μm; thickness of the seventh layer (solid) was 1.54 mm. Optical properties measured at 830 nm were similar to soft tissues with absorption coefficient of 0.48 cm^{-1} ,

reduced scattering coefficient of 12.5 cm^{-1} , and anisotropy factor of 0.88. The flow of scattering fluid through this phantom modelling blood flow at specified velocities was provided by using a dispenser of drugs (MLW Lineomat, Germany).

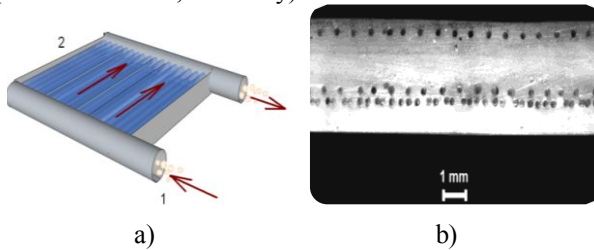


Fig. 3 Phantom: schematic illustration: 1-suspension of particles ($d \sim 7-9 \mu\text{m}$), 2-phantom (a); image of phantom cross-section (b).

Under the assumption of purely ordered flow, the speckle contrast K can be defined as follows [24-26]:

$$K = \frac{\sigma_s}{\langle I \rangle} = \left[\frac{\tau_c}{2T} \left\{ \sqrt{2\pi} \operatorname{erf} \left(\frac{\sqrt{2T}}{\tau_c} \right) - \frac{\tau_c}{T} \left(1 - \exp \left(-\frac{2T}{\tau_c} \right) \right) \right\} \right]^{1/2} \quad (4)$$

where σ_s is the standard deviation of the speckle intensity and $\langle I \rangle$ is the mean intensity, T is the exposure time of the camera, τ_c is time of correlation. Again, it is worth noting that the above equation is in actuality a cumulative distribution function of a Gaussian probability distribution function, which is characteristic to directed flows.

The simplest approach leads to a characteristic velocity v_c defined as follows [24-26]:

$$v_c = \lambda / 2\pi k \tau_c, \quad (5)$$

where λ is the source wavelength, k is the normalization factor which depends on the parameters of a Gaussian curve from Eq. (4), and the scattering properties of biological tissue or phantom. Calibration allowed us to determine the value of this coefficient as ~ 0.24 , thus we can build a theoretical curve by using Eqs. (4) and (5) and compare it with the calibration curve received for the selected exposure time of 10 ms (Fig.4).

The theoretical curve fits well to the experimental calibration curve within velocity range from 0.1 to 2.5 mm/s with the constant correction factor k . The presence of a correction factor was caused by multiple factors including the influence of static scatterers, random flow provided by upstream and downstream blood vessels, affecting value of the speckle contrast and thus causing reduction of accuracy of the flow rate estimates. These issues were discussed in the recently published papers [27-30].

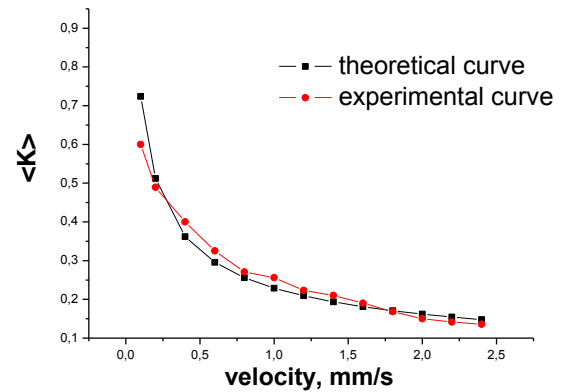


Fig. 4 Phantom studies with the selected exposure time of 10 ms: the relationship between speckle contrast and velocity of particles.

In this regard, we can introduce the concept of “reduced” velocity v' using Eqs. (4) and (5) to process phantom experimental data for contrast K at the particular exposure time of the camera T for the range of physiological velocities.

Reduced velocity v' can be associated with the velocity of blood flow determined from the speckle contrast K measurements for the further assessment of blood circulation in *in vivo* studies. Results were presented as mean $\pm$ standard error of the mean (SEM).

2.2 Animal models

We used 32 white laboratory rats weighing 300-500 g. Animals were divided into two groups: control (16 rats) and diabetic (16 rats). For modeling of diabetes in animals, we used alloxan-induced diabetes model [28, 29]. Experimental diabetes in rats was induced by a single subcutaneous injection of alloxan with a dose of 220 mg/kg body weight of the animal. Alloxan disturbed pancreas functioning leading to development of diabetes in rats [6]. Status of diabetes was confirmed by the increase of glucose level in the blood that was tested by using a commercial glucometer Accu-Chek Active (Roche Diagnostics, Germany). Average values of the glucose in the blood before the introduction of alloxan, and the day of the experiment (after 16 days) were 120 ± 16 , $403 \pm 105 \text{ mg/dl}$, respectively. We can assume that this is an initial stage of diabetes.

Under general anesthesia solution Zoletil 0.2 mL, laparoscopy was performed. Then the changes of blood flow were evaluated.

All procedures with animals were performed in strict accordance with “Rules for Conducting Qualitative Clinical Trials in the Russian Federation” (approved by the Ministry of Health of the Russian Federation and enacted on January 1, 1999), appendix 3 to Order No. 755 of the Ministry of Health of the USSR of 10.08.77, the provisions of WMA Declaration of Helsinki (2000) and the recommendations contained in the European Community Directives (No. 86 / 609EC).

In addition, vascular permeability studies were performed by applying optical clearing agent

“OmnipaqueTM-300” (a radiopaque agent for x-ray image contrasting with the active substance iohexol) and a dilute aqueous solution of “OmnipaqueTM-300” (7ml “OmnipaqueTM-300”, 3 ml of distilled water), namely, the analysis of its effects on blood flow in pancreatic vessels. The solutions were applied to the tissue site topically using a pipette in a volume of 0.5 ml.

The OmnipaqueTM is currently not yet widely used for tissue optical clearing, however a few impressive applications are already described, i.e., for subcartilaginous bone imaging by OCT [31], for clearing of ex vivo pig skin at two-photon microscopy [32], and to improve images of cerebral blood flow in newborn mice [33]. The major attractiveness of the agent is that it is a non-ionic X-ray contrast iodine-based solution that due to low osmolarity comparable with osmolarity of blood plasma and cerebrospinal fluid (CSF) does not cause tissue damage at prolonged topical application or intra-tissue injection [34].

3 Results and discussion

Results of the measurement of “reduced” velocity v' of blood flow in vessels of pancreas for control and diabetic groups of laboratory rats are shown in Fig. 5.

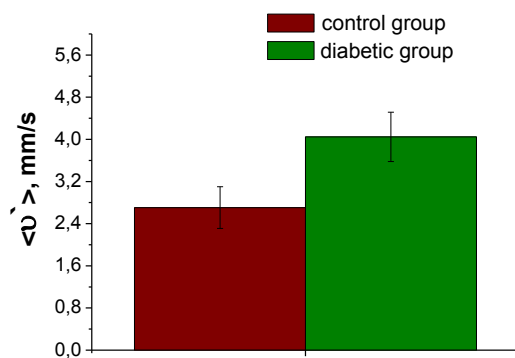


Fig. 5 Changes of blood flow in vessels of pancreas under alloxan diabetes, the difference with control is significant ($p < 0.5$).

The development of diabetes affected the change of blood flow relative to the control group of animals, the difference is significant ($p < 0.5$). From Fig. 5 it follows that the flow velocity in diabetic rats was 40% above the velocity of the control group.

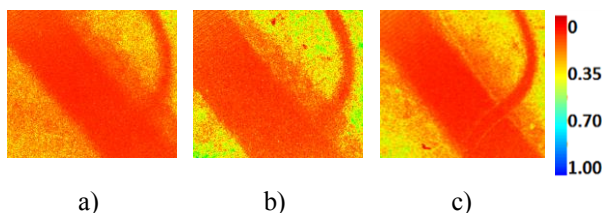
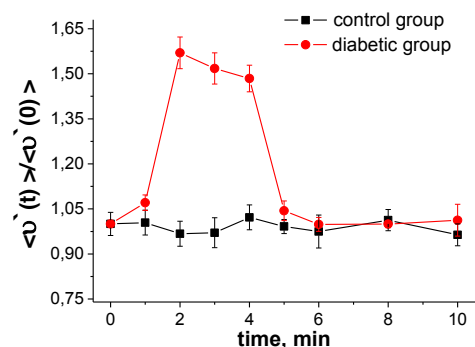


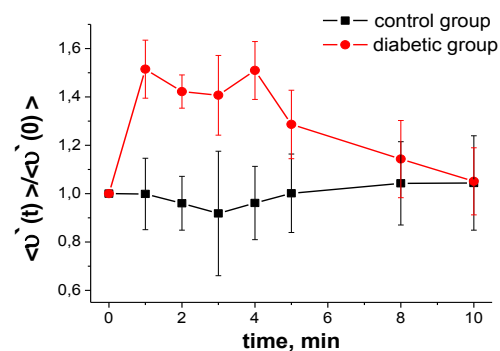
Fig. 6 Contrast images of blood vessels of pancreas after using aqueous solution of 70% “OmnipaqueTM-300”: a) without influence, b) after 1 min, c) 5 min.

Figure 6 shows the distribution of contrast of the speckle images when pancreas was subjected topically to an aqueous 70% solution of “OmnipaqueTM-300”. This is evident that use of the OCA improved the speckle contrast images of studied vessels to 5th min of its application.

Further, in Fig. 7 the normalized curves of changes of pancreatic blood flow of control and diabetic groups of laboratory animals under the influence of 100% “OmnipaqueTM-300” and aqueous solution of 70% “OmnipaqueTM-300”. Data were averaged over 8 laboratory animals for each group and for each solution, respectively.



(a)



b)

Fig. 7 Blood flow in the pancreas of rats in both groups exposed to 100% “OmnipaqueTM-300” (a) and 70% “OmnipaqueTM-300” (b).

Application of 100% “OmnipaqueTM-300” demonstrates 65%-increase of blood flow as 70% “OmnipaqueTM-300” gives 50%-increase of blood flow in the group of diabetic animals. In both cases to 10th min, blood flow velocity was completely restored. Blood flow in the control group did not show any noticeable changes. Increased blood flow after application of optical clearing agent could be caused by increased vascular endothelial permeability at diabetes even in early stages of disease. It was also found that the diameter of the vessel under the influence of the solutions did not change. Based on these results, it is worth noting that “OmnipaqueTM-300” causes some changes in the blood flow in the pancreatic vessels only in diabetic mice, and this fact should be taken into

account in the diagnostic applications of the combined technology that is proposed in this study. Thus, application of “OmnipaqueTM-300” could be used not only for getting of better speckle images of blood flow distribution in the living organ at surgical procedures, but also for monitoring of some complications related to increased vascular endothelial permeability like at diabetes.

The assessment of the effect of the agent on blood flow by LSCI opens very urgent perspectives to combine blood circulation measurements with tissue optical clearing in the course of transplantation. It is important to note that practically all OCAs are cryogenic agents used for keeping living organs before transplantation [35].

4 Conclusion

The calibration method of speckle contrast imaging using a phantom simulating directed blood circulation in the biological tissue, allows one to calculate the spatial distribution of the contrast and evaluate the dependence of contrast speckle – image relative velocity of RBC. This allows one to move from a qualitative analysis of blood flow alterations to the quantitative, namely the “reduced” velocity v'_c determined with the help of phantom calibration. Due to phantom calibration laser speckle contrast imaging may be useful for quantitative

monitoring of microhemodynamics of pancreas and other organs and study of vascular endothelial permeability at diabetes.

The ability of LSCI to measure blood flow velocity in a real time is prospective feature to be used in transplantation technologies and in emergency surgery to assess the state of internal organs and microhemodynamic pathologies. Also, one of the prospective applications of LSCI technique is a noninvasive monitoring of cerebral blood flow in the brain of small animals without craniotomy under conditions of immersion of optical clearing of the scalp and cranial tissues by means of hyperosmotic agents.

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Study of glycerol diffusion in skin and myocardium *ex vivo* under the conditions of developing alloxan-induced diabetes

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Abstract. Diabetes mellitus is one of widespread diseases, the development of which impairs the functioning of organism vital organs. The study of the processes that occur in tissues under diabetic conditions facilitates the development of diabetes diagnostics and treatment methods, as well as the prognosis of its complications. The paper reports a comparative study of glycerol diffusion in skin and myocardium of rats *ex vivo* in the normal condition and the conditions of alloxan-induced diabetes. The glycerol diffusion coefficients in tissues were determined basing on the analysis of kinetics of collimated light transmittance through tissue samples immersed in 70%-glycerol solution. As a result, it was shown that the glycerol diffusion rate in the tissues of rats with alloxan-induced diabetes is essentially decreased as compared to the control group. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Skin; Myocardium; Diffusion coefficient; Permeability coefficient; Glycerol; Alloxan-induced diabetes; Collimated transmittance; Optical clearing.

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1 Introduction

Diabetes mellitus is an endocrine disease characterised by the increased content of glucose in the organism. According to the data of the World Health Organisation in 2012 - 2013 the diabetes mellitus caused nearly five millions deaths, which made it the eighth factor of mortality in the world [1]. The worldwide number of patients suffering from diabetes mellitus in 2030 is expected to increase by 54% as compared to 2010 [2], thus moving diabetes to the seventh position in the list of dangerous diseases [3]. One-third part of all patients with diabetes have complications in the form of skin lesions, the chronic hardly healed ulcers being the severest of them, fraught with the risk of infecting the entire organism [4]. The complications of diabetes mellitus are mainly related to the glycation of the human organism proteins, which comprise a considerable part of many tissues. Protein glycation is initiated by the non-enzymatic reaction between the protein amides and the sugar carbonyl group followed by the cross-linking of proteins [5, 6]. The glycation of proteins modifies the structure of tissues, restricts their functioning [6-8], causes metabolic imbalance and, finally, impairs the organ functioning [5, 9].

Since the permeability of tissue for chemical agents is determined by its structure [10], the variation of the diffusion behaviour and rate in the tissue reflects the structure change, which can be used as a biomarker [11], e.g., a biomarker of the tissue protein glycation degree. In turn, the degree of tissue glycation in available organs, such as skin, mucous and submucous membranes of hollow organs can be used to diagnose the tissue condition in hardly accessible organs, such as myocardium, retina, brain, thus predict the vitally dangerous complication of diabetes.

To quantify the diffusion rate of an agent in the tissue, one can use the method of tissue optical clearing [10] based on the action of a biocompatible hyperosmotic chemical agent (immersion agent) on the tissue. It is well known that tissues possess sufficiently strong light scattering due to the inhomogeneity of refractive index of their components. The action of the immersion agent on the tissue causes partial replacement of interstitial fluid by the agent and temporal dehydration of the tissue, which leads to refractive index matching between the tissue components and temporary tissue packing. Finally, both

processes reduce the light scattering in the tissue. The rate of light scattering reduction in the tissue is determined by the rate of the agent diffusion.

The present paper is devoted to the comparative study of glycerol diffusion rate in skin and myocardium of rats in normal condition and under the conditions of model alloxan-induced diabetes, the aim of the study is to determine the relation between the optical and diffusion properties of skin and myocardium in the course of model diabetes development.

2 Materials and methods

The studies were carried out using *ex vivo* samples of skin and myocardium of white outbred male laboratory rats having the weight of 500 ± 20 g. Hair from the skin surface was preliminarily removed by depilatory cream «Veet» (Reckitt Benckiser, France). The skin was treated with saline to remove the residual cream from the surface of the samples. The skin samples were extracted using surgical scissors, and then the subcutaneous fat hampering the penetration of hydrophilic agents into the dermis was removed from them. The samples of myocardium *in vitro* were prepared using a scalpel after the preliminary freezing of the whole heart of a rat. Before the measurements, the myocardium samples were washed in saline to remove the blood excess from the samples. In each series of experiments, ten samples were used, each of them having the area of nearly 10×15 mm².

The laboratory animals were divided into the control group and the diabetic one. The development of model diabetes in the rats of the diabetic group was induced by a single intramuscular injection of alloxan (Acros Organic, Belgium) [4, 9, 12, 13], mixed with saline in the proportion of 10 mg of alloxan per 100 g of the rat body mass. The mean values of free glucose in the blood taken after fasting from the caudal vein were measured by glucometer "Accu-Chek Performa" (Roche Diagnostics, Germany). Before alloxan injection, on the 7-th day after injection and on the day of experiment (on the 15-th day after the injection) the free glucose concentration amounted to 128 ± 18 , 411 ± 131 , and 350 ± 147 mg/dl, respectively. Thus, glucose level in blood increased by nearly 3 times, which is characteristically for diabetes. No alloxan was injected to the rats of the control group.

The experimental protocol on animal research was compiled as per the Helsinki Declaration, 2000 and approved by V.I. Razumovsky Saratov State Medical University ethical committee.

The aqueous 70%-solution of glycerol, used as an immersion agent, was prepared by mixing the dehydrated glycerol ("Trading depot No.1 of Chemicals", Russia) with the distilled water. The refractive index of the solution $n=1.427\pm0.001$ was measured at the wavelength 589 nm by the Abbe refractometer IRF-454B2M (LOMO, Russia).

Glycerol solutions of different concentrations are frequently used for optical clearing of tissues [11, 14–16]. Our choice of concentration for the glycerol solution was determined by its relatively low viscosity (22.5 cP) in comparison with the dehydrated glycerol (1410.0 cP) [17] and yet sufficiently high refractive index of 1.427 [17] (Table 1). Moreover, the studies of the efficiency of skin optical clearing using aqueous glycerol solutions of different concentrations [11, 14, 16] have shown that the concentration of 70% is close to optimal.

Table 1 Refractive index and viscosity of glycerol solutions of different concentration [17].

Concentration of glycerol solution, %	40	50	60	70	80	90	100
Refractive index	1.384	1.398	1.413	1.427	1.443	1.458	1.474
Viscosity, cP	3.7	6.0	10.8	22.5	50.1	219.0	1410.0

The spectra of collimated transmittance of the tissue samples were measured using the multichannel spectrometer USB4000-Vis-NIR (Ocean Optics, USA). The tissue sample was fixed at the plastic plate having the area $3.5\times1.5\text{ cm}^2$ with a hole in the centre having the area $8\times8\text{ mm}^2$ and placed in the glass cuvette having the volume 5 ml filled with the glycerol solution. The cuvette was installed between two fibre-optical cables QP400-1-VIS-NIR (Ocean Optics, USA) with the inner diameter 400 μm . To provide the beam collimation, the collimators 74-ACR (Ocean Optics, USA) were fixed at the ends of fibres by the standard connectors SMA-905. The halogen lamp HL-2000 (Ocean Optics, USA) was used as a source of light. The setup is schematically shown in Fig. 1. All measurements were performed at room temperature ($\sim 20^\circ\text{C}$).

using the electronic balance (Scientech, USA) with the accuracy of $\pm 1\text{ mg}$.

The measured kinetics of collimated transmittance of tissue samples were used for calculating the efficiency of their optical clearing, the coefficients of glycerol diffusion in tissues, and the permeability coefficients of biological tissues for glycerol. The method of estimating the diffusion coefficients for chemical agents used in the present study is described in detail in Refs. [10, 18]. In the calculations we allowed for the fact that glycerol diffuses into the skin mainly from the dermis side, while in the case of myocardium glycerol penetrates into the sample from both sides of the sample. To estimate the skin permeability coefficient P for glycerol (the case of one-sided diffusion), we used the expression [19]:

$$P = \frac{D}{l}, \quad (1)$$

where D is the glycerol diffusion coefficient in the tissue, l is the tissue sample thickness.

The permeability coefficient of myocardium (the case of two-sided diffusion) was calculated using the following expression [19]:

$$P = \frac{\pi^2 D}{l}. \quad (2)$$

The optical clearing efficiency for a tissue sample was determined at different wavelengths as the relative change of the scattering coefficient of the tissue under the action of the immersion agent:

$$\text{OC}_{\text{eff}} = \frac{\mu_{s0} - \mu_{s\text{min}}}{\mu_{s0}}, \quad (3)$$

where μ_{s0} and $\mu_{s\text{min}}$ are the initial and the minimal value of the scattering coefficient of the tissue sample determined from the values of attenuation coefficient ($\mu_t = \mu_a + \mu_s$). The latter was calculated from the

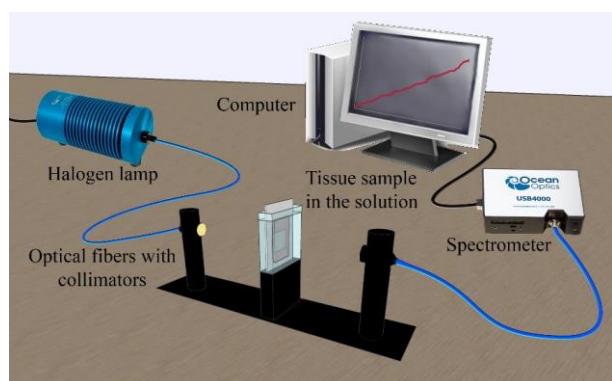


Fig. 1 Schematic diagram of the experimental setup for the measurement of collimated transmittance of *ex vivo* tissue samples.

The thickness and weight of the samples were measured before and after their placing into glycerol solution. For the thickness measurement, the samples were placed between two microscope slides. The measurements were performed at five points using a micrometer with the accuracy of $\pm 5\text{ }\mu\text{m}$ and then averaged. The weight of the samples was measured

values of collimated transmittance using the Bouguer-Lambert law, $\mu_t = -\ln(T_c / I)$, where T_c is the collimated transmittance of the tissue sample, I is the sample thickness, μ_a is the absorption coefficient of the tissue. In the calculations, we used the values of the absorption coefficient presented in Ref. [20]. Since for both skin and muscle tissue, the absorption coefficient is much smaller than the scattering coefficient [20], we did not consider the possible change of the absorption coefficient due to the osmotic dehydration of the tissue in our calculations.

3 Results and discussion

Figure 2 presents the images of skin (a, b) and myocardium (c, d) samples before (a, c) and after (b, d) the optical clearing in 70%-glycerol solution. The figure clearly demonstrates the effect of optical clearing of the studied tissues, i.e., the increase of light transmission in the visible range.

Figure 3 presents the typical spectra (a, b) and the kinetics of collimated transmittance (c, d) of rat skin samples of the control (a, c) and diabetic (b, d) groups during the optical clearing in the 70%-glycerol solution. In both cases, it is well seen that the collimated

transmittance increases and reaches saturation. However, from the comparison of the presented plots it can be seen that it takes more time (approximately 100 minutes) for the diabetic rat skin sample, as compared with that from the control group (nearly 70 minutes) to achieve nearly the same level of optical transmittance.

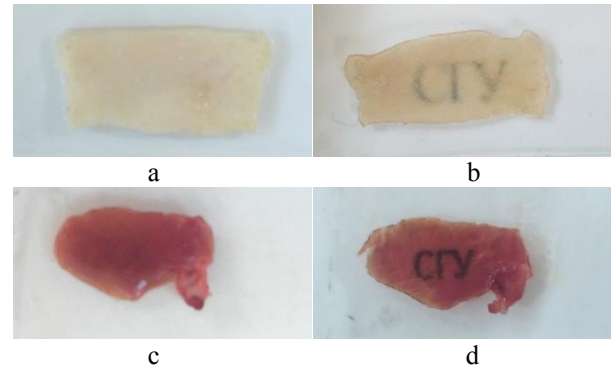


Fig. 2 Images of skin (a, b) and myocardium (c, d) samples before (a, c) and after (b, d) the optical clearing in 70%-glycerol solution.

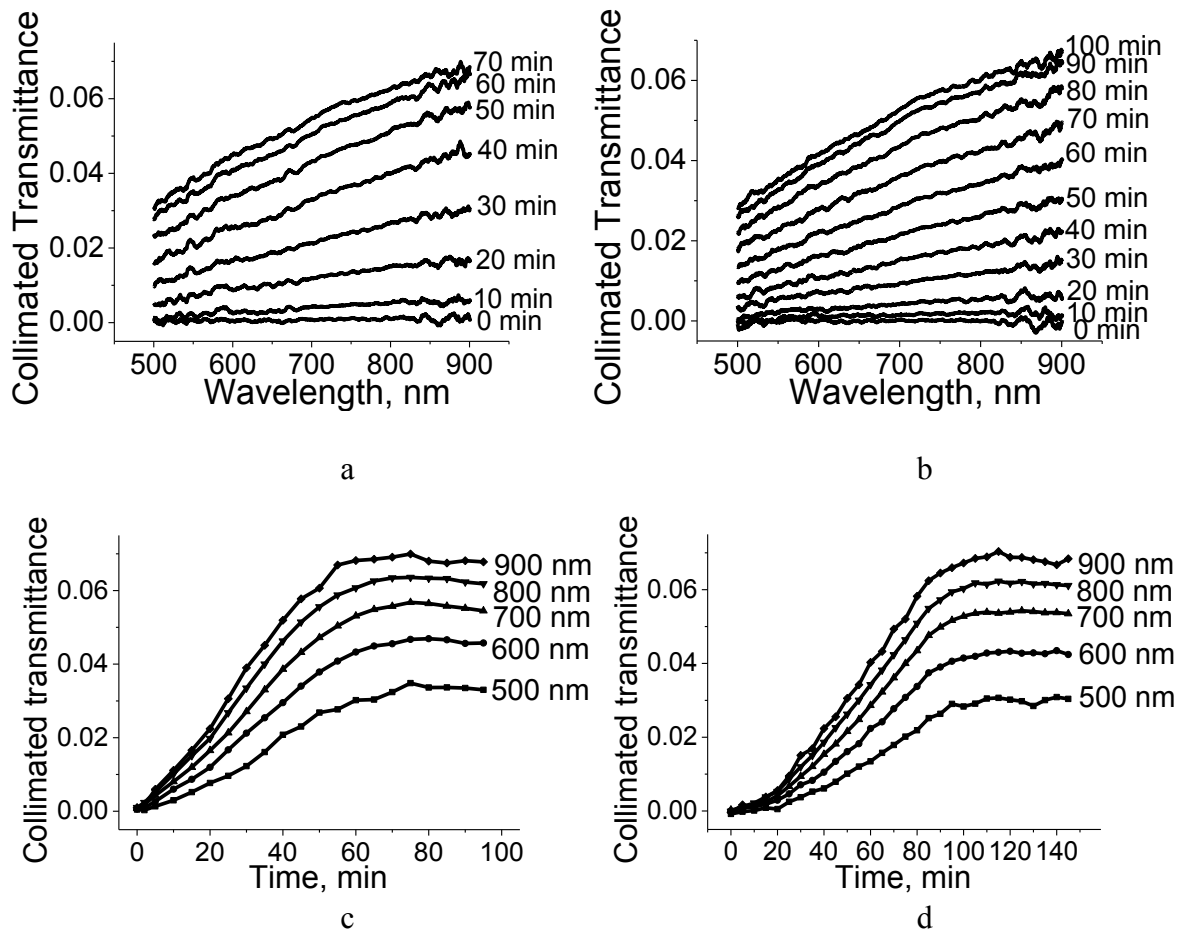


Fig. 3 Spectra (a, b) and kinetics (c, d) of collimated transmittance for the rat skin samples from the control (a, c) and diabetic (b, d) groups during the optical clearing with 70%-solution of glycerol.

