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Using gold nanorods labelled with antibodies under the photothermal action of NIR laser radiation on *Staphylococcus aureus*

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Abstract. **The effect of NIR laser radiation (808 nm) and gold nanorods on the cells of two strains of *Staphylococcus aureus*, one of them being methicillin-sensitive and the other being methicillin-resistant, is studied. Nanorods having the dimensions 10 × 44 nm with the absorption maximum in the NIR spectral region, functionalised with human immunoglobulins IgA and IgG, are synthesised. It is shown that the use of nanoparticles in combination with NIR irradiation leads to killing up to 97% of the population of microorganisms.**

Keywords: *NIR lasers, nanoparticles, antibodies, staphylococci.*

1. Introduction

During the recent five years the number of research papers, devoted to studying the biological properties of nanoparticles conjugated with immunoglobulins, has sharply increased [1–4]. These complex compounds are used in diagnostics and therapy of various pathologic conditions [3]. It is expected that the area of their application will be getting still wider.

Thanks to their small dimensions and molecular structure, the nanoparticles of various metals and their oxides possess a number of unique physical and chemical properties that determine their use in biosensors, microscopy, diagnostics of pathologic conditions and infection diseases, laser therapy, etc. Modern technologies allow for the production of nanoparticles of designed size and shape with the characteristics adapted to each particular field of application [4–9].

Antibodies are small (150 kDa) protein molecules, the components of immune system neutralising infection agents (viruses, bacteria) and their toxins and providing the activation of immune cells [10].

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Joining nanoparticles and antibodies in a functional complex allows for a combination of the unique properties of nanoparticles and the selectivity of antibodies for purposeful impact on a biological target and recognising its specific features [2, 5]. One of the most promising ways of using such conjugates is the addressed delivery of medical preparations in combination with the local laser-induced heating of cells and their components. Creating such conditions is possible, when the cells are subject to the synergetic effect of the NIR radiation and gold nanoparticles having a tunable plasmon resonance in the NIR spectral region. Such therapy provides not only the selective destruction of definite cells (addressed impact involving antibodies, chemical and thermal damage involving nanoparticles), but also the regeneration of surrounding tissues as a result of stimulating effect of NIR radiation [11–13]

As a rule, the monoclonal antibodies with the external Fab-fragment (antigen binding fragment), specific for each definite protein entering the composition of the target object, are used to functionalise the nanoparticles [2, 3, 12–21]. However, in the therapy of infection diseases it is rather promising to use inverted molecules of antibodies, where the exterior position is occupied by the constant Fc-fragment [2, 12]. In this case an inverse recognition is possible, i.e., the target 'recognises' the antibody rather than the antibody 'recognises' the target. A specific feature of the selective interaction with Fc-fragments of human immunoglobulins is inherent in a number of microbial surface or secreted proteins (protein A of staphylococcus, protein G of streptococcus), evolutionally formed to prevent the immune system response [22, 23].

The aim of the present study was to investigate the efficiency of selective photothermal action of the NIR laser (808 nm) radiation on the methicillin-sensitive (MS) and methicillin-resistant (MR) strains of *Staphylococcus aureus*, the selectivity of which is provided by using the specific labels in the form of gold nanorods, functionalised with human immunoglobulins A and G with exterior-oriented Fc-fragments.

2. Materials and methods

As model objects we have chosen two strains of the *Staphylococcus aureus* bacteria, MS and MR (L.A. Tarasevich State Research Institute of Medical Biological Preparations Standardisation and Control, Moscow). The staphylococci were cultivated at the temperature 37 °C on the universal dense nutrient medium GRM-agar (