Figure 4 presents the typical time dependences of the collimated transmittance of rat myocardium samples from the control and diabetic groups in the course of optical clearing. Similar to the case of skin (Fig. 3), in both cases we observed the growth of collimated transmittance, and in the control group the optical clearing of myocardium samples achieves the saturation faster (approximately in 20 minutes of immersion) than in the diabetic group (more than 30 minutes). At the

appropriate wavelengths, the transmittance reaches nearly the same final values in both control and diabetic groups. In the wavelength range 500-600 nm the transmittance has relatively low values, which is due to the light absorption by myoglobin. However, in this range the growth of transmittance due to the optical clearing, i.e., the reduction of light scattering, is also observed.

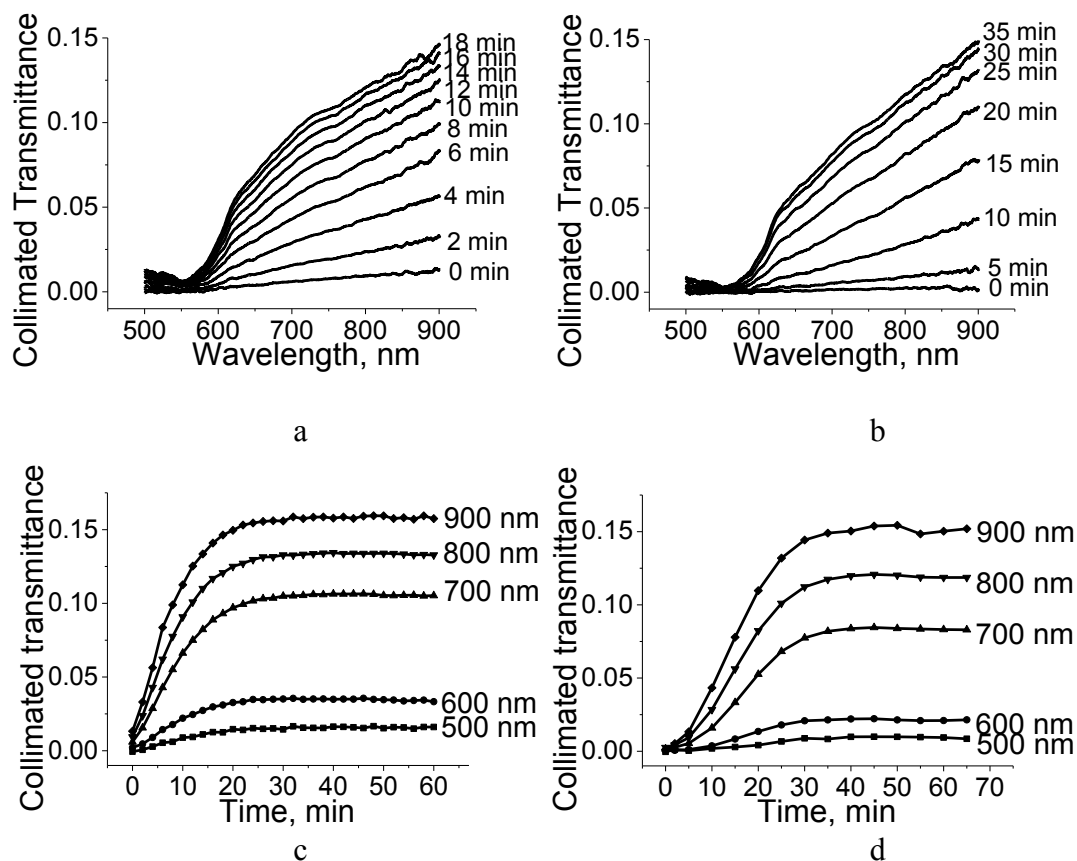


Fig. 4 Spectra (a, b) and kinetics (c, d) of collimated transmittance of rat myocardium samples from the control (a, c) and the diabetic (b, d) groups during the optical clearing with 70%-glycerol solution.

Table 2 summarises the mean values of thickness and weight of the tissue samples before (l_0 and W_0) and after (l and W) the optical clearing, the glycerol diffusion coefficients (D), and the permeability (P) of the tissue samples for glycerol, as well as the optical clearing efficiency in the tissue samples (OC_{eff}) at different wavelengths for the control and the diabetic groups. The reduction of weight (see Table 2) of skin and myocardium samples and thickness (for myocardium) is a result of glycerol-induced sample dehydration, contributing the optical clearing. It is interesting to note that the loss of weight is more expressed in the tissue samples from the control group of rats. The loss amounts to nearly 29% of the initial mass for both the skin and the myocardium in the control group, while in the diabetic group the weight loss is ~24% for the skin and ~26% for the myocardium.

The insignificant thickness increase of the skin samples lies within the measurement error and was neglected. However, the obviously different behaviour of the thickness of skin and myocardium samples indicates the difference of structural organisation of these tissues and, correspondingly, the difference in their response to the osmotic action of glycerol. As compared to myocardium, the skin reacts on the transverse compression much slower and weaker, and hence the osmotic effect in skin manifests itself in stronger longitudinal compression, which leads to insignificant (within the measurement error) increase of the sample thickness. The used method of measuring the collimated transmission with relatively small diameters of the light beam in the samples allows one to neglect the sample area variation (the longitudinal compression) in the algorithm calculating the diffusion coefficient.

Table 2 Mean values of the thickness and weight of tissue samples before (l_0 and W_0) and after (l and W) optical clearing, the glycerol diffusion coefficient (D), the tissue permeability (P), and the efficiency of optical clearing (OC_{eff}) at different wavelengths for the skin and myocardium samples of rats from the control (c) and diabetic (d) groups.

Tissue	c/d	l_0/l , mm	W_0/W , mg	D , cm^2/s	P , cm/s	OC_{eff} (600 nm)	OC_{eff} (700 nm)	OC_{eff} (800 nm)
Skin	c	$0.53 \pm 0.11 /$ 0.55 ± 0.13	$271 \pm 68 /$ 183 ± 21	$(8.33 \pm 2.60) \times 10^{-7}$	$(1.68 \pm 0.88) \times 10^{-5}$	0.58 ± 0.09	0.56 ± 0.04	0.57 ± 0.05
	d	$0.56 \pm 0.04 /$ 0.57 ± 0.07	$270 \pm 32 /$ 203 ± 73	$(6.77 \pm 2.11) \times 10^{-7}$	$(1.20 \pm 0.33) \times 10^{-5}$	0.60 ± 0.07	0.64 ± 0.04	0.65 ± 0.06
Myocardium	c	$0.68 \pm 0.11 /$ 0.51 ± 0.09	$210 \pm 37 /$ 146 ± 35	$(7.90 \pm 3.61) \times 10^{-7}$	$(11.8 \pm 6.1) \times 10^{-5}$	0.51 ± 0.07	0.59 ± 0.06	0.61 ± 0.07
	d	$0.58 \pm 0.06 /$ 0.47 ± 0.07	$214 \pm 41 /$ 153 ± 36	$(5.14 \pm 2.10) \times 10^{-7}$	$(8.60 \pm 3.21) \times 10^{-5}$	0.53 ± 0.02	0.64 ± 0.05	0.66 ± 0.06

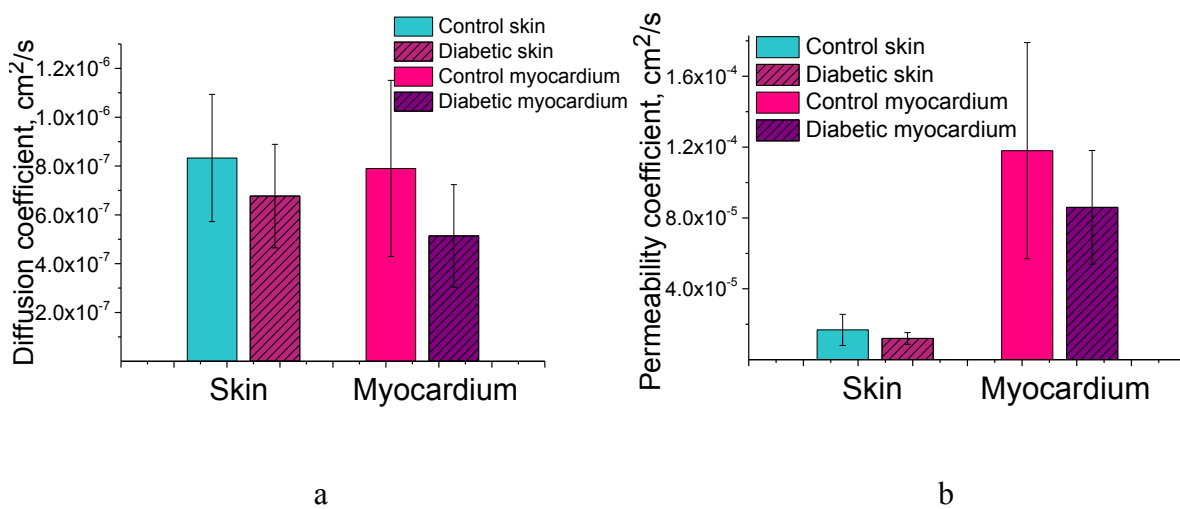


Fig. 5 Glycerol diffusion coefficients in skin and myocardium (a) and permeability coefficients (b) of the studied tissues from diabetic and control groups.

Figure 5 presents the histogram of glycerol diffusion coefficient in the skin and myocardium of rats from the control and the diabetic groups. It is clearly seen that in rats with the model alloxan-induced diabetes the measured diffusion and permeability coefficients take smaller values.

The reduction of the glycerol diffusion rate in the tissue under the conditions of developing diabetes may be related to its structural modification, including the degree of fibril packing, the cross-linking of proteins, and the change of free and bound water content in the skin [6-8, 21-23]. Obviously, the change of free and bound water balance reflects the change in the structure of fibrils and interfibrillar space and, correspondingly, the diffusion rate for the clearing agent molecules in the tissue. The authors of Ref. [22] demonstrated the increase of the scattered light intensity and, therefore, the increase of diffuse reflectance in the glycated tissue, and the authors of Refs. [12, 24] reported the increase of water content in the tissue, which allows to judge on the processes of the relevant tissue modifications during the diabetes development. Different reflectance of light by

intact and diabetic skin, caused by glycation of tissue proteins, is discussed in the review paper [25]. The change of the tissue optical properties is a consequence of its structural modification. It was shown that the glycation leads to the loss of axial packing of the collagen-I fibrils due to the twisting and distortion of the matrix by the glycation adducts [21-23, 26]. The increased water content in skin, found in the patients with diabetes mellitus [24], the intercellular and intermuscular edema [12] can also cause increased scattering of light in the tissue.

Thus, the impairment of the tissue permeability (due to the formation of interfibrillar links) should be related to the impairment of metabolism, which can, probably, lead to the development of larger-scale complications in a diabetic patient.

In Refs. [9, 12] the effect of developing alloxan-induced diabetes on the internals of laboratory animals was studied. The authors of Ref. [9] report the morphologic changes of different degree of severity in the tissues of liver, kidneys, spleen, and pancreas in rats on the 15-th day after the alloxan injection as compared

to the control group of animals. In particular, they observed the decreased accumulation of glycogen in liver, the necrosis of individual cells in kidneys, and in the pancreas, the fibrous perivascular indurations were found and the size and the number of pancreatic islands was reduced.

The structural changes of myocardial tissues in laboratory rats under the model diabetes were studied in Ref. [12]. During the first three weeks after the alloxan injection the morphological manifestations of the myocardium restructuring under the development of alloxan-induced diabetes were revealed. As a result, the authors observed dissociation, destruction, hypertrophy, fragmentation of cardiomyocytes, thinning, twisting, and destructive changes of muscular fibrils of the myocardium. The increased number of collagen fibres by 2.8 times as compared to the intact group of animals and the accumulation of protein in the muscle cells were also found, which lead to the increased viscosity of sarcoplasm, hardness and strength of myofilaments, subject to the increased mechanical load.

Thus, the data of Refs. [9, 12, 21-23, 26] confirm our conclusion that in the glycated tissues the deceleration of glycerol diffusion process takes place. The value of the glycerol diffusion coefficient in the rat myocardium, obtained in the present work for the control group $((7.90 \pm 3.61) \times 10^{-7} \text{ cm}^2/\text{s})$ is close to the glycerol diffusion coefficient in porcine myocardium $((7.71 \pm 4.63) \times 10^{-7} \text{ cm}^2/\text{s})$, measured earlier in Ref. [27]. The glucose diffusion coefficient in the bovine abdominal muscle tissue, measured *in vitro* using OCT, appeared to be $(2.98 \pm 0.94) \times 10^{-6} \text{ cm}^2/\text{s}$, the sample thickness in this case being increased [28] due to the pH difference of the used solutions. In the study of optical clearing of rat muscle tissue using 60%-ethylene glycol solution, the water diffusion coefficient $D_{\text{water}} = 3.12 \times 10^{-6} \text{ cm}^2/\text{s}$ was found as for flow induced by the hyperosmolarity of ethylene glycol solution [29]. When using the aqueous 54%-glucose solution as an immersion agent, the diffusion coefficient of the induced water flow in the muscle tissue had the close value, $D_{\text{water}} = 3.22 \times 10^{-6} \text{ cm}^2/\text{s}$ [29]. Thus, the dehydration seems to be faster than the glycerol diffusion in skin and myocardium. The discussed difference in the diffusion rate of agents in the muscle tissue is caused by the difference in the physical properties of agents, the structure of the studied tissues, and the methods of measuring the diffusion rate of the agent.

The skin permeability for glycerol (at using 70%-glycerol solution) measured in the present paper $((1.68 \pm 0.88) \times 10^{-5} \text{ cm/s})$, is higher by an order of magnitude obtained in the studies of human skin *in vivo*, $(1.67 \pm 0.04) \times 10^{-6} \text{ cm/s}$ [30], with the 40%-glycerol solution. The reason of higher permeability observed in the present work is the greater (almost by two times) osmolarity of more concentrated glycerol solution, which means that free water becomes to be involved in the total flow in the tissue. Water has much higher diffusion rate than glycerol [14]. The experimental

conditions (*in vivo* and *ex vivo*) and the methods of optical measurements (collimated transmittance and OCT) also contribute to these differences. Obviously, when the solution is applied to the skin surface *in vivo*, the glycerol penetrates into the skin from the epidermis side, which inhibits the diffusion of glycerol into skin due to its protective properties [31]. On the contrary, in the present work the action of the solution on the skin occurs mainly from the dermis side, which facilitates the process of its optical clearing.

Table 2 also presents the efficiency of optical clearing of tissue samples at different wavelengths using the 70%-glycerol solution. It can be seen that the efficiency of optical clearing is higher in diabetic tissues than in control ones, which is caused by smaller initial optical transmittance of the diabetic tissue determined by the increased light scattering [18]. The fact that diabetic tissue scatters light stronger than a normal one can be explained by three reasons: i) the increased number and irregular packing of collagen fibres in myocardium [12], i.e., the increased scattering cross section and number of scatterers; ii) the increased refractive index of collagen and other proteins due to the glycation [32, 33]; iii) the increased level of free glucose, holding the water in the interfibrillar space. The last reason is due to the fact that in the presence of diabetes the formation of hydrated shell of proteins is implemented not only via the interaction of water molecules with glycosaminoglycans of the interstitial matrix [34, 35], but also via the interaction of water with glucose molecules, each of them known to bind up to ten water molecules [36]. All these factors lead to the additional tissue hydration and, correspondingly, to the reduction of the refractive index of the interstitial fluid, which increases light scattering. The efficiency of optical clearing is higher because the values of the minimal scattering coefficient $\mu_{s\text{min}}$ achieved in the skin of rats from both control and diabetic groups are close, while the initial scattering coefficient μ_{s0} is higher for the skin of rats from the control group (see Eq. (3)). Physically it means that because of hindered diffusion of the agent and water molecules in diabetic samples the process is slower, the volume of the impregnated tissue increases, and the process of clearing is finished later, when the balance of all flows is established, but with nearly the same resulting tissue transparency in the both control and diabetic groups.

The influence of glucose molecules also explains the smaller loss of weight in diabetic tissues (see Table 2), since the glucose in these tissues possesses higher hygroscopicity as compared to the glycerol, each molecule of which binds only six molecules of water [37]. Thus, the glucose contained in the diabetic tissues inhibits the osmotic dehydration of tissues caused by glycerol.

It is well seen from Table 2 that for the tissues of the diabetic group the efficiency of optical clearing increases with the growth of the wavelength λ : slightly for skin, from 0.60 ± 0.07 ($\lambda = 600 \text{ nm}$) to 0.65 ± 0.06 ($\lambda = 800 \text{ nm}$), and essentially greater for myocardium,

from 0.53 ± 0.02 ($\lambda=600$ nm) to 0.66 ± 0.06 ($\lambda=800$ nm). For control sample of myocardium, the efficiency of optical clearing also increases with the growth of wavelength, but the effect is expressed weaker than in the diabetic tissue. At the same time, optical clearing efficiency of control skin does not depend on the wavelength. Generally, such behaviour can be explained by the fact that the difference between the refractive indices of the scatterers and the interstitial fluid, saturated with glycerol, decreases with the growth of the wavelength [38], leading to better matching of refractive indices, the reduction of light scattering, and the increase of the tissue optical clearing efficiency. Somewhat greater increase of optical clearing efficiency with the growth of the wavelength in diabetic tissues can be, probably, related to the behaviour of dispersion dependences of the refractive index of tissue scatterers, caused by the difference of their hydration degree.

comparative study of diffusion rate in skin and myocardium).

4 Conclusion

In the reported studies, we have found that the optical clearing of rat skin and myocardium is slower under the conditions of alloxan-induced diabetes. The glycerol diffusion rate in skin and myocardium and the permeability of diabetic tissues for glycerol are smaller as compared to the control group. The obtained values of glycerol diffusion coefficient in skin and myocardium of the rats from control group are higher than in the diabetic group by 1.23 and 1.54 times, respectively. The permeability of skin and myocardium in the control group of rats is higher, than for the model diabetes by 1.4 and 1.3 times, respectively. However, the optical clearing efficiency of diabetic tissues is higher, than that of healthy ones.

Thus, it is established that the diffusion of glycerol molecules in the tissues of rat myocardium and skin is hampered by the development of alloxan-induced diabetes. This result offers a possibility of developing a method for early diagnostics of diabetes mellitus complications, in particular, for assessing the degree of myocardial lesion evidence by the stage of skin glycation in the process of diabetes development and treatment. It is experimentally proved that for both studied tissues of the diabetic group the increase of the optical clearing efficiency with the growth of the wavelength is considerably higher than in the control group.

Acknowledgments

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Laser modification of liquid nutrient medium and antibiotic solutions with the assessment by the bacteria growth dynamics

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Abstract. The processes of laser photoinduced modification of nutrient media and antibiotic solutions are considered. It is shown experimentally that the growth of colonies number is slower on the irradiated medium. The impact of laser radiation on the antibiotic activity is also studied experimentally. It is found that the laser-induced photomodification can affect the antimicrobial activity of the medicinal preparations. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Bacteria, antibiotic, Laser activation, Culture medium.

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1 Introduction

The experimental studies devoted to the applications of laser radiation for medical purposes are intensely carried out from the beginning of 1970-s. To date a huge amount of data is accumulated that allows the conclusion that the laser irradiation of blood or other tissues causes the changes, which can be assessed quantitatively by the biochemical indicators of blood, by the angiospasm, and by the time of different wound healing [1]. However, in spite of the large number of techniques of using the laser radiation in medicine, the most part of researchers consider the human organism as a black box, in which the action having a certain intensity and duration produces a certain standard response. The studies devoted to the mechanisms of laser impact are few, which is explained both by the complexity of the studied system, and by the multilayer structure of the response.

Nevertheless, there are three key ideas that should be taken into account in the description of the laser biostimulation mechanism.

First, it is known that the therapeutic effect of laser radiation weakly depends on the wavelength [2]. The first studies were devoted to the application of radiation from helium-neon laser (red light), then, with the appearance of available semiconductor sources the infrared radiation began to be widely used, and at present there are papers on the application of blue and green light. Therefore, it is necessary to decide, whether there is some unique acceptor in the organism that possesses such wide absorption range, or there are many substances, able to absorb and transform the energy of laser radiation, and an individual acceptor is relevant in each particular case.

Second, it is experimentally shown [1, 2] that the local impact of laser radiation leads to the response of the entire organism rather than is restricted to a local response, which is traced in the biochemical indicators of functioning of all systems. Therefore, there should exist something responsible for the generalisation of the impact.

Third, the studies devoted to the impact of laser radiation on the living objects of different organisation level (bacteria, plants, animals, humans) show, that a recordable response is observed in all cases. Therefore, the mechanism of laser interaction with the biological objects should possess considerable universality.

With the above specific features taken into account, we can suppose that the laser radiation is able to interact with organic macromolecules, modifying their spatial structure (conformation). In the human organism, the laser radiation action to the protein molecules that can changes their second- third- and fourth-order structure, and not affecting the first-order structure, i.e., the sequence of amino acids.

It is known that the reactivity of a macromolecule is directly related to its spatial structure, which is determined by the presence of an active centre, the interaction of the macromolecule with its aqueous environment, and the interaction with other

macromolecules [3]. At present it is proved that the substances diluted by the liquids form associates of molecules at the expense of the electrostatic interaction forces [4, 5]. The molecules included in an associated lose its mobility and the active centres can overlap with the adjacent molecules. The larger size of the associate, the larger ratios of its mass to surface and, therefore, the molecule have to weaker processes of assimilation and reactivity.

In the frameworks of this concept, the laser radiation is absorbed by the associate rather than by a single molecule, which causes the reformation and decay of the associate into smaller fragments. Since in any multicomponent solution the associates of different configurations are formed, they can absorb the energy in a very broad range, which explains the similarity of effects arising under the laser impact with different wavelength.

On the other hand, if the interaction occurs at the "radiation-associate" level, then its results must be observable not only in living systems, but also in model media, such as solutions of proteins, blood plasma, biological liquids, organic solutions for growing bacterial cultures, etc. In Refs. [6-10] it is shown that in relation to the protein and bile solutions this statement finds its experimental confirmation.

The purpose of the present work was to determine the impact of laser radiation on the recordable changes in organic solutions by the example of nutrient media used for bacteria cultivation and the antibiotic solution.

2 Experiment

2.1 Materials and methods

Two series of experiments were carried out. In the first series, we assessed the influence of the laser irradiation on the nutrient medium by the dynamics of the bacterial growth. In the second series, we studied the impact of laser radiation on the antibiotic activity.

To study the dynamics of the *Bacillus subtilis* microorganism growth the continuous cultivation was used. The microbial suspension was prepared from the single-day culture grown on the meat peptone agar (MPA) at the temperature of 37 °C.

Then 1 ml of microbial suspension was added to 100 ml of nutrient medium cooled to 45 °C, the components were mixed and poured into three Petri dishes. After the agar hardening all dishes were put into a thermostat at the temperature 37 °C. The assessment of microbial growth was performed every 12 hours by counting the number of colonies, starting from the zero point. The colonies of microorganisms grown in the nutrient medium without laser irradiation were used as control samples.

The semiconductor laser diode served as a radiation source. The laser impact on the nutrient medium in the regime of "light boiler" was implemented before the addition of the suspension of microorganisms. The exposure time was 120 and 300 seconds, the power of

radiation was about 100 mW, the wavelength was 532 nm, and the total irradiation energy was nearly 5 J/g.

In the second series the antibiotic *Vancomycin* irradiated by the laser and not exposed to laser radiation were added to the nutrient medium using the method of diffusion into agar. Using a sterile cylinder with the diameter 0.5 cm, three holes were made in the dishes with the hardened medium at equal distance from each other and from the edges of the dish. Then the dishes were grouped in pairs, and the holes were filled with physiological solution (one hole in the first dish and one hole in the second dish), the non-exposed antibiotic (two holes in the first dish), and the antibiotic exposed during 120 seconds and 300 seconds (two last holes in the second dish). In 24 hours, we measured the diameters of the zones of *Bacillus subtilis* growth inhibition with the accuracy to 1 mm using the millimeter paper, which is a standard procedure for the assessment of antibiotic activity [11].

2.2 Results and discussion

In the first series of experiments that allow the assessment of the impact of the laser irradiation of the nutrient medium on the bacterial growth dynamics, the first growth was observed in 12 hours after the experiment zero point. The number of colonies in the control samples and in the laser exposed ones was practically the same. In 24 hours from the zero moment the number of bacteria grown in the control dishes was greater and amounted to 31 CFU, while after the 120 s of laser irradiation it was smaller by 29% and after the 300 s exposure it was by 16% smaller. In 48 hours after the zero point the number of colonies in the control samples amounted to 48 CFU, in the samples irradiated during 120 seconds it was smaller by 6% (45 CFU) and for those irradiated during 300 seconds it was smaller by 17% (40 CFU). Figure 1 presents the curve of *Bacillus subtilis* growth in the control sample and in the medium exposed to the laser irradiation. The spread of colony numbers does not exceed 12% of the presented mean values.

In the second series, we studied the impact of laser radiation on the antibiotic activity by measuring the zones of suppressed growth. The measurements were performed in 24 hours after placing the nutrient medium with the bacterial culture into the thermostat. Around the holes filled with physiological solution for control, we observed intense growth of bacteria. For the unexposed antibiotic, the inhibition zone was 38 mm. For the antibiotic exposed to the laser radiation during 120 seconds, the inhibition zone was 42 mm, which is by 10.5% greater than without the laser exposure. For the antibiotic exposed during 300 seconds, the growth inhibition zone was 43 mm, i.e., greater by 13% as compared with the intact antibiotic. The spread of the determined zone size did not exceed 8% of the mean values.

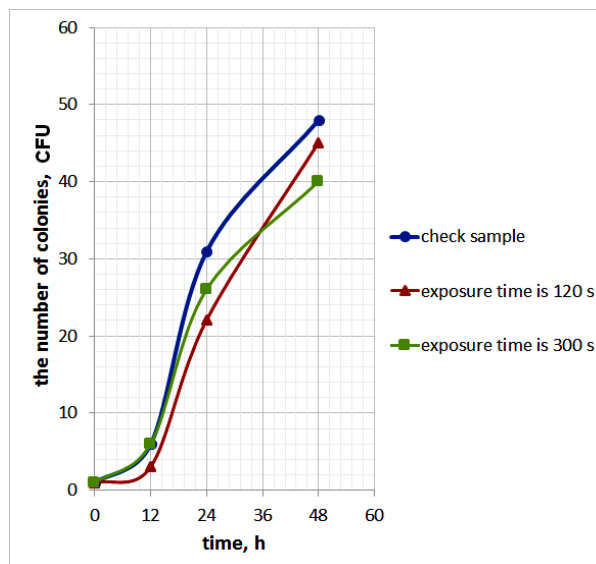


Fig. 1 Curves of *Bacillus subtilis* growth in the control samples and after the laser irradiation during 120 and 300 seconds.

The mechanism of the observed effects can be thought as follows. As mentioned above, the organic molecules in solutions form associates mainly stabilised by hydrogen bonds. Besides that, on the surface of an associate the coating of water molecules is formed, which additionally stabilises the system. In the absence of external perturbations, the associate is rather stable. However, since the energy of its bonds is not high, even a small external perturbation can bring it out of the equilibrium position. In our experiment, the laser radiation deliver the energy that makes the parts of the associate move, and this energy is sufficient to bring the structure out of equilibrium, break the present hydrogen bonds and lead to its decay.

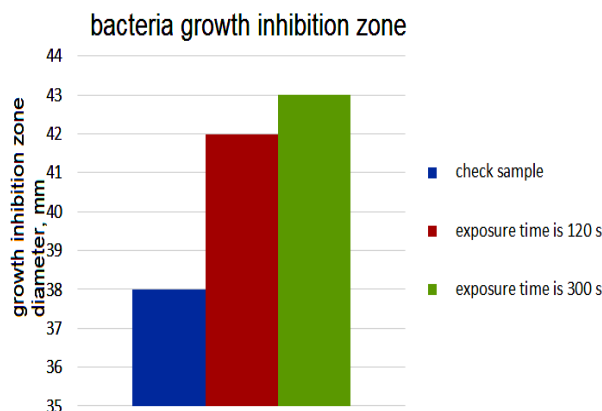


Fig. 2 Zones of *Bacillus subtilis* growth inhibition for the intact antibiotic and the antibiotic irradiated during 120 and 300 seconds.

In the experiment with antibiotic, the activation effect observed in the experiment is explained as follows. The *Vancomycin* antibiotic acts because its molecule is bound to the bacterial membrane (or to the

proteins on the membrane surface). Ideally, each molecule must be bound to one bacterium. However, since the antibiotic is stored in the form of a powder and solve in water or physiological solution immediately before the injection, the solution contains associates rather than single molecules. As a result, the efficiency of binding the antibiotic to bacteria is reduced, since a few molecules are bound to one bacterium instead of many bacteria. One can suppose that under the impact of laser radiation the associates are broken into smaller fragments, so that the reactivity of the antibiotic is increased, and, therefore, its efficiency is increased, too.

The data in Fig 2 can serve as a base for the standard determination of antimicrobial activity of antibiotics using the method of diffusion into agar after the laser impact [11].

3 Conclusion

Thus, it is experimentally found that the laser irradiation essentially affects the structure of a liquid, disintegrating the molecular associates. This changes the reactivity of molecules in the solution. In particular, the laser irradiation can increase the antimicrobial activity of antibiotics, as well as affect the parameters of bacterial growth in the irradiated nutrient medium.

Reaction of cyclopentadienyl and methyl radicals

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Abstract. Geometries and potential energies of reagents, products, and intermediate states for the reaction between cyclopentadienyl (C₅H₅) and methyl (CH₃) radicals are found by means of ab initio quantum mechanical methods CCSD(T)/cc-pVTZ-f12, B2PLYPD3/AUG-CC-PVDZ and B3LYP/6-311G. Basing on the analysis of the found energy, structural and kinetic characteristics of the compounds involved, the reaction paths leading to the formation of fulvene and benzene, the simplest aromatic compound, are determined. The reaction path begins from the formation of the intermediate compound, methylcyclopentadiene, followed by tearing-off a hydrogen atom from it: C₅H₅ + CH₃ → C₅H₅CH₃ → C₅H₄CH₃ + H. The subsequent monomolecular transformations of C₅H₄CH₃ are closed by the formation of either fulvene (via the loss of one hydrogen atom from the methyl group) or benzene (via the stages of transforming the pentamers ring into a hexamers one and tearing-off a hydrogen atom). The rate constants found in the paper using the software package MESS show that the rate of benzene formation is always higher than that for fulvene within the temperature interval 500-2250 K. Since fulvene can also isomerize into benzene, the reaction C₅H₅ + CH₃ is an important supplier of the initial bricks for building polycyclic aromatic hydrocarbons dangerous for living systems. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Combustion, methyl, cyclopentadienyl, benzene, fulvene, PANs, quantum chemistry calculations, reaction pathways, rate constant.

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1 Introduction

Polycyclic aromatic hydrocarbons (PAH) comprise a considerable part of harmful emission from different fossil fuel power plants and serve as predecessors in soot formation [1-3]. They hugely affect the environment and human health. Appeared in the air and soil, they can be inhaled by humans or consumed with food, which leads to various health problems. PAH are very toxic by their nature, their prolonged effect is related to such diseases as skin allergy, immune deficit, asthma and other lung diseases, destruction of red blood cells, tumours, preterm birth, diseases of kidneys and liver, and valvular defect in neonates.

Methyl and cyclopentadienyl are among the key radicals in the PAH and soot formation. The methyl radical can be formed by partitioning of complex hydrocarbons or by their interaction with oxygen, while the cyclopentadienyl is turned out in the reactions of benzene and phenyl with atomic oxygen [6], in the pyrolysis of phenoxy radical [3-6], phenol [6], cyclopentadiene [7], etc. The reaction $\text{CH}_3 + \text{C}_5\text{H}_5$ is interesting because it yields the first aromatic compound, benzene, that is an initial brick in building more complex compounds, such as PAH, soot, carbon nanoparticles, fullerene, etc. There are strict standards on emission of these compounds for different power plants using hydrocarbon fuels [8]. At present, the search for hydrocarbon fuel burning regimes, in which the formation of aromatic compounds is suppressed basing on the detailed analysis or the processes that occur in the burning zone is carried out [9]. The reaction

between methyl radical and cyclopentadienyl occupies an important position among the kinetic reaction schemes of the formation of aromatic compounds.

The reaction of CH_3 with C_5H_5 was studied in theoretical papers [10-13]. Refs. [10, 11] show that the final products of this reaction are fulvene and benzene. Melius et al. [12] theoretically studied the isomerisation of fulvene using the method BAC-MP4. Jasper et al. [13] reported a detailed consideration of the fulvene-to-benzene isomerisation process catalysed by atomic hydrogen. However, in these papers, not all ways leading to the formation of benzene were considered, and no rate constants of elementary processes, depending on temperature and pressure, were presented.

In this paper, we present the geometries of the initial and final products involved in the reaction as well as of the intermediate and transition compounds, calculated by means of the density functional theory (DFT), B2PLYPD3 and B3LYP methods. For all found structures, the energy values are corrected using ab initio quantum chemistry calculations. Basing on the obtained data, we determine the optimal reaction paths for the system $\text{C}_5\text{H}_5 + \text{CH}_3$, leading to the formation of benzene and fulvene. Using the corrected energies and optimised geometries, we calculated the rate constants for a number of basin monomolecular reactions in the multistep process of interaction between the two radicals at the temperatures 500-2500K.

2 Methodology

We used ab initio quantum mechanical methods of high level to study the possible products of the reaction $\text{CH}_3 + \text{C}_5\text{H}_5$. The optimised structures and energies of the reagents, products, intermediate and transient states involved in the reaction were found at the first stage using the widespread hybrid method of density functional theory B3LYP with the basis set 6-311G**. Using the same method, we calculated the vibrational frequencies and zero-point energies (ZPE) for all the structures.

At the second stage the energies, the optimised geometries and vibrational frequencies for all structures were found using more up-to-date density functional method B2PLYPD3 with the basis set AUG-CC-PVDZ in order to compare the two methods applied to the system $\text{CH}_3 + \text{C}_5\text{H}_5$.

At the last stage the total energies of the involved structures were corrected using the method of couple clusters with the single and double excitations taken into account (CCSD(T)) with the basis set cc-pVTZ-f12. The used method allows the energy calculations with the accuracy to 1-3 kcal/mole. The B3LYP and B2PLYPD3 calculations were carried out using the Gaussian software packages [14-16], while the CCSD(T) calculations were performed using the Molpro software package [17].

Basing on the corrected total energies and geometry of reagents, products, intermediate and transient states, we calculated the rate constants at different pressures and temperatures, using the software package MESS, based on the Rice-Ramsperger-Kassel-Marcus statistical theory [18].

3 Results and discussion

Figure 1 presents the reaction paths of interaction between the CH_3 and C_5H_5 radicals. The energies of the transient states TS_i , the intermediate states and the products, calculated using three methods, are presented relative to the total energy of the initial reagents in the kcal/mole units. As a result of the interaction at the first stage the methylcyclopentadiene $\text{C}_5\text{H}_5\text{CH}_3$ molecule is formed. Further transformations are related to the possible transition to the initial products or with the hydrogen atom tearing-off and the formation of $\text{C}_5\text{H}_4\text{CH}_3$ or $\text{C}_5\text{H}_5\text{CH}_2$ molecules. In Ref. [10] it was shown that the first path is less energy-consuming, therefore, below we will consider the path with the formation of the molecule $\text{C}_5\text{H}_4\text{CH}_3$ (1). Then the path via the transient state (barrier) TS_6 with the decay into fulvene $\text{C}_5\text{H}_4\text{CH}_2$ (2) and the hydrogen atom. An alternative path from the structure $\text{C}_5\text{H}_4\text{CH}_3$ is related to regrouping the atoms into the molecule $\text{C}_5\text{H}_5\text{CH}_2$ (3) with the activation barrier TS_5 of 54.1 kcal/mole. In further transformations either the structure $\text{C}_5\text{H}_5\text{CH}_2$ yields a fulvene molecule and a hydrogen atom with the transient barrier TS_1 of 61.1 kcal/mole, or the expansion

of the ring begins leading to the formation of the $\text{C}_5\text{H}_5\text{CH}_2\text{RE}$ (4) structure with the transient barrier TS_2 of 37.9 kcal/mole. After passing the barrier TS_3 of 40.99 kcal/mole the bond between carbon atoms is broken and the cyclohexadienyl structure $\text{C}_6\text{H}_6\text{H}$ (5) is formed. Finally, the hydrogen atom tearing-off leads to the formation of the benzene ring C_6H_6 (6) after passing the barrier TS_4 (31.1 kcal/mole). The relative energy of the final state of this chain in the form of fulvene amounts to 56.1 kcal/mole, while in Ref. [10] it was equal to 54.2 kcal/mole. The ab initio method chosen here, providing higher precision as compared with G2M (rcc, MP2) used in Ref. [10], allowed higher accuracy of relative energies of the structures, involved in the reaction (Table 1).

Table 1 Relative corrected energies of reagents, transient states, and reaction products obtained using the method CCSD(T)/cc-pVTZ-f12.

Molecule	Relative energy, kcal/mole
$\text{CH}_3 + \text{C}_5\text{H}_5$	0.0
$\text{C}_5\text{H}_5\text{CH}_3$	-70.4
$\text{C}_5\text{H}_4\text{CH}_3$ (1)	1.4
TS_5	54.1
$\text{C}_5\text{H}_5\text{CH}_2$ (3)	29.6
TS_1	61.1
TS_2	37.9
$\text{C}_5\text{H}_5\text{CH}_2\text{RE}$ (4)	31.5
$\text{C}_5\text{H}_4\text{CH}_2$ (2)	56.1
TS_3	41.0
$\text{C}_6\text{H}_6\text{H}$ (5)	4.3
TS_4	31.1
C_6H_6 (6)	24.7

Table 2 Rate constants for the reactions $\text{C}_5\text{H}_4\text{CH}_3 \rightarrow \text{C}_5\text{H}_4\text{CH}_2$, $\text{C}_5\text{H}_4\text{CH}_3 \rightarrow \text{C}_6\text{H}_6$ under the pressure of 1 atm.

T (K)	k (s^{-1})	
	$\text{C}_5\text{H}_4\text{CH}_3 \rightarrow \text{C}_5\text{H}_4\text{CH}_2$	$\text{C}_5\text{H}_4\text{CH}_3 \rightarrow \text{C}_6\text{H}_6$
500	6.01×10^{-13}	3.10×10^{-11}
600	9.51×10^{-9}	2.64×10^{-7}
700	9.73×10^{-6}	1.64×10^{-4}
800	1.79×10^{-3}	1.97×10^{-2}
900	0.11	0.87
1000	2.76	16.76
1125	72.74	318.91
1250	981.79	3.29×10^3
1375	7.94×10^3	2.11×10^4
1500	4.26×10^4	9.36×10^4
1650	2.07×10^5	3.76×10^5
1800	6.93×10^5	1.08×10^6
2000	2.29×10^6	3.02×10^6
2250	6.62×10^6	7.39×10^6

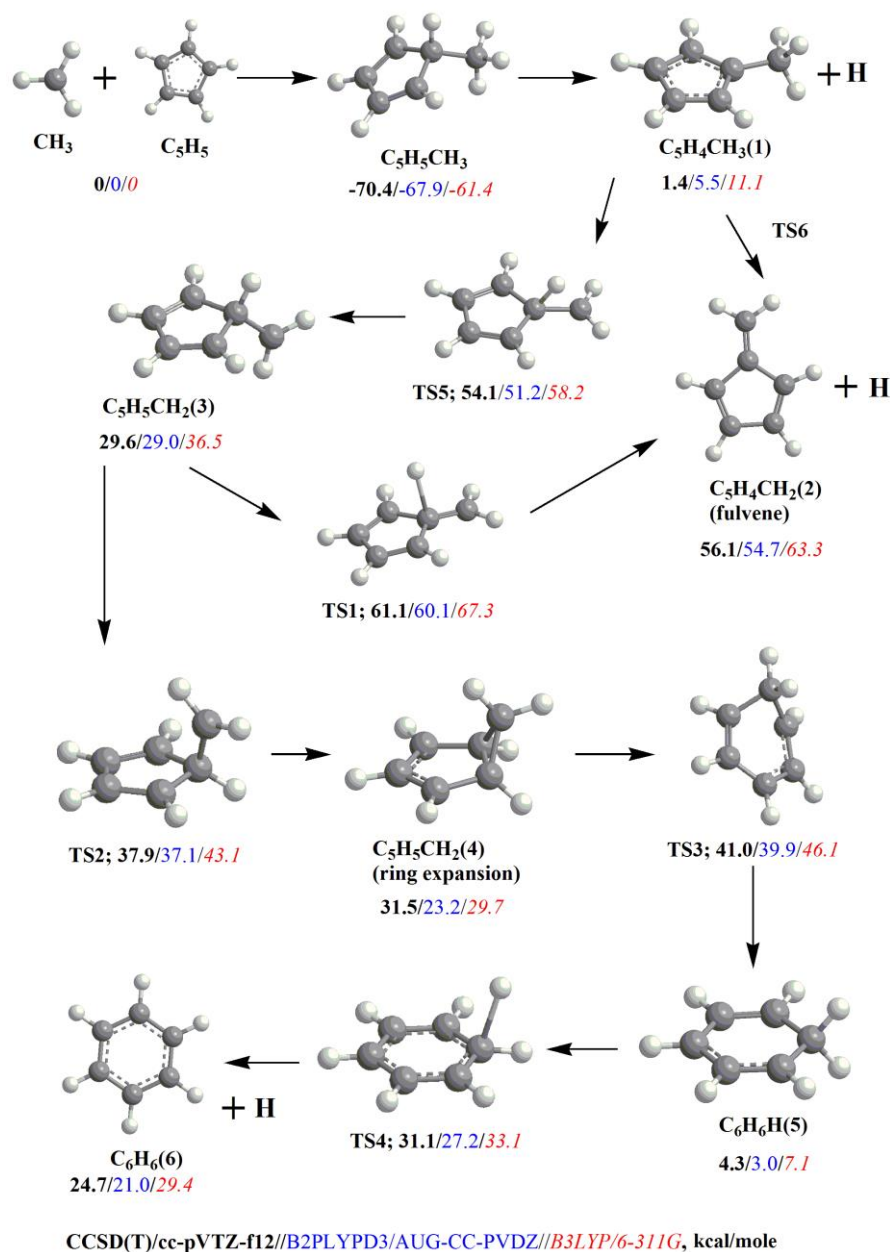


Fig. 1 Reaction paths, structures and relative energies for the reaction of interaction between methyl and cyclopentadienyl radicals. The figures below each structure indicate the relative energy, calculated using the methods CCSD(T) with the basis set cc-pVTZ-f12 (boldface type), B2PLYPD3 with the basis set AUG-CC-PVDZ (normal type, blue colour), B3LYP with the basis set 6-311G** (italic type, red colour), respectively.

Table 2 presents the rate constants for the sequence of monomolecular transformations from $\text{C}_5\text{H}_4\text{CH}_3$ to $\text{C}_5\text{H}_4\text{CH}_2$ and from $\text{C}_5\text{H}_4\text{CH}_3$ to C_6H_6 under the pressure of 1 atm., calculated using the software package MESS [16] that provides kinetic accuracy. From the obtained values, one can see that under the increase of temperature the rates of formation of fulvene and benzene molecules grow exponentially. Under the pressure of 1 atm. the rate of benzene formation exceeds that of the fulvene at all temperatures, considered in the present work. At the temperatures below 1000 K the rate of monomolecular transformations in the chain $\text{C}_5\text{H}_4\text{CH}_3 \rightarrow \text{C}_6\text{H}_6$ is higher by more than an order of magnitude, while in the temperature interval 1000-2250

K the rate constants for both reaction paths have the same order of magnitude.

4 Conclusion

As a result of the present work, the $\text{CH}_3 + \text{C}_5\text{H}_5$ reaction paths are found, the energies of the compounds are determined with higher accuracy, the general picture of fulvene and benzene formation is considered, and the rate constants for the reactions $\text{C}_5\text{H}_4\text{CH}_3 \rightarrow \text{C}_5\text{H}_4\text{CH}_2$, $\text{C}_5\text{H}_4\text{CH}_3 \rightarrow \text{C}_6\text{H}_6$ at different temperatures and the pressure of 1 atm. are calculated for the first time. Basing on the analysis of the obtained energy and kinetic characteristics, three reaction paths were

revealed, two of them leading to the formation of fulvene and one to the formation of benzene. The rate of benzene formation is always higher than that of fulvene over the entire temperature range implemented in combustion chambers. Fulvene is known to be capable of further isomerisation into benzene [13], which makes the reaction $\text{CH}_3 + \text{C}_5\text{H}_5$ a potential source of PAH in combustion chambers of different power plants using hydrocarbon fuel.

The balance between the accuracy and the available computation facilities is commonly achieved by using two-level calculations, where the geometry of reagents, transient and intermediate states are found using the DFT and their energies in optimised geometries are calculated using the CCSD(T) method. The DFT method B3LYP is most frequently used to find the

structural parameters of the compounds at the first level. However, the DFT methods developed in recent years allow the calculation of geometry and vibrational frequencies with the similar or somewhat better accuracy than B3LYP, in particular, the method B2PLYPD3 that allows for the dispersion corrections and, therefore, describes distant interactions better than B3LYP. As follows from our data, presented in Fig. 1, the values of energies calculated using B2PLYPD3 are in much better agreement with those calculated using the ab initio method than the results of B3LYP. Thus, our data show that the DFT method B2PLYPD3 has successfully passed the test in application to the particular system $\text{CH}_3 + \text{C}_5\text{H}_5$.

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Spectral method of photoplethysmogram processing for screening of atherosclerosis

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Abstract. The paper is devoted to the analysis of the existing indicators for the diagnostic of arterial vessels condition and the development of new ones based on the contour analysis of the pulse wave. The performed analysis of the techniques used to process the pulse wave signal has shown that the classical approach to the morphological analysis of pulse wave in terms of stiffness index and reflection index does not provide sufficient diagnostic accuracy due to the instable detection of the reflected wave maximum. A new technique of contour analysis based on spectral transformation of a sequence of replicated single fragments of the pulse wave is developed. The comparative analysis of the contour analysis methods has shown that the proposed method detects the changes of the pulse wave caused by diseases in the form of increased difference between the indicator values. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Pulse wave contour; Reflection index; Stiffness index; Form factor; Harmonic factor.

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1 Introduction

Nowadays the pathologies of cardiovascular system occupy the leading position among all human diseases. The main cause of cardiovascular diseases (CVD) is the deformation of blood vessel walls, the atherosclerosis that arises due to the malfunction of lipid and protein metabolism and is accompanied by the deposition of cholesterol. The localisation of vascular pathology determines the consequences of the disease development, e.g., the atherosclerotic lesion of coronary arteries causes the development of ischemic heart disease (IHD). The atherosclerosis of brain vessels disturbs brain blood circulation, the possible consequences of which are the ischemic stroke and intracerebral bleeding [1].

Thus, the timely diagnostics of atherosclerosis development risk could make it possible to avoid multiple complications and fatal cases. Among the existing methods of clinical diagnostics of atherosclerosis, one can mention the angiography, the ultrasonic dopplerography, the laboratory examinations, and the analysis of pulse waves (PW), recorded using the sphygmographic or plethysmographic sensors [2]. The angiography is an invasive method of vascular examination that requires special preparation of the patient and the absence of contraindication. Hence, this diagnostic method is inapplicable to screening. The ultrasonic dopplerography allows the estimation of blood flow velocity and the visualisation of the vessel structure [3]. The laboratory examination of the vascular condition consists in determination of the level of endothelial dysfunction markers in blood [4].

The method most suitable for screening of atherosclerosis is the analysis of pulse wave signal, which is a noninvasive, relatively cheap and safe test, free of the patient's discomfort.

The aim of the present paper is to develop new approaches to the formation of diagnostic indicators for the assessment of arterial vessels condition.

2 Morphology of pulse wave

The pulse wave contour results from overlapping two waves. The direct one (the systolic wave A_1) is formed by the systolic blood volume transferred directly from the left ventricle to the arterial vessels of upper extremities during the systole. The reflected one (the diastolic wave A_2) is due to the reflection of the blood volume, transferred to the lower extremities via the aorta and the main arteries and directed back into the ascending aorta and then to the arterial vessels of upper extremities [2,5,6].

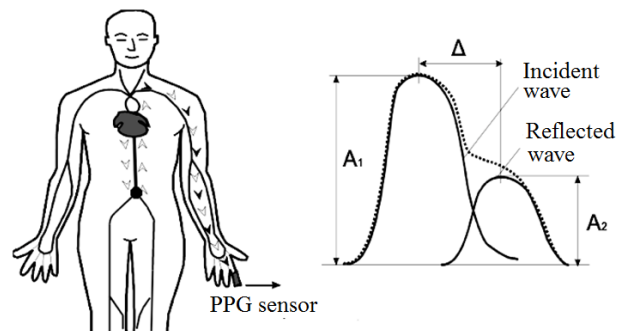


Fig. 1 Process of pulse wave formation.

The process of PW contour formation is illustrated in Fig. 1, where $\blacktriangleright$ represents the direct pulse pressure wave, $\blacklessgtr$ shows the reflected pulse pressure wave, $\bullet$ is the aorta femoral bifurcation point, A_1 is the amplitude of the direct pulse wave, A_2 is the amplitude of the reflected pulse wave, Δ is the duration of pulse wave reflection.

The duration Δ of pulse wave reflection is determined by the propagation velocity given by the Moens-Korteweg equation:

$$v = \sqrt{\frac{Eh}{\rho d}}, \quad (1)$$

where v is the propagation velocity of the pulse pressure wave, E is the Young modulus of the arterial wall, indicating its elasticity, h is the thickness of the arterial wall, ρ is the blood density, and d is the artery diameter.

The parameters h , ρ , and d are nearly similar for typical of blood vessels, so that the PW propagation velocity is mainly determined by the vessel elasticity [7]. Table 1 based on the data of Ref. [8] shows that with the decrease of vessel elasticity (increase of the Young modulus) the delay of the reflected pulse wave decreases. The presented values of the Young modulus were calculated basing on the results of ultrasonic scanning of common carotid arteries and common femoral arteries [8].

Thus, with increasing arterial stiffness, the reflected wave comes back faster, and its contribution is transferred from the diastolic component of the PW to the systolic one. The increased rigidity of arteries can appear not only with age, but also at earlier stages of life due to kidney diseases or diabetes mellitus. Figure 2 presents the classification of PW contours according to Dawber [9]. Class 1 is characterised by the expressed dicrotic notch, clear distinction between the direct and reflected wave, and different slope angles. Class 2 is characterised by the absence of notch, horizontal

Table 1 Wave delay time (Δ) depending on the Young modulus (E).

	Carotid artery (length 8 mm, diameter 6.3 mm, thickness 1.75 mm)			Femoral artery (length 56.6 mm, diameter 8 mm, thickness 1 mm)		
	healthy	control	sick	healthy	control	sick
E, kPa	522.15	825.44	1072.43	620	975.94	1122.76
Δ, ms	21.38	17.01	14.92	206.94	164.94	153.78
Blood density, kg/m^3	1036					

separation of direct and reflected wave, and different slope angles. In Class 3 the notch and the change of slope angle are absent.



Fig. 2 Modification of the pulse wave shape with age and in the presence of vascular diseases.

2.1 Methods of contour analysis

To date the processing of pulse wave signal is performed using the following amplitude-time parameters of the PW characteristic points:

- the reflection index (RI) that characterises the reflection wave evidence and its contribution to the increase of the pulse arterial pressure
- $RI = \frac{A_2}{A_1} \cdot 100\%$; (2)
- the stiffness index (SI) determined by the velocity of pulse wave propagation

$$SI = \frac{h}{\Delta}, \quad (3)$$

where h is the height of the patient

The methods of single or double differentiation of the signal are also in use (see Fig. 3) [10].

The first derivative allows the determination of the diastolic peak position (the point where the first derivative is zero), the reflection time (the time Δ between the systolic and the diastolic peaks, i.e., the points where the derivative changes its sign), and the PW rise time (the systolic push, the time during which the PW signal ascends from foot to peak).

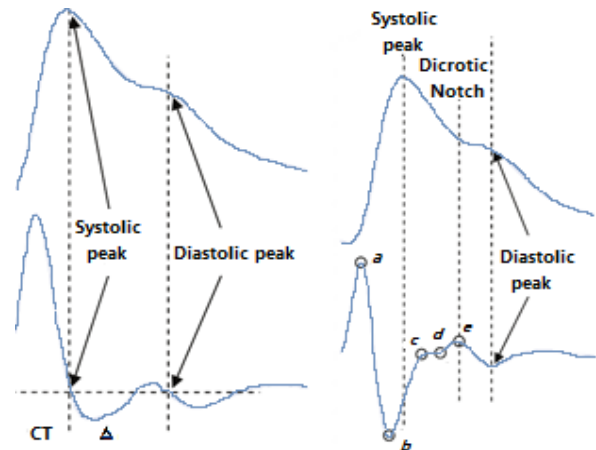


Fig. 3 The first and the second derivative of PW.

The second derivative of the pulse signal indicates the acceleration of the blood pulsation in the vessel. The contour of the second derivative of the pulse wave is formed by five sequential waves a , b , c , d , and e that differ from each other in shape and amplitude. The relation of amplitudes of these waves is given by the coefficients that describe the vascular system condition [7, 10]. The diagnostic indices determined basing on the analysis of the first and the second derivative are presented below:

- The coefficient (b/a).
- The form factors (FF) [11]

$$FF = \frac{\sigma_{x''}/\sigma_{x'}}{\sigma_{x'}/\sigma_x}, \quad (4)$$

where

$\sigma_{x''}$ is the standard deviation of the second derivative of the considered fragment of the pulse wave signal from the mean value of the second derivative;

$\sigma_{x'}$ is the standard deviation of the first derivative of the considered fragment of the pulse wave signal from the mean value of the first derivative;

σ_x is the standard deviation of the considered fragment of the pulse wave signal from the mean value of the signal.

To study the existing methods of contour analysis by means of the certified computer photoplethysmograph ELDAR (ZAO "Novye Pribory", Russia), we formed an

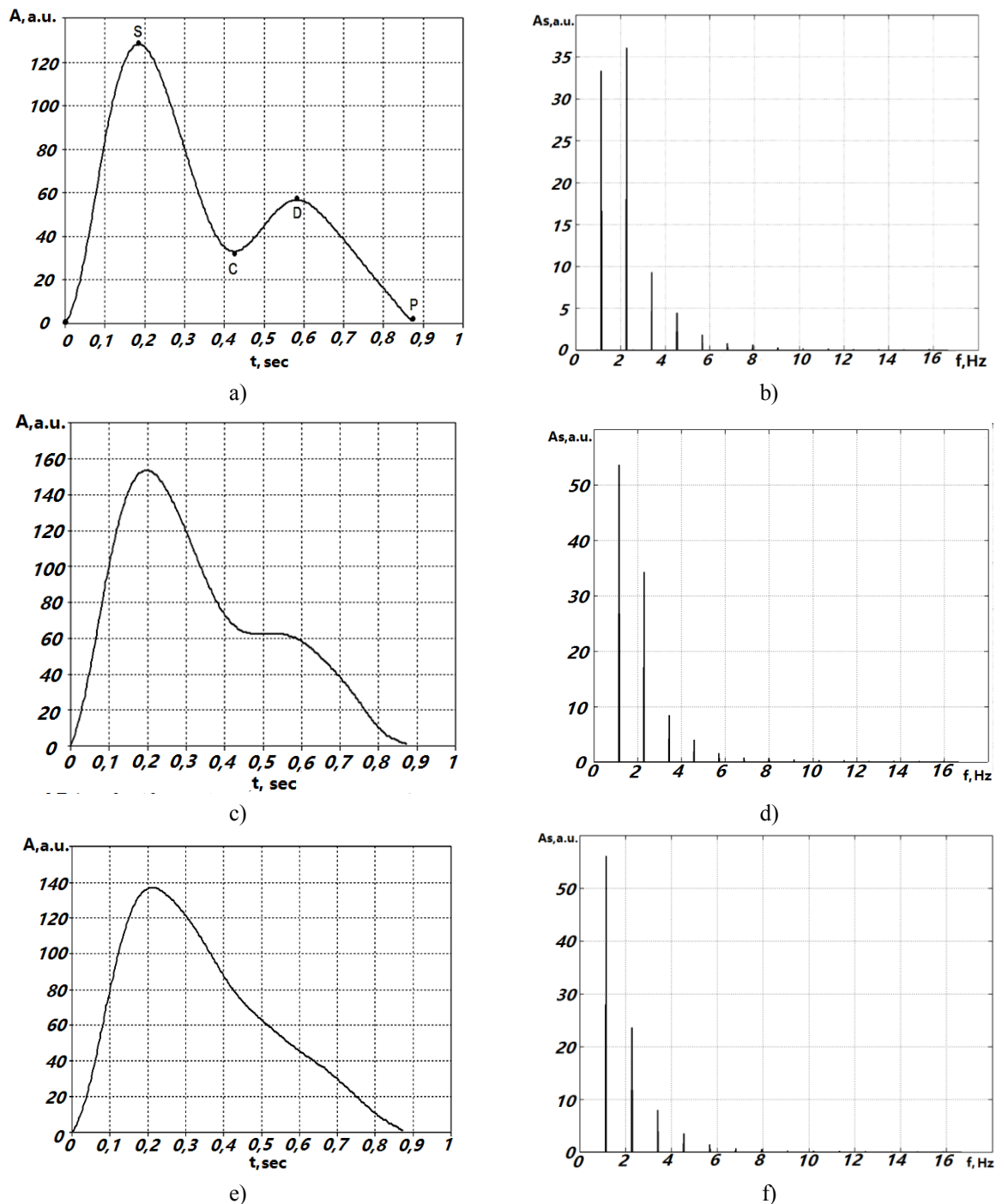


Fig. 4 Typical fragments of pulse wave signal (a, c, e) and the amplitude spectrum of replicated fragments (b, d, f).

experimental array of pulse wave signals, recorded in groups of volunteers without diseases of vascular system, with age-related changes of vascular wall, and with cardiogenic pathologies. All patients confirmed their voluntary consent for being included in the examination. The preliminary analysis of the obtained signals revealed the presence of the first three types of the pulse waves.

2.2 Results

The transition from time domain to frequency domain by means of the spectral Fourier transform allows clearer assessment of the PW shape. To get the spectral assessment of the PW contour morphology, we applied the fast Fourier transform operations to the signal, obtained by sequential replication of single fragments of PW with the steady component removed. To form a fragment of the pulse wave, we used the amplitude-time detector of reference points, characterised by small detection error under the conditions of noise and

Table 2 Diagnostic indices.

PW type	Reflection index, %	Stiffness index, m/s	Form factor	b/a	Harmonic factor
Norm	54±13	7.5±0.73	1.91±0.19	-0.79±0.24	0.93±0.17
Risk	61±15	9.8±1.21	1.48±0.16	-0.80±0.18	1.58±0.22
Pathology	Impossible to determine		1.21±0.12	-0.81±0.26	2.45±0.2

interference having different intensity and origin [12]. In the present amplitude-time detector, the characteristic points used to form a singular fragment were the points of maxima for the first derivative of the original signal. In this way, we avoid the detection of dicrotic maximum.

The analysis of the obtained spectral characteristics of the signal, formed by a sequence of different typical contours, has shown the presence of certain differences in the structure of amplitude spectrum (Fig. 4).

As an appropriate index to assess these differences, we proposed the harmonic factor, defined as the ratio of the first harmonic amplitude As_1 and the second harmonic amplitude As_2 in the spectrum:

$$KS = \frac{As_1}{As_2}. \quad (5)$$

The block diagram of the algorithm for complex processing of a photoplethysmogram (PPG) implemented in the Matlab software package is presented in Fig. 5.

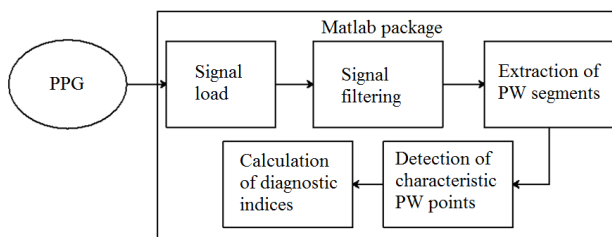


Fig. 5 Structure of the algorithm for photoplethysmogram processing.

3 Conclusion

The calculated diagnostic indices aimed to assess the arterial vessels condition basing on the PW contour analysis are presented in Table 2.

With age, when the stiffness of blood vessels increases, or in the presence of pathology, the reflected wave propagates faster and stronger overlaps with the systolic component of PW. The bimodal shape of the signal transforms into a unimodal one (see Fig. 2, Class

3). Such reshaping makes the detection of the dicrotic maximum hardly possible.

The stiffness index and the reflection index do not allow one to obtain reliable diagnostic information in the group of volunteers with the pulse wave shape of the third type (Fig. 2). The coefficient (b/a) characterises only the systolic part of PW. The harmonic factor based on the spectral analysis characterises the diastolic component better and allows the assessment of the signal shape change. The first two harmonics used to calculate the harmonic factor are less sensitive to noise. Thus, we get a more precise diagnostic parameter, in comparison with the form factor that takes the entire signal into account, as well as the entire spectrum of the signal. Moreover, the harmonic factor demonstrates the largest difference between three types of waves, compared to other indices.

The spectral analysis of the sequence of replicated PW fragments allows the assessment of the arterial vessels condition basing on the contour analysis. The main advantage of this method is that it is not necessary to detect precisely the amplitude-time parameters of the PW dicrotic notch. However, it is important to extract the pulse wave fragment correctly.

On the mechanism of the gallstone nucleus formation and the impact of laser radiation on its growth

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Abstract. Results of the experimental study of laser radiation impact on the process of crystal formation in bile preparations are presented. The possible mechanism of laser effect on the crystal formation process in the volume of biological fluids, in particular, the human bile, is discussed. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Biological solution; Laser; Cluster; Pathogenic mineral formation; Biominerals; Bile.

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1 Introduction

Processes of pathogenic mineral formation are known to occur in the human organism leading to the growth of stones, e.g., in the urinary system, dental and salivary stones. To date the growth of gallstone morbidity rate is recorded in many countries, and the number of patients in the world at least redoubles during each decade. Therefore, the issues of diagnostics, treatment and prophylactics of stone formation remain actual [1].

Cholelithiasis is a polyetiologic disease characterised by the formation of stones in bile passages. The major site of stone formation due to developing cholelithiasis is the gallbladder, but they can also appear in all other parts of the biliary system [2].

The following types of gallstones are differentiated [3]:

1. Homogeneous stones (cholesteric, pigmental (bilirubin), calciferous).
2. Mixed stones (80% of all gallstones). The core consists of organic substance, coated by layers of three major elements: cholesterol, bile pigment, and calcium salts.
3. Complex stones, a combination of both forms, are observed in 10% of all cholelithiasis cases. The stone core contains cholesterol, and its shell has mixed composition (calcium, bilirubin, cholesterol).

The bile is a liquid with complex composition. Besides the cholesterol, it includes bile acids and their salts, phospholipids, conjugated bilirubin, protein and inorganic ions [3]. Combined with cholesterol, these substances make it water-soluble, forming the so-called macromolecular (lipid) complex that plays the role of a transport system (in the form of complex micelles and vesicles) and provides the transfer of all components of bile and the conservation of its colloidal stability [4]. Under the normal physiological conditions, all components of bile are completely soluble in it. The disturbance by some factors of the colloid system stability that keeps its components in soluble state,

leads to the transition of some components into unsoluble state and sedimentation. If the process progresses, the stones are formed [2].

Although the change in the stability of the bile colloid system is of great importance for the explanation of gallstone formation, the mechanism of this process cannot be considered as completely studied [5]. There is no unique concept explaining the stone formation in the gallbladder. It can be considered as established that the factors that disturb the colloid stability include infections, stagnation of bile, and impairment of cholesterol metabolism. To none of these factors the exclusive significance can be attributed [2, 3]. There are also other biochemical processes that violate the bile colloid equilibrium [2].

On the other hand, the capability of many biological liquids (saliva, blood, urine, bile) to crystallise is used in the diagnostics of diseases. In such diagnostics different patterns formed as a result of the crystallisation of a liquid droplet on a substrate (crystallograms) are analysed [6].

In Ref. [7] the planar crystallisation of human bile was studied experimentally. It was shown that the morphology of crystallograms depends on the destabilisation stage of the bile colloid structure and the remoteness of the appearance of biliary pathology symptoms.

The authors of Ref. [8] studied the influence of laser radiation on the processes of planar crystallisation of blood serum. It was shown that the laser irradiation of the liquid leads to the reduction of the mean size of the planar crystals (lamellar rays) compared to the samples not exposed to laser radiation. The effect of laser radiation on protein solutions was studied in Ref. [9]. It was shown that as a result of the exposure of protein solution to laser radiation the craquelure structure (rupture pattern) of its dried film is changed. The mean size of the elements in such pattern is reduced as compared to the non-irradiated solution. It is worth noting that the possibility to affect polymer gels of

biological macromolecules was also shown in the course of hologram recording in gelatine gel-colloid systems, in particular, at the expense of the exposure of gel-colloid layer to the infrared (IR) radiation [10].

The experimental *in vitro* studies of the effect of the helium-neon laser (the wavelength 630 nm, the power 5, 10, and 15 mW) on the physicochemical properties of bile have shown that in the bile samples from the patients with cholelithiasis the laser irradiation reduces the viscosity and pH of the solution and increases the nucleation time [11].

However, according to the present data about the influence of laser radiation on a biological liquid planar crystallisation processes and even on the nucleation in the bile volume, the laser radiation is still not used to prevent mineral formation in the hepatobiliary system. Probably, this is due to the experimental data deficit about the laser radiation impact on the processes of crystal formation in the bile volume, the insufficient development of theory about the mechanism of such impact, as well as the incomplete understanding of the stone nucleation process itself.

At present, the high-energy laser radiation is used for the fragmentation of gallstones (laser lithotripsy) [12], while the low-energy laser radiation has found application in the therapy of inflammation diseases of hepatobiliary system, e.g., cholangitis [13]. Some inflammatory processes are due to the cholelithiasis.

Therefore, the study of physicochemical processes of stone nucleation in colloid and gel-like media is still important both for deeper understanding of gallstone formation and searching for the methods to prevent lithogenesis.

The aim of the present study is to consider the possible mechanism of crystal nucleation in colloid and gel-like media without boundary surfaces and the experimental study of laser radiation impact on the processes of lithogenesis in the volume of these media, in particular, human bile.

2 Biomaterial nucleus formation

Basing on the general knowledge of the colloid structure and biochemistry, as well as on the results of the studies of crystallisation dynamics in bile samples [5], we assume that a few mechanisms of the biomaterial nucleus formation are possible.

2.1 Associates of macromolecules

The crystal appears and the growth in size process begins due to the appearance of a nucleus. The nucleation mechanism for the formation of crystals on a solid substrate is known [14], but in contrast to the crystal formation on a substrate, in the real biotissue there are no distinct nonuniformities like interfaces between media. Therefore, the nucleation may be initiated by certain mechanical inclusions or local inhomogeneities (clots) in the gel solution. The studies of crystallisation dynamics and crystal morphology in the course of sedimentation in samples of natural bile

and in model media, carried out in Ref. [5], have shown that the rate of nucleation is determined by the degree of bile inhomogeneity, which manifested itself in the form of clots having the size to 0.5 mm. It was shown that just on the clots the mass crystallisation of cholesterol and carbonate phases occurs. In the clot-free space, the stones appeared significantly later [5].

It is known that the solidification of gel solution is accompanied by the formation of intermolecular hydrogen bonds, i.e., the gelation occurs [15, 16]. Even in semidiluted solutions of polymers (with the polymer concentration 1% and less) the macromolecules in the statistical coil molecule conformation, overlap, penetrating into each other with their chain fragments. The chains of adjacent macromolecules can interact and form "bridges" between macromolecules.

The intermolecular bonds are weak. They permanently decay under the impact of thermal fluctuations and appear at new sites. Therefore, such bonds are typically formed all over the volume of the gel solution and can hardly give rise to stone formation. In the worst case, the entire solution can approach the glassy state under the conditions of growing polymer concentration [15, 17].

Therefore, to our opinion, the formation of stone nucleus is more probable due to the entanglement of adjacent chains of macromolecular coils [15, 17]. Such entanglement of chains can occur also for different other macromolecules, such as proteins and organic molecules. The quasi cross-linkage of macromolecules is topologically more stable than the usual bridge bonds. They join two macromolecules into a united coil molecule conformation. With time in such heterogeneous gel solution, the quasi cross-linkage can serve as a preferable site of sedimentation for other macromolecules, including those having the quasi-crystalline globular conformation. Finally, the microscopic stone is formed that plays the role of a nucleation centre for a big stone.

2.2 Fringed micelles

It is possible that the mechanism of inhomogeneity formation is based on the appearance of a fringed micelles in the bioorganic solution, which can be a gallstone nucleus. The fringed micelle is a macromolecule, in which the crystalline and amorphous segments alternate [18]. As a result of intermolecular interaction that arises between such macromolecules the formation of an intermolecular fringed micelle becomes possible, in some regions of which the macromolecules are packed parallel to each other (the crystals with unwound chains), i.e., the conditions for partial crystallisation are created. These highly ordered regions can be interrupted by the regions with irregular arrangement of molecules. Since the length of macromolecules exceeds that of crystallites, the same polymer chain participates in the construction of a few crystallites, i.e., it spreads through a few crystalline and amorphous regions. Thus, the individual crystallites are linked via the amorphous regions [19].

Most probably, a single macromolecule, even fold into a fringed micelle, can change or invert its conformation. However, if several such molecules join to form a cluster, it is more difficult to transform them into a coil molecule state. That is why the formed cluster can play the role of a stone nucleus.

3 Laser radiation impact on the solutions of bile preparations

3.1 Experimental technique

To clarify the effect of laser radiation on the processes of crystal formation in bulk bioorganic media we experimentally studied the influence of laser radiation on the formation of crystal nucleation in the preparation of bile using the method of bile crystal growth in silica cuvettes. The experimental protocol on biomedical research was compiled as per the Helsinki Declaration, 200.

In the experiment we used the following preparations: i) white bile (free of pigment), gallstones after cholecystectomy in the case of gallbladder hydrops; ii) natural bile, gallstones after cholecystectomy in the case of cholecystitis.

The gallstones were hanged on a nylon thread in the silica plane-parallel cuvette with mat walls, filled with bile, so that the stone had no contact with walls or bottom. The cuvettes were closed with cover glass.

For the experiment, two cuvettes with white bile and two cuvettes with natural bile were prepared. During the experiment, one cuvette with white bile and one cuvette with natural bile were irradiated with laser light. The second cuvette of each pair was not exposed to laser radiation. The cuvettes were kept under the atmospheric pressure $p = 705\text{--}735$ torr and the temperature 35°C , insignificantly differing from that of gallstones inside the human organism.

The gallstone that was neither placed in bile, nor exposed to any external influences played the role of a control sample.

For the bile irradiate we used the semiconductor laser with the wavelength 532 nm, the power 30 mW, and the beam diameter 5 mm. In the experiment the laser radiation, incident on the depolished glass wall of the cuvette, was scattered and affected the bile with the sample merged in it. The bile was exposed to radiation 5 minutes every day. The experiment was carried out during 65 days and was immediately finished after the complete evaporation of bile from the cuvette.

3.2 Results

After the experiment, the following objects were visually studied: i) the gallstone kept in cuvette and not exposed to laser radiation; ii) the gallstone kept in cuvette and exposed to laser radiation; iii) the cuvette with bile remainders not exposed to laser radiation; iv) the cuvette with bile remainders exposed to laser radiation.

In the preparations of natural bile, no qualitative changes were noticed both in the bile remainders in the cuvettes and in the sample gallstones that were kept in theses cuvettes. The studied samples of gallstones from both cuvettes were identical to the control sample.

In the preparations of white bile, strong qualitative changes were observed.

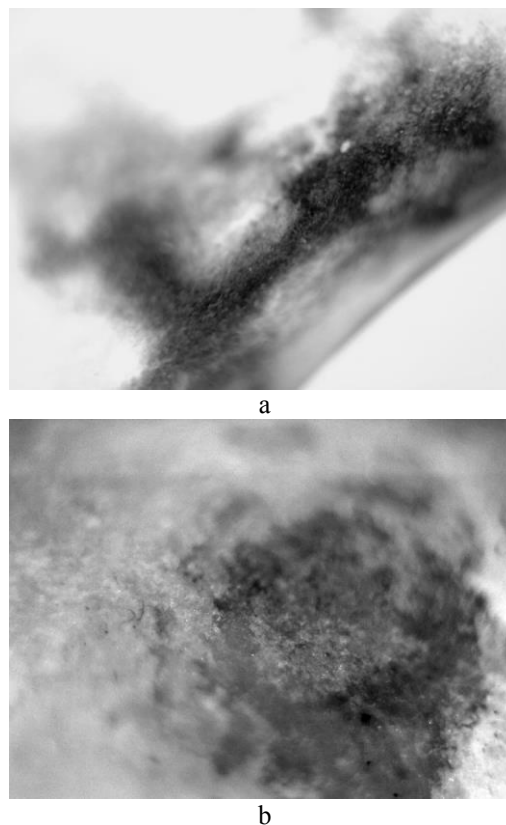


Fig. 1 Deposition on the gallstone kept in the cuvette with bile, not exposed to laser radiation: a) nonuniformity distribution of the deposition; b) surface defects. The magnification was $60\times$.

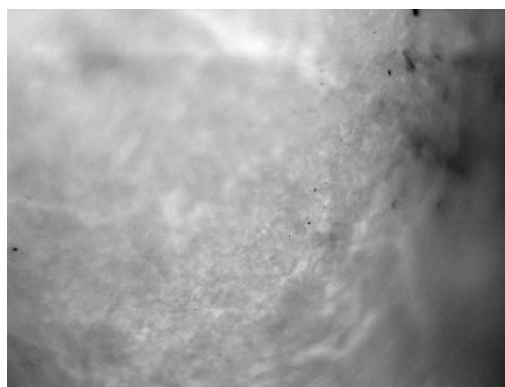


Fig. 2 The surface of white coating on the gallstone kept in the cuvette exposed to laser radiation. The magnification was $60\times$.

In contrast to the control samples and the samples from the cuvettes with natural bile, the gallstones from both cuvettes were coated with white deposition. The

sample from white bile, not exposed to laser radiation had nonuniformity deposition with surface defects well seen in the microscope (Fig. 1).

The gallstone kept in the bile exposed to laser radiation had uniform coating without surface defects (Fig. 2). The coating thickness was nearly 0.1-0.2 mm.

During the visual investigation of the cuvette that contained white bile we found parallelepiped-shaped crystals having the thickness of nearly 0.2-0.3 mm at the bottom (Fig. 3).

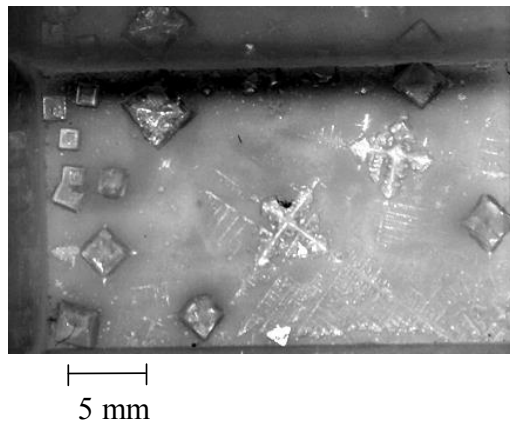


Fig. 3 The crystals grown at the bottom of the cuvette with bile, not exposed to laser radiation.

One of the crystals was extracted for detailed study. The microscopy revealed the presence of concentric rings around one centre, conceivably the nucleus (Fig. 4).

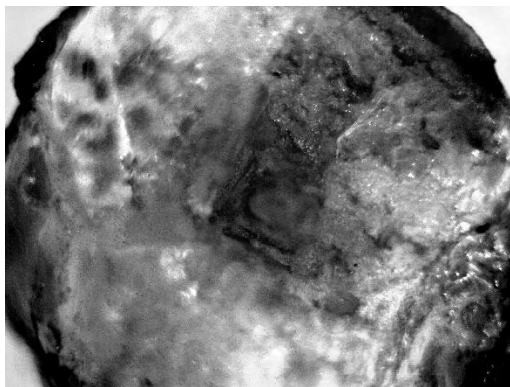


Fig. 4 The crystal with rings around a certain centre observed by means of microscopy (the image is magnified and additionally contrasted).

The study of the crystal in polarised light has shown the presence of anisotropy properties. When the crystal was placed between two crossed polarisers, the observed field of view was cleared in the region of the crystal.

During the visual study of the cuvette, in which during the experiment the white bile exposed to laser radiation was kept, we have found a thin transparent layer of homogeneous viscous colloid liquid with yellowish colour and the thickness of nearly 0.2 mm at

the bottom of the cuvette. No crystals like those that appeared in the unexposed cuvette were found. Between two crossed polarisers we found slight triangle-shaped yellowish clearings in the field of view along the bottom edges near the walls. The clearings were caused by small crystalline formations having the thickness of nearly 0.2 mm and the longitudinal dimensions considerably smaller than those of the crystals found in the non-exposed cuvette.

3.3 Discussion

Our results allow the assumption that the laser radiation affects in some way the processes of crystal formation in the volume of white bile.

Our hypothesis is that the mechanism of such influence of the electromagnetic radiation on the solutions of organic substances, including the colloid ones, can be related to the specific behaviour of the interface monomolecular layers of polar solvent near the surface of macromolecules or particles. If the monomolecular hydrate film on the colloid particle surface consists of dipolar molecules, densely covering the particle surface, with similar charged ends directed to the particle, then the film forms a double electrical layer. This double layer is spontaneously polarised (due to the interaction between water molecules themselves and of water molecules with the particle), and the degree of this polarisation grows with the particle or macromolecule size [20]. The unavoidable spontaneous polarisation of the 2D layer of polar molecules on the particles surface occurs, and a gigantic dipole can arise, which agrees with the experimental data [20].

Thus, the possible mechanism of the laser radiation impact on the mineral formation in the bile solution consists in the specific features of the interaction of electromagnetic waves with the organic liquids, namely, in the solution the large clusters that could be the nucleation centres are divided into smaller associations. Due to this reason, in the cuvette exposed by laser radiation the homogeneous viscous mass with minor crystalline inclusions was left, while in the cuvette not exposed to laser radiation the large-size crystals were formed.

The difference between the stones in the uniformity and homogeneity of deposition is most probably also because under the laser exposure, the molecular clusters are broken into smaller parts, and smaller groups of molecules implement the sedimentation. Thus, the deposition layer acquires more uniform thickness than in sedimentation of larger molecular clusters.

Possibly the structure of the liquid, modified by laser radiation will return to the initial conformations after the impact termination via a chain of intermediate structure modifications, rather than instantaneously. In the case of biological molecules, this process can take macroscopic time (minutes or hours). Therefore, the liquid structure changes initiated by the laser radiation could be conserved during long time under the multiply repeated laser impact. This is particularly important, when the laser radiation acts on biological liquids in the human

organism *in vivo* and demonstrates clearly expressed positive effect [21].

4 Conclusion

Thus, the possible mechanism of gallstone nucleation can be related to the appearance of local inhomogeneities (clusters) of the gel solution, in which the adjacent chains of different proteins and organic molecules interacting with each other with the participation of water can form stable links. The experimental results allow the assumption that the exposure of white bile by laser radiation affects to the crystal formation processes in it.

The hypothetic mechanisms of the effect are the division of larger molecules associates into smaller ones in the field of laser radiation, which, in turn, reduces the viscosity of the solution and inhibits the stone growth in the gel-colloid medium.

The practical significance of the obtained results consists in hypothetic possibility of using the laser radiation for prophylactics of pathogenic stone formation in the hepatobiliary system. More confident statements will be possible if further experiments will show the stable inhibition of stone formation in the preparations of natural bile, including the dependence on the parameters of the used laser radiation.

OCT study of skin optical clearing with preliminary laser ablation of epidermis

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Abstract. We report the results of the experimental study of optical immersion clearing of laboratory animals skin *in vivo* with preliminary laser ablation of epidermis. It is shown that the ablation of the skin surface leads to the local edema that reduces the optical detection depth immediately after the impact. However, the water evaporation from the damaged epidermis causes the optical clearing of skin, comparable with that caused by polyethylene glycol in intact skin. It is shown that the preliminary ablation of the skin surface before the application of immersion agent does not lead to significant increase of the optical detection depth as compared to the sole effect of ablation or immersion. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: skin laser ablation; optical coherence tomography; optical detection depth; immersion optical clearing.

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1 Introduction

Skin has a complex inhomogeneous structure causing a strong light scattering, which limits the optical methods applicability to the diagnostics and treatment of various diseases [1, 2]. The development of methods enhancing the optical clearing of skin is an urgent problem that has been a subject of multiple studies [3, 4]. However, the diffusion of immersion agents into the skin is hindered by the existence of epidermal barrier. To overcome the barrier function of the epidermis, different methods are used, including the chemical dissolving of the lipid layer [5, 6], the mechanical damages [7], the effect of ultrasound [6-9], etc. Among these methods, the laser microablation of epidermis can be used to solve the problem. In the literature, the results of applying microablation to enhance the skin permeability for different medical preparations and particles are presented [10, 11]. However, the effect of the epidermis microablation on the optical detection depth in the case of monitoring the process of skin optical clearing using the optical coherence tomography (OCT) is studied insufficiently.

OCT is widely used to study the skin optical clearing, since it is a relatively simple and convenient instrument for monitoring the changes of optical probing depth and attenuation coefficient in the process of the immersion agent penetration into the skin [9, 12-14].

The aim of the present work is to study the effect of the epidermis ablation *in vivo* on the optical detection

depth using OCT in the process of skin immersion optical clearing. The paper presents the results of the comparative study of the immersion clearing of the intact skin and the skin with preliminary ablation of the surface epidermis layer.

2 Methods and Materials

As a source of radiation for skin ablation we used the erbium laser Palomar Lux2940 (Palomar Medical Technologies Inc., Burlington, MA, USA) generating single-spoke pulses at the wavelength 2940 nm with the energy of 1 J, the pulse duration being 5 ms, and the spike duration being 200 μ s. In the experiments, we used the regime of ablation of the upper layer of skin using a wide beam at the area 6×6 mm² to the depth not exceeding 50 μ m. As an illustration, Fig. 1 shows a fragment of skin with the epidermis ablation.

As an optical clearing agent, we used polyethylene glycol 300 (PEG-300, the molecular weight 300 dalton, Sigma-Aldrich, Germany). The refractive index of PEG-300 at the wavelength 930 nm is equal to 1.456. The measurements were carried out using the multiwave Abbe refractometer DR-M2/1550 (ATAGO, Japan).

The studies were performed *in vivo* in 15 laboratory albino rats, divided into three groups of 5 animals in each: group I – laser ablation alone, group II – application of PEG-300 alone, and group III – ablation followed by PEG application. The age of the animals was 1 year; the mass was 200-300 g. The animals were subjected to anaesthesia by the intramuscular injection

of Zoletil 50 (Virbac, France), the dose of 0.18-0.2 ml. The hair from the skin surface was removed using the depilatory cream «Veet» (Reckitt Benckiser, France). The studies were approved by the Ethics Committee of V. I. Rasumovsky Saratov State Medical University of the Ministry of Health of Russian Federation (protocol No. 6 of 01.03.2016).

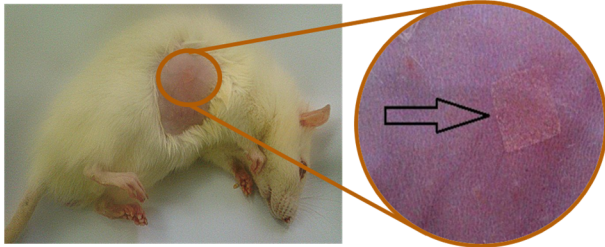


Fig. 1 Photograph of a rat and the magnified image of the skin area with the ablated upper layer fragment (pointed by an arrow).

To monitor the optical detection depth (ODD) we used the spectral optical coherence tomograph OCP930SR (Thorlabs, USA) having the following parameters: the central radiation wavelength 930 ± 5 nm, the radiation bandwidth at the half-maximum level 100 nm, the longitudinal and transverse resolution in air 6.2 and 9.6 μm , respectively. The tomograms of the processed zone were recorded before the ablation, immediately after ablation and PEG-300 application, and then each 5 minutes during 1.5-2 hours.

Basing on the analysis of the OCT images at each moment of time, the ODD was determined, i.e., the

optical distance between the skin surface and the lower bound of the useful signal, in three regions in the ablation zone. The obtained data were averaged over all animals in the group. The root-mean-square deviation was calculated.

3 Results and Discussion

Figure 2 (a-c) shows the typical OCT images of the intact rat skin from the group I, in 30 minutes, and 1.5 hours after the ablation. The rectangle selects the region 101 pixels wide, which nearly corresponds to 300 μm , over which the A-scans were averaged to measure the ODD. For the present series of images at the initial moment of time the ODD amounted to 520 ± 10 μm (Fig. 2a), then in 30 minutes it became 468 ± 10 μm (Fig. 2b). The reduction of ODD is related to the slight edema in the region of ablation due to the diffusion of lymph and water from the surrounding interstitial fluid to the site of the damage, which is a natural reaction of the organism to the skin injury. However, in 1.5 hours, the increase of the ODD to 626 ± 10 μm due to the water evaporation from the ablation zone and tissue shrinkage was observed (Fig. 2c). The arrows point at the epidermis layer. One can clearly see that the ablation and consequent dehydration of tissue decreases the optical thickness of the epidermis. The mean estimated optical thickness of the epidermis amounted to 57 ± 5 μm , 30 minutes later it was 42 ± 6 μm , and in 1.5 hours it was 36 ± 5 μm .

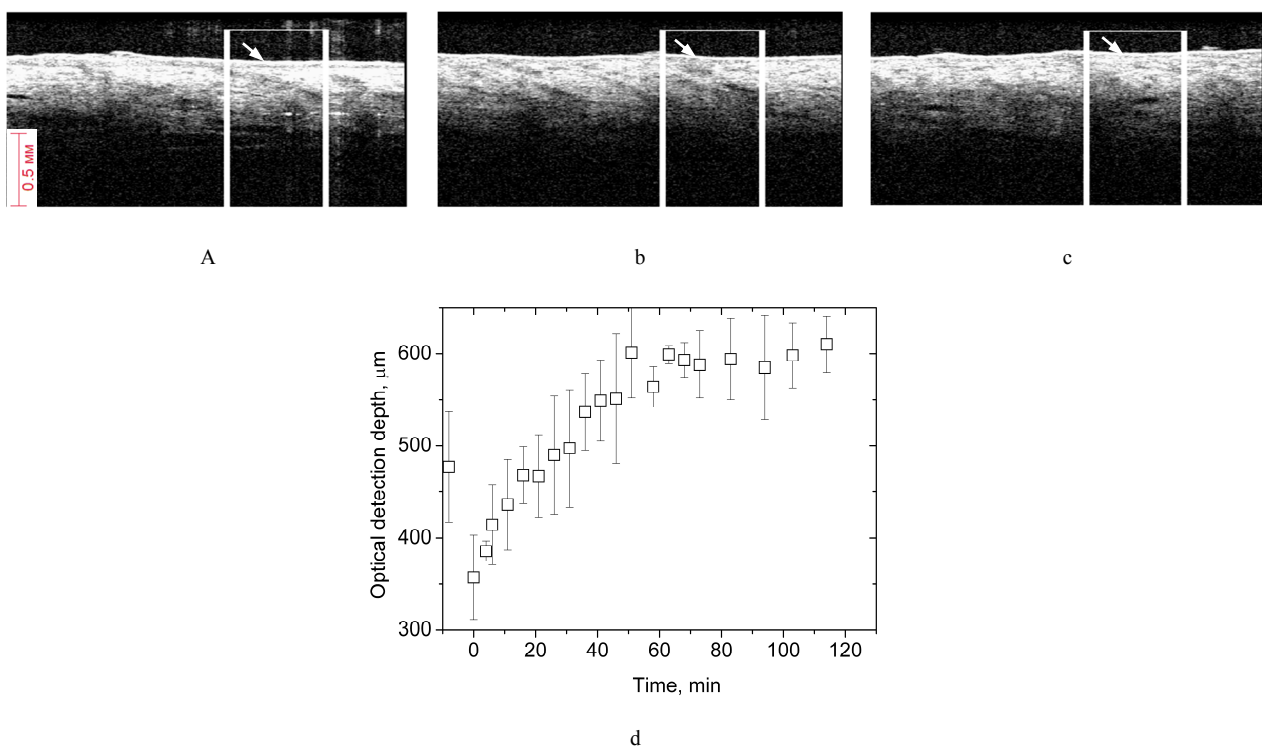


Fig. 2 OCT images of the rat skin from the first group: a – before the beginning of experiment, b – in 30 minutes, c – in 1.5 hours after the beginning of the experiment. The rectangle selects the zone of averaging A-scans of the OCT signal used to determine the optical detection depth. The arrows point at the epidermis layer. The time dependence of the averaged ODD (d).

The kinetics of the averaged ODD is presented in Fig. 2d. The averaging was performed over 15 skin regions in five experimental animals. The vertical segments show the root-mean-square deviation. The zero abscissa corresponds to the beginning of the ODD kinetics measurement after the ablation, and the negative abscissa corresponds to the intact skin ODD. In the figure, it is clearly seen that at zero time the value of ODD is significantly smaller than before the ablation, which is a manifestation of sharp growth of light scattering by the tissue. The restoration of the ODD value to the level, corresponding to the intact skin, occurred during 30–40 min, and then the ODD increased and reached the maximal value in nearly 50–60 min after the ablation. Then up to the end of observation the ODD remained practically unchanged, which means that no more water is evaporated from the skin surface because of the formation of a dense layer in the region of damaged tissue. Sufficiently large deviation of ODD values within the group is, apparently, due to the high sensitivity of the method to insignificant breathing movements of the animal during the observation process.

Figure 3 (a–c) presents the typical OCT images of the rat skin from group II before the impact, in 30 minutes, and in 1.5 hours after applying the immersion agent. At the initial moment of time the ODD amounted to $557 \pm 10 \mu\text{m}$ (Fig. 3a), and 30 minutes later it was $572 \pm 10 \mu\text{m}$ (Fig. 3b). The increase of ODD occurred at the expense of the reduction of the scattering in the tissue due to the diffusion of the immersion agent into the interstitial space and the matching of the refractive indices of the interstitial fluid and collagen and elastin fibres of dermis. The ODD growth continued during the entire experiment, and in 1.5 hours the ODD reached $686 \pm 10 \mu\text{m}$ (Fig. 3c). The mean optical thickness of the epidermis in this case increased from $87 \pm 4 \mu\text{m}$ to $93 \pm 7 \mu\text{m}$, since due to the penetration of propylene glycol into the interstitial space, the mean refractive index of the epidermis increased.

Figure 3d presents the kinetics of the mean value of the ODD. One can clearly see that the ODD reached its maximal value in nearly 70–80 min, and then stayed almost unchanged or even decreased. This effect can be due to the gradual reduction of the immersion agent concentration because of its diffusion to the surrounding tissue beyond the observation zone.

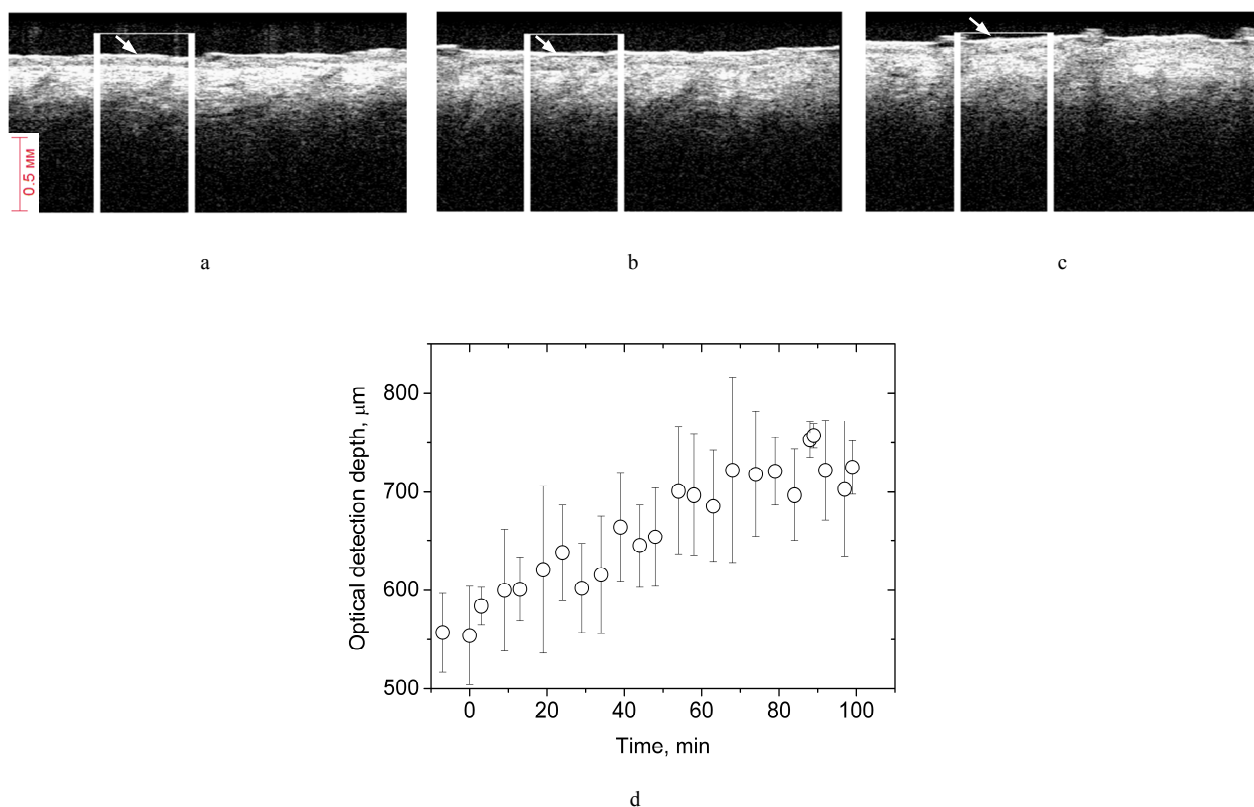


Fig. 3 OCT image of the rat skin from group II: a – before the beginning of experiment, b – in 30 minutes, c – in 1.5 hours after the beginning of experiment. The rectangle selects the zone of A-scans averaging and measuring the optical detection depth. The arrows point at the epidermis layer. Time dependence of the averaged ODD (d).

Figure 4 (a–c) shows the typical OCT images of the intact rat skin from group III before the impact, in 30 minutes, and in 1.5 hours after the ablation and application of immersion agent. At the initial moment the ODD amounted to $512 \pm 10 \mu\text{m}$ (Fig. 4a), and in 30

minutes it was $635 \pm 10 \mu\text{m}$ (Fig. 4b). The mechanism of optical clearing incorporated two competing processes. On the one hand, the scattering increased due to the influx of water and lymph from the surrounding interstitial fluid to the site of injury. On the other hand,

the scattering decreased due to the impact of the immersion agent; the growth of its concentration in the interstitial fluid matched the refractive indices of the interstitial fluid and the collagen fibres of the dermis. As a result, in 1.5 hours the ODD increased to $701 \pm 10 \mu\text{m}$ (Fig. 4c). The estimated optical thickness, first,

decreased from $72 \pm 6 \mu\text{m}$ to $63 \pm 5 \mu\text{m}$ because of the partial removal of the epidermal layer. Then it gradually grew to achieve $78 \pm 6 \mu\text{m}$ (in 1.5 hours) because of the increase of the mean refractive index of the tissue due to the penetration of polyethylene glycol, like in the group II.

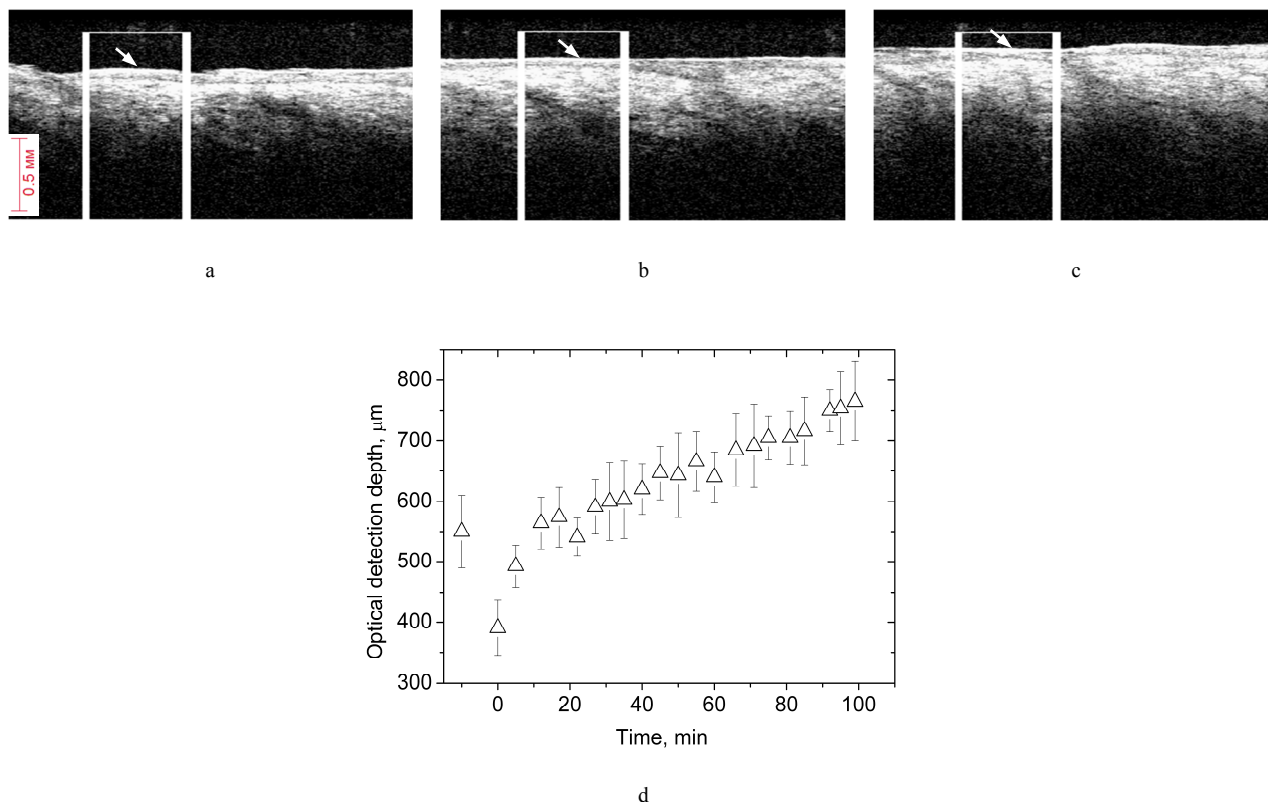


Fig. 4 OCT image of the rat skin from group III: a – before the beginning of the experiment, b – in 30 minutes, c – in 1.5 hours after the beginning of the experiment. The rectangle selects the zone of A-scans averaging and determination of the optical detection depth. The arrows point at the epidermis layer. Time dependence of averaged ODD (d).

The kinetics of the group-averaged ODD presented in Fig. 4d shows that, like in the first group, the growth of the ODD began from the lower values, as compared to the second group, due to the appearance of skin edema because of its damage. However, the restoration of the ODD to the initial values occurred significantly faster (during nearly 15-20 minutes) than in the first group. In the present case, no water evaporated from the skin surface, since the surface was covered with a layer of PEG-300. The ODD growth may be attributed only to the reduction of skin scattering because of immersion.

Figure 5 presents the time dependence of ODD for three groups of experimental animals in the process of observation, normalised to the skin ODD before the ablation and/or the application of PEG-300 (the negative abscissa values). The moment of time $t = 0$ corresponds to the beginning of the recording of the optical clearing kinetics. The deviation of values with respect to the normalised mean value is not shown here for clarity, since the curves are rather close to each other.

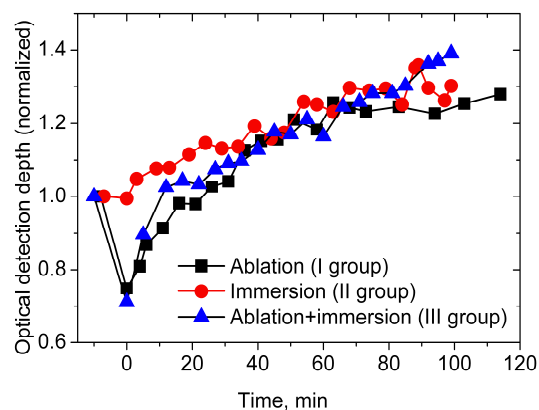


Fig. 5 Time dependence of ODD normalised to that of the intact skin. The symbols correspond to the experimental data.

Basing on the presented results one can estimate the reduction of the detection depth due to the ablation as 25-30%, which to our opinion is related to the increased light scattering in the skin at the ablation zone due to the appearance of edema. From the comparison of curves, corresponding to groups I and III it is seen that the

application of PEG-300 to the skin surface leads to insignificant lowering of the ODD, apparently, related to the probe radiation shielding by the layer of immersion agent.

From the comparison of kinetic curves, it also follows that the rate of optical clearing in groups I and III is higher than in group II, since the slope of the first and the third curve in the region from 0 to approximately 60 min is greater than that of the curve corresponding to the second group. One can explain this fact by the protective properties of the epidermis that inhibit the penetration of PEG-300 into the skin. It is also clearly seen that the slope of the third curve is maximal. Besides that, the comparison of the ODD variation kinetics shows that nearly in an hour after the ablation in the first and the second group the ODD becomes stabilised and remains practically unchanged, while in the animals of group III the ODD continues to increase, which demonstrates the dominant influence of PEG-300 on the ODD.

The increase of skin permeability both for water, leaving the skin in the process of evaporation, and for the preparations applied to its surface, due to the reduction of the epidermis thickness or to the epidermis removal by ablation, was reported in Refs. [15-18]. The appearance of skin edema in the zone of fractional microablation was detected using the backscattering spectra [19]. It was noticed that the residual edema stayed for 24 hours after the ablation. However, the kinetics of measurement of the OCT optical detection depth under the simultaneous formation of post-ablation edema and tissue immersion is studied in this paper for the first time. We have shown that after the ablation of the epidermis using a wide beam, the edema arising as a

protective reaction of the organism to the injury, significantly reduces the depth of light penetration into the skin. To our opinion, this is due to the increased light scattering by dermis, mainly due to the reduction of the refractive index of interstitial fluid, i.e., the additional mismatch of refractive indices of dermis components caused by the influx of the interstitial fluid and lymph to the site of injury.

4 Conclusion

The results of the study of optical clearing of rat skin in vivo based on the kinetics of the OCT optical detection depth have shown that the epidermis ablation does not increase the ODD during 100-110 minutes. Moreover, at the initial stage (30-40 minutes) it even leads to the ODD reduction by 25-30%. Thus, although the permeability of epidermis for the immersion agent increases, the light scattering by dermis increases because of the edema, which inhibits the optical clearing of skin. This effect does not allow one to use the wide-beam ablation to increase the rate of optical clearing of skin. The reduction of edema degree and the increase of the skin optical clearing rate could be possible by using the fractional microablation that produces a matrix of microscopic channels in the skin [10]. However, this hypothesis requires experimental testing.

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Raman and autofluorescence analysis of human body fluids from patients with malignant tumors

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Abstract. In this study we measured Raman and autofluorescence spectral features of blood and urine from patients with various cancers. A total number of 26 blood samples from patients with lung cancer and 12 blood samples from patients with other cancers, and also 10 urine samples from patients with lung cancer and 9 urine samples from patients with other tumors were tested. The processing of experimental data and definition of informative bands for body fluid spectral analysis were performed on the bases of PLS-DA method. Wherein, there is no significant correlation between the most informative criteria for blood and urine. This fact shows that simultaneous study of blood and urine samples can increase the analysis informativeness. In general, the developed approach of body fluids analysis may become the basis of an inexpensive, quick and reliable method of lung cancer screening. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Raman spectroscopy; autofluorescence; lung cancer; biofluidity homeostasis; blood; urine.

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1 Introduction

According to the data from World Health Organization, a cancer rate steadily increases. Moreover, most common cancer is a lung cancer [1]. To decrease the mortality rates associated with cancers one has to develop a simple and reliable method for early cancer detection. Currently, X-ray, tomographic, endoscopic and cyto-histological techniques are widely used for diagnosing lung cancer. However, application of some diagnostic methods is ineffective in the early stages of tumor growth [2]. Therefore, effective diagnostics requires the development of new methods for early cancer detection.

The incidence of registered cancer cases leads to a progressive deterioration of patient's health associated with the weakening of immunity, progressing of cachexia and changes in the internal organs functionality [3]. Since these changes provoke alterations in body fluids homeostasis, it is possible to use the component composition analysis of urine, blood, saliva and other body fluids for cancer detection. Presently, the biochemical analysis [4-6] and the analysis of tumor markers [7, 8] are widely used for the body fluids cancer diagnosis. However, biochemical analysis has poor informative ratio as a result of the poor specificity of the examining body fluids components in a certain cancer localization detection. Analysis of tumor markers is much more informative than biochemical analysis, but the applicability of the most tumor markers in screening studies is also limited by low specificity, and generally tumor markers are used for the disease course monitoring.

Today in addition to the laboratory methods a variety of physical methods of analysis [9] may be successfully utilized for examining the body fluids component composition. Physical methods have such advantages as simplicity of sample preparation, wide dynamic range and great versatility in comparison with chemical methods of analysis. Therefore, body fluids analysis with optical methods can become a successful alternative to existing laboratory methods. Raman Spectroscopy (RS) and autofluorescence (AF) analysis

[10] allow for detecting homeostasis changes in the body fluids at the molecular level. These techniques are successfully used in different branches of clinical medicine and in the experimental studies of the body fluids composition for cancer detection in various locations.

For example, application of RS resulted in 92.3%, 79.5% and 86.4% sensitivity and 85.7%, 91.0% and 80.0% specificity respectively in monitoring blood composition in patients with oral cancer [11], stomach cancer [12] and colorectal cancer [13]. RS of urine allowed for the detection of the prostate cancer [14] with 100% sensitivity and 89% specificity. Moreover, it is possible to increase the diagnostic accuracy of tumor detection by combining simultaneously several body fluids analyses. Thus, the utilization of combined AF analysis of blood plasma, cellular components acetone extract, sputum and urine helped to achieve 90% accuracy of lung cancer detection [15].

A low level of cancer detection with only one diagnostic method and in the analysis of only one type of body fluid requires to carry out more complex analysis and use a combination of RS and AF for the simultaneous study of body fluids (such as urine and blood). It is important to note that in the majority of studies reviewed above spectral features of body fluids have been examined in order to classify of a healthy group and a precancerous (or cancerous) group of only one localization. The aim of this paper is to study the application of body fluids RS and AF analysis for cancer location determination.

2 Materials and Methods

2.1 Experimental setup

The study of body fluids spectral features was performed with the experimental setup shown in Fig. 1. The excitation of collected spectra was performed by the Luxx Master LML-785.0RB-04 laser module (central wavelength 785 nm). The RPB785 fiber-optic Raman probe allows for focusing the exiting radiation,

collecting and filtering the scattered radiation. The collected signal was decomposed into a spectrum using a high-resolution Shamrock SR-500i-D1-R spectrograph with integrated cooled up to -65°C ANDOR DU416A-LDC-DD digital camera.

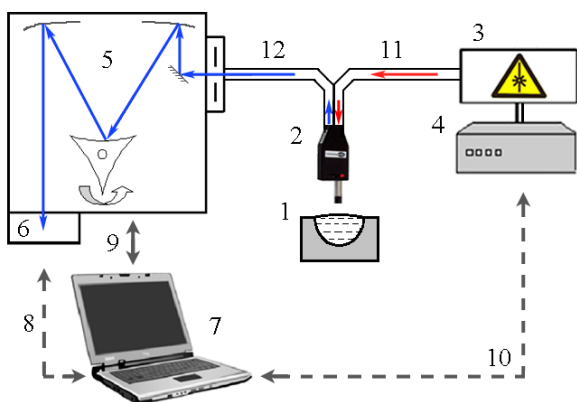


Fig. 1 Experimental setup: 1 – cuvette with body fluid sample; 2 – Raman probe RPB785; 3 – laser module; 4 – power supply; 5 – Spectrograph; 6 – Cooled digital camera; 7 – PC; 8, 9, 10 – electric cables; 11 – excitation fiber; 12 – collection fiber.

The test body fluids were placed in the PMMA cuvette with an aluminum coating. The cuvette geometry (depth 6.5 mm, radius of deepening curvature 19 mm) was optimized to match the working distance of probe focusing lens. The Raman probe was normally positioned on the axis of the deepening.

2.2 Body fluid samples and experimental preparation

The blood and urine samples were collected from patients with cancers of different localizations. The collected samples were placed in sterile test-tubes and were stored at $+2 - +4^{\circ}\text{C}$ before the analysis. The analysis of collected body fluids was performed within 60 h after sample collection. Patients of Samara Regional Clinical Oncology Dispensary with malignant tumors or benign tumors were enrolled in this study. Patients with systemic diseases and patients taking any medical antitumor drugs were excluded from the study.

We performed our study for two cohorts of patients. The first cohort included 26 blood samples from patients with lung cancer, 12 blood samples from patients with other tumors (2 benign tumors, 6 stomach cancers, 3 mediastinum cancer, 1 kidney cancer), 10 urine samples from patients with lung cancer, and 9 urine samples from patients with other tumors (2 benign tumors, 1 stomach cancer, 5 mediastinum cancer, 1 kidney cancer). For the second cohort of 10 samples from patients with lung cancer and 3 samples from patients with other tumors (1 benign tumor, 1 stomach cancer, 1 kidney cancer) the simultaneous recording of spectral properties of urine and blood was carried out. The performed studies were approved by the ethical committee of Samara State Medical University.

2.3 Spectra processing

All spectra were recorded in the spectral range 780-950 nm, the exposure time being 20 seconds. A sequential recording of three spectra for each tested sample was performed. The final spectrum was received from averaging the three recorded spectra. The total time of the final spectrum recording was 3 minutes. The recorded spectra were processed by the method proposed by Zeng et al [16] for AF and Raman signals separation.

The raw spectrum of the urine sample is presented in Fig. 2. The spectrum contains a wide decreasing AF part and narrow Raman peaks. AF was approximated by a tenth order polynomial function. The Raman component of the spectrum was obtained by subtracting the AF component from the raw spectrum. Further analysis of RS and AF spectra was performed independently.

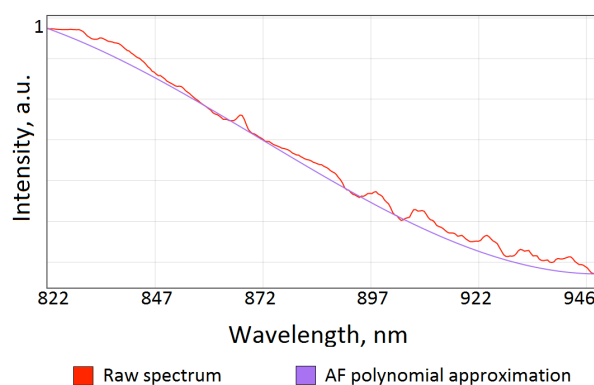


Fig. 2 Raw spectrum of urine.

The processing of experimental data and calculation of posterior lung cancer determination from other types of tumors by body fluid spectral characteristics were performed on the bases of regression analysis. Prior to regression analysis, the raw spectral data were centered, smoothed by the Savitsky-Golay filter, and normalized by using standard deviation of a normal variate method (SNV) [17]. Data centering decreases the model rank by one, and is applicable in uniform model cases. The SNV method subtracts mean value from each spectrum and divides each signal value by the standard deviation of the whole spectrum. The SNV method is used for leveling the experimental data dispersion [17].

The recorded spectra of body fluids may contain hidden links between different spectrum bands, due to the contribution of the same chemical components to these bands. Which results in the appearance of multiple correlations (collinearities). Consequently, the analyzed spectral data is multicollinear, therefore, the projection methods are required for such data analysis. Since we have *a priori* information about exact cancer type corresponding to each study sample, it is recommended that training and classification problem should be solved. The most popular approach to such problems is the discriminant analysis method with regression on latent structures – PLS-DA [18].

The regression problem is solved by the PLS method, further it allows for the regression prediction application in classification of new samples. The regression problem solution by PLS method was performed by the following algorithm: a two-dimensional matrix of spectra (predictors block) and a one-dimensional matrix of diagnoses (responses block) are decomposed into a matrix of scores, a matrix of loadings and a residual matrix. The matrix of loadings provides information about the role of variables, the matrix of scores provides information about the data similarity and correlation. After model construction the k-fold cross validation was used for accuracy testing. Cross validation was carried out as follows: 10% of the samples are excluded from the data set, a model is built to the excluded part and then applied to the remaining part of samples. Then the excluded part of the samples is returned, and the cycle is repeated 9 times with excluding of the following samples parts.

The spectrum informative bands during the regression model construction were determined by the analysis of the variable importance in projection (VIP) [19]. VIP allows for evaluating individual variables from the predictors block influence on the PLS model. The higher the VIP-score of an individual variable is, the more significant it is in model construction. Variables with a low VIP-score are less important, and may be regarded as candidates for exclusion from the model. The VIP distribution makes it possible to define the most informative spectral bands in the blood and urine spectra for constructing a regression model for classification of patients with lung cancer and patients with other tumors.

Multivariate analysis was carried out with using the TP^Tcloud^{beta} software module (<https://tptcloud.com>). The statistical processing of the results, analysis of the correlation dependence and calculation of the Pearson correlation coefficient were performed in the IBM SPSS Statistics ver. 23 software package.

3 Results

3.1 Raman spectra of blood

The Raman spectra of blood are presented in Fig. 3. As shown in Fig. 3, blood samples of patients with various tumors have qualitatively coinciding spectra. Differences are observed in the intensity amplitude of the individual spectral bands. Human body fluids have a complex chemical composition; therefore the shape of body fluids Raman spectra and certain spectral bands intensities depend on the contribution of molecular vibrations of several components. This set of blood spectra was a subject to the multivariate analysis for constructing regression model. Fig. 4 shows the VIP-scores of Raman spectra matrix of blood samples for the constructed regression model of lung cancer detection among tumors of other localizations.

Results shown in Fig. 4 allow for defining the most informative spectral bands in the constructed regression

model for discriminating the lung cancer from other tumors during the analysis of blood Raman spectra. For example, the spectral band $790\text{--}820\text{ cm}^{-1}$ corresponds to glutathione [20]. Oncological diseases are followed by a change in the relative amount of neutrophils, which stimulates oxidative stress in the patient's blood.

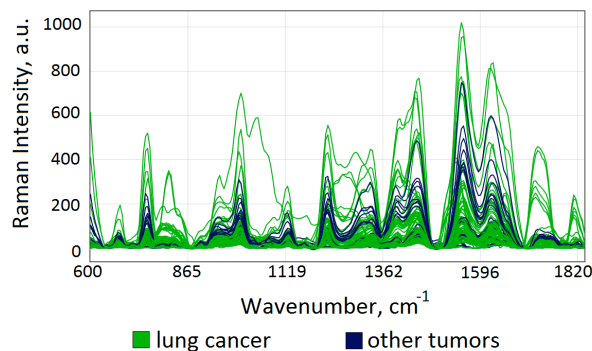


Fig. 3 Raman spectra of tested blood samples.

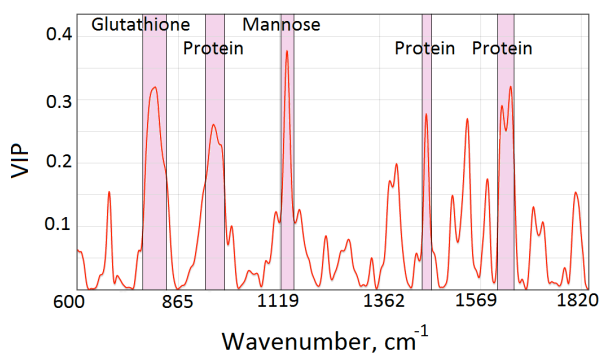


Fig. 4 VIP-scores of blood samples Raman spectra matrix.

Changes of glutathione concentration causes the antioxidant activity decrease of plasma, therefore, this decrease may be regarded an informative criterion for assessment of the organism oxidative stress [21]. Cancer tissues are characterized by increased proteolysis and increased concentration of acute phase proteins [22]. Informative Raman bands associated with these changes are $946\text{--}970\text{ cm}^{-1}$ (proteins), $1465\text{--}1475\text{ cm}^{-1}$ (lipids, proteins), $1640\text{--}1660\text{ cm}^{-1}$ (proteins, phospholipids) [23 - 25]. The intensity of the spectral band $1135\text{--}1140\text{ cm}^{-1}$ is proportional to the mannose concentration [26].

The metabolic imbalance of minor sugars and changes in the mannose concentration lead to changes in the glycoproteins synthesis and changes in glycosylation [27]. As a result, the organism produces "abnormal" immunoglobulins, and the immune system ability to identify "abnormal" cells decreases. The constructed regression model enables to discriminate the lung cancer from other tumors by blood sample spectral characteristics analysis with 84.9% *a posteriori* probability. To improve our research informativity spectral characteristics of urine samples from cancer patients were analyzed.

3.2 Spectral characteristics of urine

The porphyrins (nitrogen-containing pigments) accumulate in sites of active cell division and excrete with urine [28]. Alterations in the AF urine spectrum reflect changes and metabolic imbalance of porphyrins. Therefore, the AF intensity of urine can be used as an informative criterion of oncopathology growth. The AF spectrum of porphyrins has features in red and near-infrared spectral ranges [29], so the excitation of the AF spectra by 785 nm laser allows to evaluate the presence of porphyrins in the test sample. The raw spectra of urine samples from patient with stomach cancer and patient with lung cancer at different exposure times of laser radiation are shown in Fig. 5 (a, b).

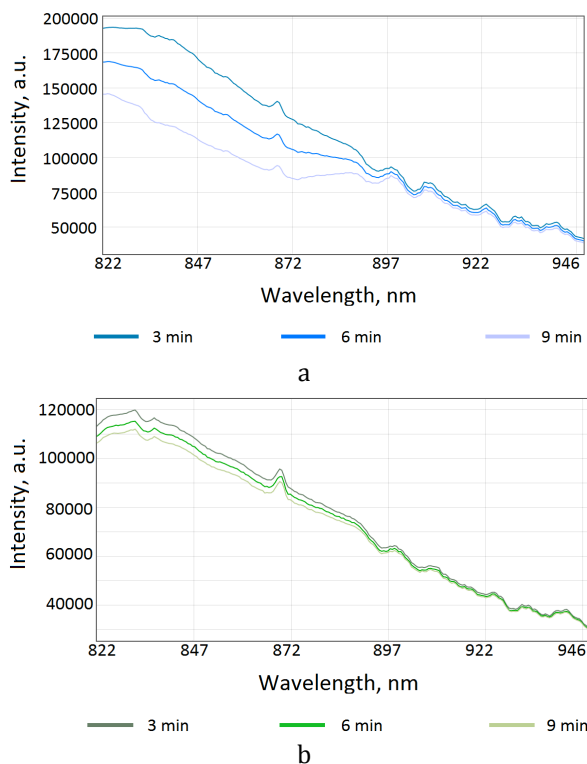


Fig. 5 Urine samples spectra: a) stomach cancer, b) lung cancer. All spectra were obtained as a result of three consequent measurements.

Fig. 5 demonstrates changes in AF intensity and changes in photobleaching process for patients with different diagnoses. The photobleaching mechanism for various porphyrins in urine is quite complicated, since photosensitizing porphyrins may interact with various photo-oxidizing molecules in biological fluids [30]. A standardized spectra recording was performed to correctly estimate AF; the sample irradiation time being 3 minutes. Approximation curves of urine AF for test samples are shown in Fig. 6. Features of various urine samples AF are caused by porphyrin metabolism changes and interaction of porphyrins with various organic molecules [29, 30].

On the basis of AF, approximating curves set the regression model was built. For the model obtained the

a posteriori probability of lung cancer determination from other tumors was 83.3% for obtained model. Thus, it is necessary to study the Raman spectra of urine samples from cancer patients towards improving informativity of the analysis of urine spectral characteristics for detecting lung cancer. Raman spectra of urine samples are presented in Fig. 7.

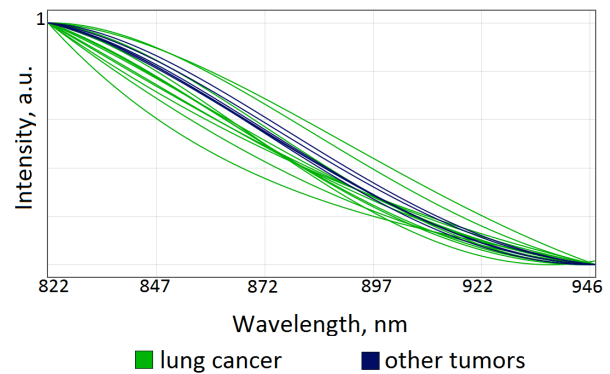


Fig. 6 Polynomial approximation of urine samples AF.

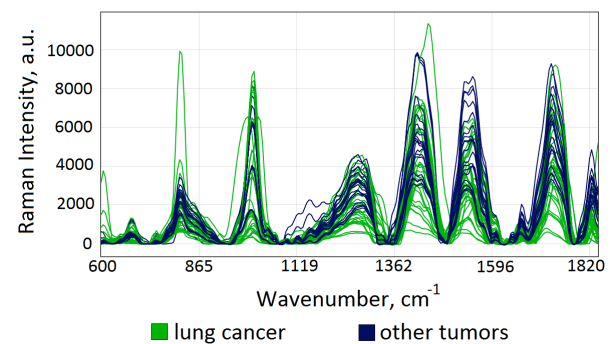


Fig. 7 Raman spectra of tested urine samples.

A multivariate analysis based on the obtained urine Raman spectra was carried out, and a regression model was built. VIP-scores of urine Raman spectra samples for the constructed regression model of lung cancer detection are shown in Fig. 8.

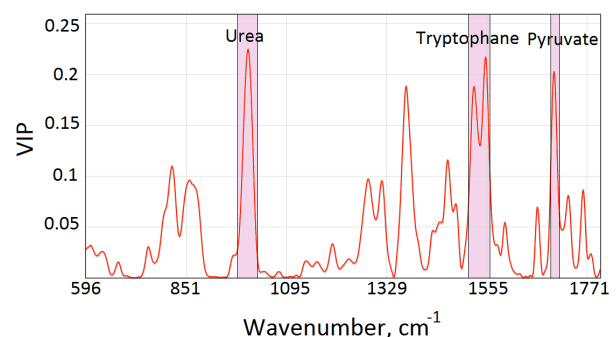


Fig. 8 VIP-scores of urine samples Raman spectra matrix.

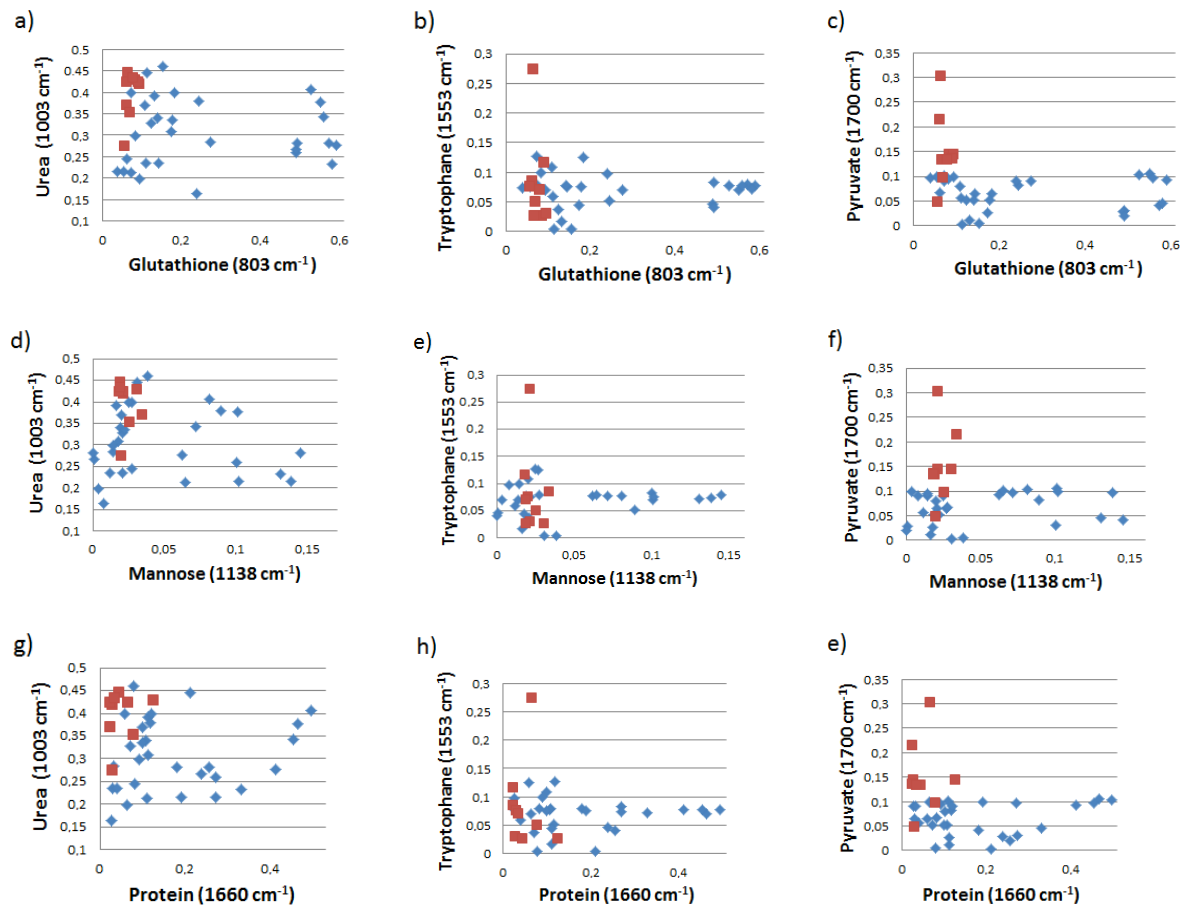


Fig. 9 Phase plane discriminant analysis of lung cancer patients ($\diamond$) and patients with other tumors ($\square$): a – urea (1003 cm^{-1}) in urine and glutathione (803 cm^{-1}) in blood; b – tryptophan (1553 cm^{-1}) in urine and glutathione (803 cm^{-1}) in blood; c – pyruvate (1700 cm^{-1}) in urine and glutathione (803 cm^{-1}) in blood; d – urea (1003 cm^{-1}) in urine and mannose (1138 cm^{-1}) in blood; e – tryptophan (1553 cm^{-1}) in urine and mannose (1138 cm^{-1}) in blood; f – pyruvate (1700 cm^{-1}) in urine and mannose (1138 cm^{-1}) in blood; g – urea (1003 cm^{-1}) in urine and protein (1660 cm^{-1}) in blood; h – tryptophan (1553 cm^{-1}) in urine and protein (1660 cm^{-1}) in blood; i – pyruvate (1700 cm^{-1}) in urine and protein (1660 cm^{-1}) in blood.

Fig. 8 demonstrates the most informative spectral bands in the regression model constructed for discriminating the lung cancer from other tumors in the analysis of the urine Raman spectra. These bands are $1000\text{--}1015\text{ cm}^{-1}$ (urea) and $1525\text{--}1560\text{ cm}^{-1}$ (tryptophan, proteins) [31, 32]. Progress of oncopathology growth is followed by an increased proteolysis, which corresponds to the changes in $1525\text{--}1560\text{ cm}^{-1}$ band intensity. Synthesis of ammonia during proteolysis in the body leads to further ammonia fermentation in the liver with the formation of urea. Urea is the nitrogen metabolism end product in the proteins metabolism and it may be a criterion for protein metabolism evaluation in the body cells [33].

Tumor cells are characterized by a high glucose intake. In this case, there is an anaerobic glycolysis. The marker of increased glycolysis is lactic dehydrogenase (LDH) [34]. LDH affects the pyruvic acid concentration corresponding to the spectral band $1690\text{--}1705\text{ cm}^{-1}$ (pyruvate) [32]. The constructed regression model allows for discriminating the lung cancer from other

tumors during the urine spectral characteristics analysis with the *a posteriori* probability of 93.9%.

3.2 Combined analysis of urine and blood spectral data

Improving the proposed approach accuracy for lung cancer determination is possible by combining spectral analysis data of blood and urine. A two-dimensional distribution of intensities proportional to the previously described changes of Raman spectra components in blood and urine is shown in Fig. 9 (a-i).

The *a posteriori* probability of lung cancer determination for the selected Raman bands of urine and blood spectra shown in Fig. 9 laid down between 76.2% and 94.9%, wherein proteins (1660 cm^{-1}) in blood and pyruvate (1700 cm^{-1}) in urine are the most informative combination of blood and urine components, which is indicative of lung cancer.

We estimated the correlation between the main informative Raman bands of urine and blood. Urine is a

Table 1 Pearson coefficients matrix for pair correlations between the most informative criteria for lung cancer separation from other tumors.

	$I_{bl.803}$	$I_{bl.955}$	$I_{bl.1138}$	$I_{bl.1471}$	$I_{bl.1556}$	$I_{bl.1660}$	$I_{ur.1003}$	$I_{ur.1375}$	$I_{ur.1525}$	$I_{ur.1553}$	$I_{ur.1700}$
$I_{bl.803}$	1	0.426	0.468	0.009	0.434	0.786	-0.172	-0.102	-0.289	-0.067	-0.282
$I_{bl.955}$	0.426	1	0.113	0.351	0.322	0.330	0.077	0.235	0.054	-0.095	-0.061
$I_{bl.1138}$	0.468	0.113	1	-0.122	0.283	0.602	-0.201	-0.120	-0.316	0.017	-0.47
$I_{bl.1471}$	0.09	0.351	-0.122	1	-0.188	-0.031	-0.017	-0.101	0.153	-0.126	-0.284
$I_{bl.1556}$	0.434	0.322	0.283	-0.188	1	0.361	-0.257	0.120	-0.312	-0.179	-0.336
$I_{bl.1660}$	0.786	0.33	0.602	-0.031	0.361	1	-0.064	0.114	-0.089	-0.075	0.161
$I_{ur.1003}$	-0.172	0.077	-0.201	-0.017	-0.257	-0.064	1	0.187	0.204	-0.046	0.257
$I_{ur.1375}$	-0.102	0.235	-0.120	-0.101	0.120	0.114	0.187	1	0.552	-0.213	0.028
$I_{ur.1525}$	-0.289	0.054	-0.316	0.153	-0.312	-0.089	0.204	0.552	1	-0.401	0.003
$I_{ur.1553}$	-0.067	0.095	0.017	-0.126	-0.179	-0.075	-0.046	-0.213	-0.401	1	0.627
$I_{ur.1700}$	-0.282	-0.61	-0.047	-0.284	-0.336	-0.161	0.257	0.028	0.03	0.627	1

product of blood filtration through the kidneys. Consequently, increasing blood components concentration to a certain reabsorption threshold can lead to a change in concentration of the corresponding components in urine. Therefore, the urine component concentration can correlate with the blood component concentration. As it follows from the VIP distribution, the most informative Raman bands for lung cancer determination are: $I_{bl.803}$ (glutathione), $I_{bl.955}$ (proteins), $I_{bl.1138}$ (mannose), $I_{bl.1471}$ (proteins, lipids), $I_{bl.1556}$ (tryptophan), $I_{bl.1660}$ (proteins, phospholipids) in blood analysis; and $I_{ur.1003}$ (urea), $I_{ur.1375}$ (arabinose), $I_{ur.1525}$ (proteins, tryptophane), $I_{ur.1553}$ (tryptophane), $I_{ur.1700}$ (pyruvate) in urine analysis. Here $I_{bl(ur),i}$ is the Raman intensity on the i -th band of blood (bl) or urine (ur) spectra. Table 1 shows pair correlations between the most informative criteria for discriminating the lung cancer from other tumors of both test body fluids are presented in. Significant correlations (p -value < 0.01) are in bold type.

It follows from Table 1 that there is no correlation between the most informative criteria of lung cancer detection for both test body fluids. Consequently, simultaneous analysis of the several body fluids spectral characteristics may improve the accuracy of the proposed lung cancer detection method. Significant correlations between $I_{bl.803}$ – $I_{bl.955}$, $I_{bl.803}$ – $I_{bl.1138}$, $I_{bl.803}$ – $I_{bl.1660}$ criteria can be explained by the presence of glutathione. Glutathione spectrum has strong Raman peaks at 953 cm^{-1} , 1143 cm^{-1} , 1660 cm^{-1} wavenumbers [32], and therefore, glutathione contributes to the corresponding blood spectra bands. Likewise, a significant correlation between $I_{bl.1556}$ – $I_{bl.803}$ is probably related to the fact that the tryptophan Raman spectrum has peaks at 803 cm^{-1} and 1556 cm^{-1} wavenumber

region and tryptophan may contribute to the corresponding spectral bands [32].

4 Discussion and Conclusions

On the basis of blood sample experimental data multivariate analysis the Raman bands intensity changes are proportional to the concentration changes of glutathione, mannose and proteins, and these bands may be an informative criteria for discriminating the lung cancer from other tumors. The fluorescence intensity changes associated with porphyrins and the Raman intensity changes corresponding to urea, tryptophan and pyruvate are the most informative criteria for lung cancer and other tumors classification by urine analysis. The *a posteriori* probabilities of lung cancer separation from other tumors based on the proposed methods of blood and urine analysis are presented in Table 2.

In current study, the highest *a posteriori* probability of lung cancer detection is 94.9%. It was achieved by a simultaneous analysis of urine and blood by the RS method. Decoupled RS analysis of urine and blood allows for achieving 84.9% and 93.9% *a posteriori* probability of lung cancer detection respectively. AF urine analysis made it possible to separate lung cancer from other tumors with 83.3% *a posteriori* probability. However, the blood spectra analysis was performed for a larger number of samples, while a lower *a posteriori* probability in blood RS analysis in comparison with urine RS analysis may be associated with this fact. Therefore, additional studies with a large number of body fluid samples are necessary in order to determine the precise capabilities of the proposed method. On the other hand, there is no significant correlation between the most informative criteria of lung cancer detection

for both body fluids. This fact has shown that simultaneous study of the blood and urine samples can increase the analysis informativeness and improve the probability of lung cancer separation from other tumors by using a combination of RS and AF.

Comparison of the obtained results with those of other studies shows that the proposed optical method may become the basis for cancer screening and may be used in combination with other methods for enhancing research informativeness. For example, a non-invasive and cost-effective cancer screening method (breast cancer, cervical cancer, colon cancer, leukemia, esophageal cancer, liver cancer, bladder cancer) by fluorescence analysis was demonstrated by V. Masilamani et al. [35]. This study showed 86.7% *a posteriori* probability of various cancers detection by changes of flavoproteins and porphyrins excreted with urine.

Table 2 *A posteriori* probability of lung cancer separation from other tumors.

Body fluid (Method of analysis)	Posterior probability, %
Blood (Raman)	84.9
Urine (Autofluorescence analysis)	83.3
Urine (Raman)	93.9
Urine (Raman) Pyruvate + Blood (Raman) Protein	94.9

Adding the data of urine and blood Raman analysis allows to increase the accuracy of lung cancer detection by including the information about urea, tryptophan and pyruvate content to the analysis. G. Del Mistro et al [14] demonstrated 95% *a posteriori* probability of prostate cancer detection by urine RS analysis. The greatest spectral changes for the urine samples of the prostate cancer group are associated with changes in the 6-oxypurine content. The diagnostic study of the body fluids spectral characteristics by using RS for the oral cancer detection was demonstrated by S. Jaychandran et al [31]. Analysis of 158 urine samples, 158 blood samples and 158 saliva samples made it possible to define the differences between the healthy, precancerous and cancer groups with 90.5%, 78%, and 93.1% *a posteriori* probability respectively for each body fluid. For the studied groups, the main spectral

differences of blood samples are associated with changes in phenylalanine, lipids, collagen, purine and amide I; the spectral differences of urine samples were associated with changes in creatinine, tryptophan, indoxyl sulfate. Thus, the informativeness increase for the above mentioned studies is possible due to adding the glutathione, porphyrins and pyruvate content data to the analysis.

Besides the combined analysis of the several body fluids, the increasing of the cancer detection accuracy using body fluids spectral characteristics analysis is possible by preallotment of certain markers from tested samples as demonstrated by Shangyuan Feng et al [36]. The proposed method uses modified nucleotides separation from the urine samples by affinity chromatography with the following nucleotides RS analysis. The PLS-DA analysis of spectroscopic data allows for achieving 95% *a posteriori* probability of nasopharyngeal cancer, esophageal cancer, and a healthy group separation by urine RS analysis. This method demonstrated high accuracy; however, such analysis is complicated, since the specific substance separation from a body fluid sample requires utilizing certain ligand.

The discussed approaches to various cancer detection demonstrate that the proposed method may prove alternative to the available cancer detection techniques. The increase in the study informativeness of blood and urine may be achieved by AF and RS combined study and joint analysis of registered spectroscopic data. However, a comprehensive understanding of cancer detection possibility with the proposed method requires that the number of patients enrolled should be increased. Also it is advisable that method sensitivity and specificity be checked for detecting cancer among the patients with non-oncological diseases and healthy people. In order to do this, numerous studies with body fluid samples from people without oncological pathologies should be performed. In addition to studying the spectral characteristics of urine and blood, it is also possible that other body fluids be utilized [37, 38] as research objects for increasing the cancer detection accuracy.

Acknowledgments

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Analysis of albumin Raman scattering registration efficiency from different volume and shape cuvette

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Abstract. The cuvette shape influence on the Raman registration efficiency at small volumes of albumin (0.15-1 ml) using the PLS model was analysed. This paper examines the smallest measurement error RMSE of 3-6 g/l with a correlation coefficient $R^2 = 0.95$ was achieved for pure Raman spectra extraction by baseline correction with asymmetric least squares smoothing method for aluminum spherical type cuvette-reflectors. Its curvature center was matched with the focal spot of the exciting laser. The error of albumin concentration determination equaled about 5% of albumin normal concentration in a blood plasma using a spherical cuvette with a volume of 0.55 ml. A further decrease of the cuvette volume led to a sharp loss of the method prediction accuracy. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: albumin; raman spectroscopy; PLS.

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1 Introduction

Blood plasma comprises more than a hundred different proteins. Albumin is the most common component of blood plasma (up to 60%), its normal concentration being 35-50 g/l. It acts as a transport protein for some hormones, free fatty acids, bilirubin, supports a colloid-osmotic constancy of blood [1]. The albumin concentration changes indicate the presence of body inflammation or organ pathology.

Physicochemical methods with various reagents are usually used in clinic-based studies to determine the protein fraction concentrations in blood plasma [2]. Protein electrophoresis is one of the most common methods [3], which measures specific proteins in blood. The test separates proteins in blood on the basis of their electrical charges. After the proteins separating, the plate is placed in a solution to stain the protein bands. The staining intensity is related to the protein concentration. Blood samples with a volume of up to 5 ml are required for the protein electrophoresis. The analysis time is 1.5 to 2 hours.

In modern studies, spectral methods are utilised to reduce the protein analysis time. The methods are based on measuring optical properties of substances without additional chemical reagents. Raman spectroscopy (RS) is one of the most sensitive methods [4-6]. The main limitation of RS methods is low scattering cross section, which leads to low Raman scattering intensity. Resonance RS methods, confocal microscopic systems [4-7], surface-enhanced Raman spectroscopy (SERS) using metallic nanoparticles [8, 9] are used to increase the signal-to-noise ratio. However, these technologies are expensive and time-consuming, which impedes their use for routine analysis in medical laboratories.

Usually, the signal-to-noise ratio increase for non-resonant Raman systems [7, 10] is achieved by the signal accumulation (normal law of noise distribution). However, this effect has limitations associated with the signal saturation, for example, in [7] the registration time was 5 minutes. We propose that, the efficiency of laser radiation pumping by means of multireflection and multiple scattering in the focus area of the Raman probe be increased by using cuvette-reflectors with a certain shape. For these purposes, a quantitative analysis of albumin Raman scattering registration efficiency was performed by using cuvette-reflectors with different shape and volume (0.15-1 ml). Since most proteins, including albumin, are natural fluorophores, they have intrinsic fluorescence signal. The albumin limiting concentration also depends on the autofluorescence background removal method to obtain the pure Raman spectrum. We compared the influence of polynomial approximation and baseline correction with asymmetric

least squares smoothing on the prediction accuracy of albumin concentrations.

2 Materials and Methods

2.1 Experimental setup

The optical scheme of the experimental setup for Raman scattering registration is presented in Fig. 1. The InPhotonics RPB785 Raman probe was used to deliver probing laser radiation and collect scattered radiation. It includes elements for scattered radiation filtering. The probing laser radiation (785 nm) was delivered through fiber and a band-pass filter (BPF), which cut off the fluorescent contribution of the optical fiber, and radiation was focused by the L2 lens in the cuvette center (focal length 7.5 mm). The radiation power at the output of the L2 lens was 200 mW. The scattered radiation was collected by the same lens and directed by a dichroic (DM) and deviating (M) mirror to the detection channel through a long-pass filter (LPF) to eliminate Rayleigh component (785 nm) of the scattered radiation. The radiation (fluorescence and Raman scattering) was recorded by QE65Pro Ocean Optics spectrometer with Hamamatsu S7031-1006 detector with a resolution of 3 cm⁻¹. The registered signal was transmitted to the computer for further processing of the Raman spectra.

2.2 Study samples

The medical drug – 10% solution of human albumin (anatomical therapeutic chemical classification system B05AA01) was experimentally tested allowing for simulating a medium close to blood plasma with guaranteed reproducible optical properties. Various concentrations of the model media (5-100 g/l with 5 g/l step) were achieved by mixing albumin and distilled water using microdispenser (accuracy 20 ± 0.2 µl).

2.3 Cuvette

In the study, we used specially designed aluminum cuvette with different volume and three types of shapes: cylindrical with a flat bottom, trapezoidal bottom, and spherical bottom. The material was selected due to the high reflectivity and the absence of an intrinsic Raman spectrum. Fig. 2 shows cuvette with volumes of 0.15 ml, 0.35 ml, 0.55 ml, 0.9 ml and 1 ml.

The depth of the cylindrical cuvettes with a flat bottom was 45 mm and significantly exceeded the diameter (Ø 2 mm, volume 0.35 ml – Fig. 2a and Ø 5 mm, volume 0.9 ml – Fig. 2b) to increase the "light" volume due to the laser radiation reflection from the

side wall surface. The depth of trapezoidal bottom cuvette used as an inverse reflector was selected to commensurate with its “effective” diameter (volume of 1 ml – Fig. 2c). In the spherical type cuvette with volumes of 0.15 ml (diameter of the hole $\varnothing$ 4 mm – Fig. 2d) and 0.55 ml (diameter of the hole $\varnothing$ 8 mm – Fig. 2e) the center of bottom curvature was matched with the focal spot by the internal cavity to fix face plane position of the RS probe. It should be noted that the focal spot is displaced due to multiple scattering in the medium and its size more than 1 mm with an inhomogeneous energy distribution. The spot center is displaced closer to the source due to absorption. This geometry increases the light field energy concentration in the focal area by reflecting from the cuvette bottom and collects scattered forward radiation.

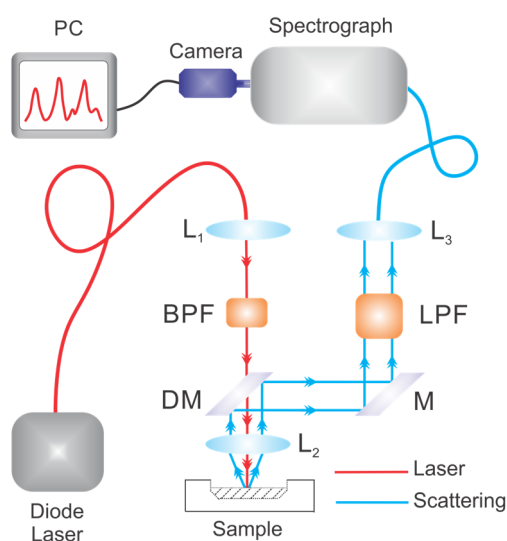


Fig.1 Optical scheme of the experimental setup.

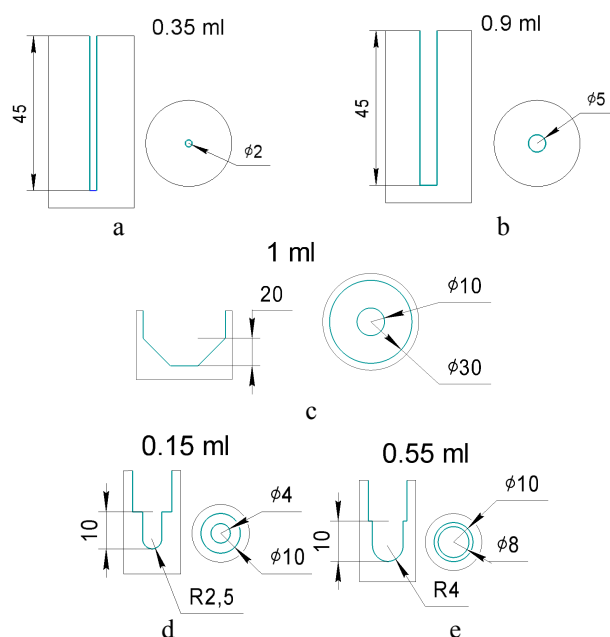


Fig. 2 Cuvettes with volumes of 0.35 ml (a), 0.9 ml (b), 1 ml (c), 0.15 ml (d), 0.55 ml (e).

2.4 Data processing methods

Projection on latent structures (PLS) method was used for the experimental data analysis [11, 12], which interprets the results based on a smaller number of bilinear components. Its goal is to predict a set of dependent variables (vector Y – albumin concentrations) from a set of independent variables or predictors (matrix X – albumin Raman spectra intensities for different wavelengths). This prediction is achieved by extracting from predictors a set of orthogonal factors called latent variables (LV) which have best predictive power.

Model performances were characterised by the root mean square error (RMSE) of calibration, prediction or cross-validation, as well as the coefficient of determination R^2 .

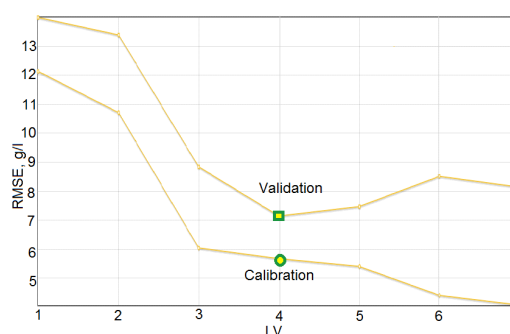


Fig. 3 An example plot of the root mean square error of calibration ($RMSE_C$) versus root mean square error of cross validation ($RMSE_{CV}$) as a function of the number of latent variables. The optimum number of latent variables corresponds to the minimum of $RMSE_{CV}$.

Fig. 3 illustrates typical RMSE dependence with latent variables increase in the analytical model. It is observed that a certain number of LV (in this example $LV = 4$) corresponds to the minimum value of RMSE of cross validation ($RMSE_{CV}$). The number of LV was determined and used to evaluate the model for each cuvette.

The registered signal includes the autofluorescence and Raman spectra, so a raw signal preprocessing was performed to background (autofluorescence) removal. Two methods of the Raman spectrum extraction were compared: polynomial approximation method and baseline correction with asymmetric least squares based on procedures implemented in the TPTcloud cloud service (<https://tptcloud.com/>). The baseline correction approach includes mean centering and data normalization using standard normal variate (snv) method [12, 13]. Savitzky-Golay (sav gol) filter was used to noise reduction [14], and background elimination was realised by baseline correction with asymmetric least squares (baseline als) [15]. Typical results of preprocessing methods application are shown in Fig. 4.

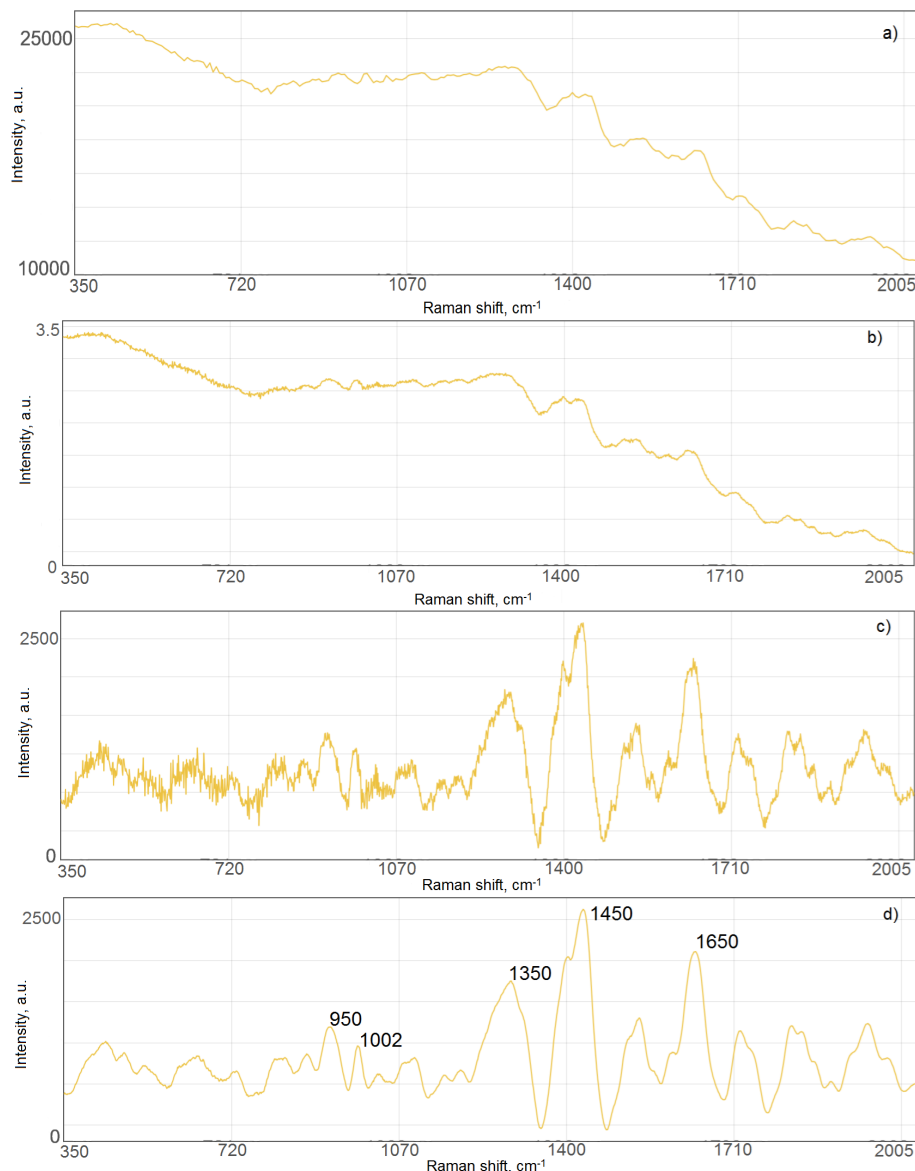


Fig. 4 Preprocessing methods results for albumin RS signals: a) raw signal, b) standard normal variate (snv), c) baseline correction with asymmetric least squares (baseline als), d) Savitzky-Golay filter (sav gol).

The polynomial method utilisation for the Raman spectra fitting is shown in Fig. 5 which includes averaging and smoothing (Fig. 5b), approximation of the averaged signal by a polynomial of the 10th degree (Fig. 5c) to obtain pure Raman spectrum (Fig. 5d). Then, the pure Raman spectra were also normalised (snv) and centered (mean center).

Validation method was applied to estimate the model performance on “independent” data. KFold cross-validation method was used [11]. The KFold splits the data onto K blocks (10 in our model). Further on, the blocks were excluded (one at a time) from the training data and used for validation of the model built on the remaining data. Validation statistics for all individual segments were then averaged.

3 Results and Discussion

For each cuvette scattering spectra were five times registered with an albumin concentration ranging from 5 to 100 g/l (step of 5 g/l). Each raw spectrum was preprocessed by two methods (polynomial and baseline als), afterwards PLS model was built for spectral data of cuvette. The main peak positions of albumin Raman spectra (Figs 4, 5) are clearly observed at wavelengths: 950 cm^{-1} (stretching mode of CC bond), 1002 cm^{-1} (phenylalanine), 1350 cm^{-1} (deformation mode of CH bond), 1450 cm^{-1} (deformation mode of CH_2 bond) and 1650 cm^{-1} (Amide I) [5].

Predictive performances of the PLS models show a strong correlation between the concentrations predicted by Raman spectroscopy and target albumin concentrations. The PLS model performances of 0.9 ml cylindrical cuvette were compared for the baseline als

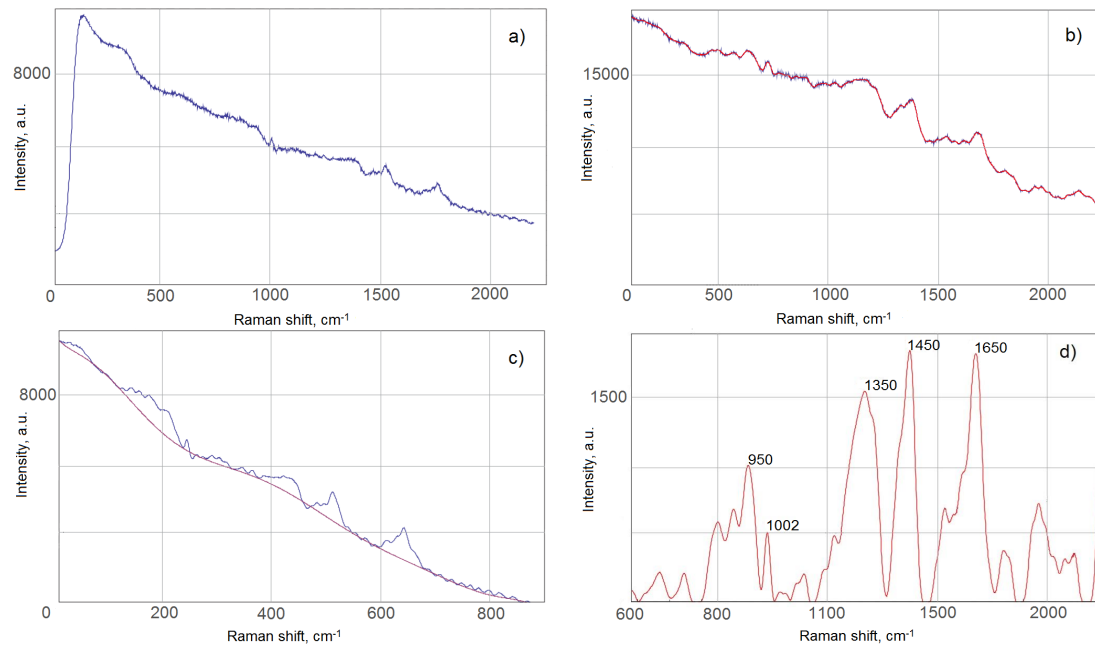


Fig. 5 Raman spectra fitting by polynomial approximation method: a) raw data, b) averaging and smoothing, c) polynomial approximation, d) pure Raman spectrum.

technique (Fig. 6) and for the polynomial technique (Fig. 7). The PLS calibration models show the minimum RMSE with $LV = 4$ for the baseline als method and with $LV = 5$ for polynomial approximation method.

The correlation coefficient (R^2) values above 0.9 were obtained for both processed methods, which indicate the results consistency. However, the baseline als method produces a slightly smaller data spread $\{LV = 4, RMSE = 5.7, R^2 = 0.96, RMSE_{cv} = 7, R^2_{cv} = 0.94\}$ compared to the polynomial method $\{LV = 5, RMSE = 6.5, R^2 = 0.95, RMSE_{cv} = 8.5, R^2_{cv} = 0.92\}$. Here the index “CV” indicates the data obtained by cross-validation. The results of the PLS albumin concentration prediction for all cuvette types are summarised in Table 1.

Comparison of the results shows that the baseline als method for pure Raman spectra extraction has an undoubted advantage over all types of cuvettes, providing smaller values of RMSE.

It is clearly seen that the cylindrical cuvette with extra depth ($\varnothing 5$ mm, volume of 0.9 ml) has similar accuracy characteristics as a shallow wide cuvette with a trapezoidal bottom of the same volume (1 ml). This fact indicates that reflection contribution of forward scattered radiation from the bottom and illumination increase due to reflection from the side surface has the same magnitude order of the measurement error reduction. This effect is particularly significant to the cuvette with the spherical bottom with respect to the curvature center matching with focal spot position of the Raman probe. In this case, it is possible to obtain the same measuring errors of albumin concentration for cuvettes with half volume (0.55 ml): $\{RMSE_{cv} = 6.3, R^2_{cv} = 0.95\}$ compared to cylindrical cuvette with a volume of 0.9 ml $\{RMSE_{cv} = 7, R^2_{cv} = 0.94\}$.

Apparently, volume of 0.5 ml, is critical because a sharp decrease of the correlation coefficient R^2 for cross-validation data is observed for both cylindrical and spherical cuvette as well as for cuvettes with a bottom; R^2 starting from this volume value becomes less than 0.9. Nevertheless, it can be noted that these trends are of different character. For example, the volume increase of the cylindrical cuvette by a factor of 2.6 leads to accuracy increase in albumin concentration determination by 58%, and for spherical type cuvettes by 102% with a volume increase by 3.7 times.

It should be noted that for the spherical type cuvette a small displacement (1 mm) of the bottom curvature center position to the source relative to focal spot center of the Raman probe decreases the determination concentration error.

It could be concerned with the biofluid refraction and the irregular distribution of a radiation energy, associated with absorption of the laser radiation.

It can be argued that the use of the spherical shape cuvette with a volume of ~ 0.5 ml makes it possible to increase the determination accuracy of albumin concentration to 3-6 g/l corresponding to 5-10% of a normal albumin concentration in a blood plasma. This value is comparable to the error of more complicated methods of Raman microscopy and equals about 2 g/l, but this technique requires the use of specialised optics [5].

4 Conclusion

The study shows that for small volume cuvette-reflectors their shape could significantly influence registration efficiency of the albumin Raman signals. In particular, the implementation of the cuvette with a spherical bottom leads to an almost twofold accuracy

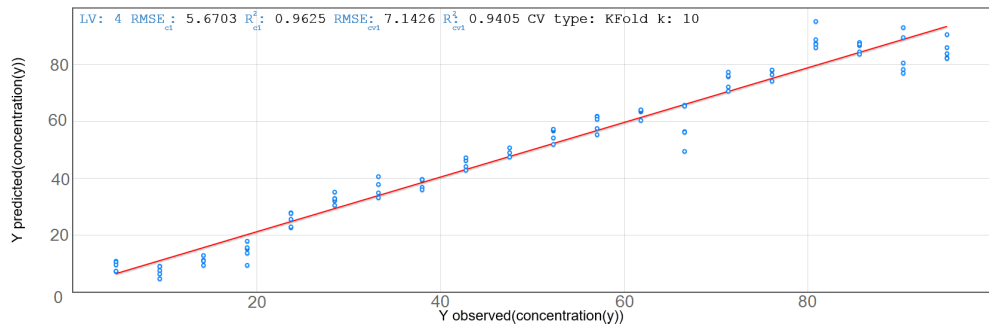


Fig. 6 Prediction results obtained using PLS regression on albumin spectra processed by baseline als, sav gol, snv and mean center for the 0.9 ml cuvette. (LV = 4, RMSE = 5.7, R² = 0.96, RMSE_{cv} = 7 and R²_{cv} = 0.94).

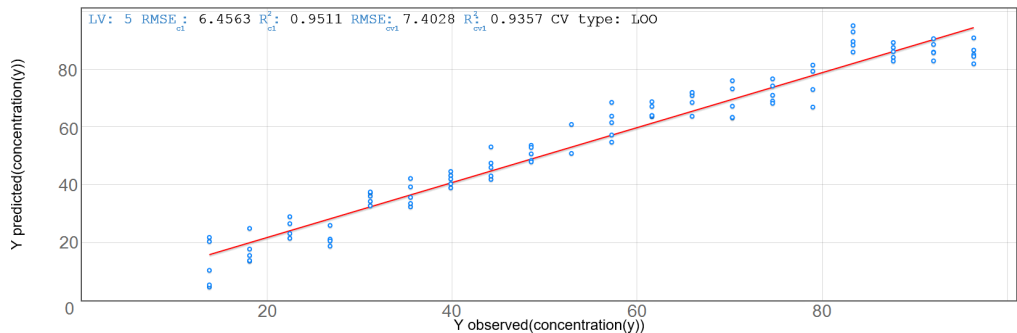


Fig. 7 Prediction results obtained using PLS regression on albumin spectra processed by polynomial approximation, snv and mean center for the 0.9 ml cuvette. (LV = 5, RMSE = 6.5, R² = 0.95, RMSE_{cv} = 8.5 and R²_{cv} = 0.92).

Table 1 The determination accuracy of albumin concentration by the PLS regression methods for cuvette of different shapes and volumes (the highest determination accuracy for the cross-validation data set is in bold type).

Cuvette type	Cylindrical		Trapezoidal		Spherical	
Cuvette volume	0.35 ml	0.9 ml	1 ml	0.15 ml	0.55 ml	
Baseline als method						
Calibration data	RMSE = 6.8 R ² = 0.94 (LV = 5)	RMSE = 5.7 R ² = 0.96 (LV = 4)	RMSE = 4.7 R ² = 0.97 (LV = 3)	RMSE = 5.9 R ² = 0.96 (LV = 5)	RMSE = 2.6 R ² = 0.99 (LV = 5)	
Cross-validation data	RMSE _{cv} = 10.6 R ² _{cv} = 0.87	RMSE_{cv} = 7 R²_{cv} = 0.94	RMSE _{cv} = 6.1 R ² _{cv} = 0.95	RMSE _{cv} = 14 R ² _{cv} = 0.75	RMSE_{cv} = 6.3 R²_{cv} = 0.95	
Polynomial method						
Calibration data	RMSE = 9.5, R ² = 0.89 (LV = 5)	RMSE = 6.5 R ² = 0.95 (LV = 5)	RMSE = 5.2 R ² = 0.96 (LV = 3)	RMSE = 8 R ² = 0.92 (LV = 4)	RMSE = 7.5 R ² = 0.94 (LV = 4)	
Cross-validation data	RMSE _{cv} = 11.6 R ² _{cv} = 0.84	RMSE _{cv} = 8.5 R ² _c = 0.92	RMSE _{cv} = 6.7 R ² _{cv} = 0.95	RMSE _{cv} = 20 R ² _c = 0.5	RMSE _{cv} = 11 R ² _{cv} = 0.86	

increase due to collection of the scattered forward Raman radiation. It was stated that RMSE of the PLS model is less than 1-5 g/l for all types of cuvettes using baseline correction with asymmetric least squares method to obtain the pure Raman spectra in comparison with the polynomial method. The smallest error (3-6 g/l) was achieved for the spherical type cuvette with a volume of 0.55 ml by matching the center of curvature

with the Raman probe focus. This accuracy allows for reliable determination of abnormal albumin concentrations in blood plasma. The volume decrease less than 0.5 ml (0.35 ml and 0.15 ml) for any shape cuvettes leads to a sharp drop of correlation coefficient (R²<<0.9), which indicates failure to obtain satisfactory results without utilization of SERS-substrates, resonant or confocal microscopic systems.

5 Conclusion

The study shows that for small volume cuvette-reflectors their shape could significantly influence registration efficiency of the albumin Raman signals. In particular, the implementation of the cuvette with a spherical bottom leads to an almost twofold accuracy increase due to collection of the scattered forward Raman radiation. It was stated that RMSE of the PLS model is less than 1-5 g/l for all types of cuvettes using baseline correction with asymmetric least squares method to obtain the pure Raman spectra in comparison with the polynomial method. The smallest error (3-6 g/l) was achieved for the spherical type cuvette with a

volume of 0.55 ml by matching the center of curvature with the Raman probe focus. This accuracy allows for reliable determination of abnormal albumin concentrations in blood plasma. The volume decrease less than 0.5 ml (0.35 ml and 0.15 ml) for any shape cuvettes leads to a sharp drop of correlation coefficient ($R^2 \ll 0.9$), which indicates failure to obtain satisfactory results without utilization of SERS-substrates, resonant or confocal microscopic systems.

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Using Fourier spectroscopy for the assessment of oxidative degradation of polypropylene medical meshes

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Abstract. We considered the effect of oxidative degradation of polypropylene (PP) on its spectral properties. For this aim, the model experiments have been performed on the oxidation of PP mesh by the exposure to UV radiation and the technique of quantitative assessment of the appeared oxygen-containing groups has been elaborated. As a result, the concentrations of carbonyl and hydroxyl groups were found and the detection ability of the technique was estimated. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: IR Fourier spectroscopy; polypropylene mesh; oxidation of polypropylene.

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1 Introduction

Due to high chemical inertness and good biocompatibility, PP is extensively used in many fields of vital activity, including medicine. However, in medical practice it sometimes happens so that the organism rejects the mesh, which leads to unwanted sequela. A hypothesis exists that this is due to PP degradation in the process of its oxidation under the effect of the living organism environment [1].

A specific feature of the study is that the object of study is a mesh used in medical practice rather than a film, as in Ref. [2]. The samples extracted from the organism and accordingly the control samples cannot be

converted into powders or films in order to reveal the structural changes, caused by the effect of living organism, without introducing possible changes related to the additional mechanical impact. In contrast to films, meshes have holes, so that only a fraction of the IR radiation incident on the sample passes through PP. This feature requires special technique to process the spectra of such meshes.

The aim of the present paper is to analyse the oxidative degradation of PP using IR spectroscopy.

2 Theoretical background and experimental technique

PP is a high-molecular polymer synthesised by stereospecific polymerisation of propylene under rather low pressure using the Natta-Ziegler catalysts. Different conditions of the polymerisation process yield the production of polymers with different molecular structure that determines their physical and spectral properties. Depending on these conditions, a few types of PP structures can be obtained that differ in the spatial localisation of methyl groups with respect to the main polymer chain: the isotactic, syndiotactic, and atactic structures [3]. For mesh production, the isotactic structure is used, since it possesses better mechanical and chemical properties.

The main instrument for experimental studies is the Fourier spectrometer FSM 2201. In the experiment, we used the PP mesh with the fibre thickness 0.1 mm. During the spectrum recording, the samples were fixed with holders placed in the cuvette section of the spectrometer. The spectra were measured in the transmission mode with the resolution 1 cm^{-1} . The studied spectral region was from 400 to 4000 cm^{-1} . Each final spectrum was obtained by averaging over 20 individual scans. Before studying each sample, the reference spectrum was recorded.

To model the oxidative degradation of PP mesh, the sample was exposed to UV radiation. The irradiation of the sample was performed using the arc mercury lamp DRT 230. The light flux power density calculated using the spectral characteristics of the mercury lamp amounted to 0.3 W/cm^2 . The exposure was executed in a few stages, after each stage the transmission spectrum of the sample was recorded. The duration of each stage was one hour. The total exposure time was 10 hours.

3 Experimental results

In the course of experiment, the PP mesh extracted from a human organism was studied. Figure 1 makes it possible to see that in the spectrum of the extracted sample at the wavenumbers 668, 2343 and 2360 cm^{-1} , corresponding to water, the decrease of absorption band intensities was observed. This is due to different absorption capability of the samples' surface. One can also observe the difference in the absorption saturation, which is explained by the normalisation of the spectra to the maximal transmission. Such normalisation is necessary for comparison, since the transmission of samples is essentially different. In the rest of the spectrum no characteristic changes, typical for functionally substituted PP were detected.

Since in the studied samples no results of oxidative degradation were found, it was decided to compare the results with the known results of the PP oxidative degradation under the exposure to UV radiation [4]. In this case, there are the oxidation results in the appearance of hydroxyl and carbonyl groups in the polymer chain.

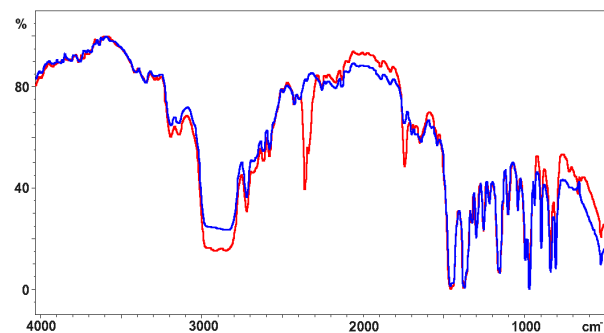


Fig. 1 Spectra of the extracted PP mesh (blue) and the initial PP mesh (red).

The analysis of the spectra has shown that in the spectrum of PP mesh exposed to UV irradiation during 10 hours a number of bands are observed that evidence in favour of the PP degradation process. However, in correspondence with the aim of the present work, we are interested only in those bands, which are related to the oxidative destruction of the polymer. The presence of these bands in the spectrum is caused by the appearance of hydroxyl and carbonyl functional substitution groups.

In the region $3300 - 3500\text{ cm}^{-1}$ of the irradiated PP mesh transmission spectrum (Fig. 2), one can observe a dip, which, according to the published data, is related to the appearance of hydroxyl groups, characteristic for the oxidation process in this polymer. We recorded the spectrum of vaporous ethanol ($\text{C}_2\text{H}_5\text{OH}$), having the hydroxyl group, and compared it with the spectrum of the studied sample. The centres of the broad absorption band (3400 cm^{-1}) in the spectra of the irradiated mesh and ethanol practically coincide.

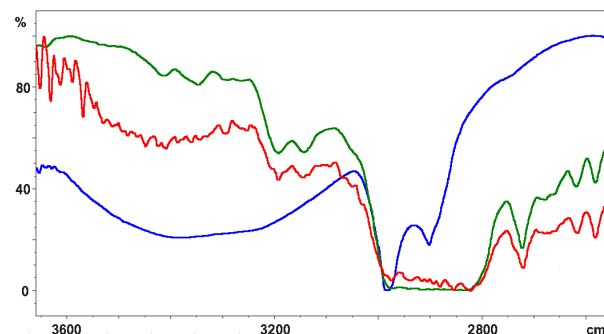


Fig. 2 Spectra of ethanol (blue), original PP mesh (green), and PP mesh exposed to UV radiation during 10 hours (red).

Besides that, in the absorption spectrum the absorption lines corresponding to the wave number 1715 cm^{-1} were found (Fig. 3). These lines were attributed to the vibrations of carbonyl groups. The origin of these lines is also related to the oxidative degradation. As a reference sample the stearin acid ($\text{C}_{17}\text{H}_{35}\text{COOH}$) was chosen, for which the presence of absorption lines in this region is characteristic.

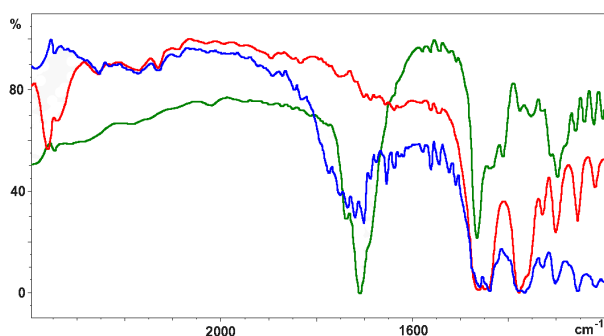


Fig. 3 Spectra of stearin (green), original PP mesh (red), PP mesh after 10 hours of exposure to UV radiation (blue).

4 Quantitative concentration assessment of hydroxyl and carbonyl groups in the pp mesh after UV irradiation

For quantitative assessment of the carbonyl (C=O) and hydroxyl (-OH) groups content in the mesh, exposed to the UV radiation, the data on the spectra of the characteristic substances known from Ref. [5] were used.

The absorption corresponding to certain vibrations can be related to the change in the optical density. The ratio of the change in the optical density of the studied sample ΔD to that of the reference sample ΔD_0 is expressed as

$$\frac{\Delta D}{\Delta D_0} = \frac{gnl}{n_0l_0}, \quad (1)$$

where g is the mesh filling factor.

To determine this factor in the spectrum, we used the region of saturated absorption. In the spectrum, one can see that the value of transmission corresponding to this saturation amounts to 80%. Correspondingly, the filling factor of the mesh equals ~ 0.2 .

Besides that, an independent estimate of g was found using the geometric characteristics of the mesh. We measured the volume of the mesh and its mass, which yielded the density equal to 0.16 g/cm^3 . Since the density of bulk PP is known (0.91 g/cm^3), we get the same filling factor of the mesh $g=0.2$.

Form Eq. (1) the concentration n is expressed as

$$n = \frac{\Delta D n_0 l}{\Delta D_0 g l_0}. \quad (2)$$

As a reference sample for the determination of concentration of carbonyl groups, we prepared a tablet

of KBr containing 10% of stearin, which corresponded to the carbonyl groups concentration $n_0 = 5 \cdot 10^{20} \text{ cm}^{-3}$. Measuring the appropriate optical densities in the reference sample and the studied one in the region of 1715 cm^{-1} , we found $n = 2 \cdot 10^{20} \text{ cm}^{-3}$ in the studied sample.

For the hydroxyl group the saturated ethanol vapours were used as a reference sample, for which the relation between the pressure and the temperature is known [5]. Making use of the equation $n = P/kT$, we get the concentration of hydroxyl groups in the ethanol $n_0 = 1,227 \cdot 10^{18} \text{ cm}^{-3}$. For calculation, the optical density at the 3400 cm^{-1} absorption line was used. The concentration of hydroxyl groups calculated from the appropriate spectra amounted to $n = 2.6 \cdot 10^{18} \text{ cm}^{-3}$ in the studied mesh.

In the obtained spectra, the signal levels in the region corresponding to carbonyl and hydroxyl groups for PP exceeded the noise level by nearly 5 times. This fact allowed the estimation of the minimal level of concentration of the groups that corresponds to the detection threshold. The obtained values are $n_{C=O} = 10^{20} \text{ cm}^{-3}$, $n_{-OH} = 1.5 \cdot 10^{18} \text{ cm}^{-3}$, respectively. In other words, the technique allows the detection of one hydroxyl group per 15000 carbon atoms and one carbonyl group per 300 carbon atoms.

5 Conclusion

As a result of this work we arrived at the following results and conclusions:

- The technique of PP spectra recording was developed and the identification of spectral lines was carried out.
- The destruction of samples modelled by means of UV irradiation has shown that this process indeed is accompanied by oxidative reactions with the attachment of carbonyl and hydroxyl groups to the polymer chain. The concentrations of these groups were quantitatively estimated.
- The detection thresholds of the carbonyl and hydroxyl groups were assessed. It is shown that these values allow reliable detection of the appearance of the oxidation reaction products in PP, e.g., in the process of the polymer UV irradiation.
- For PP meshes degraded due to the action of living organism environment the concentration of products of oxidative reactions appeared to be smaller than the detection threshold, which made it impossible to judge unambiguously on whether the oxidative reactions are the dominant mechanism of PP degradation or not.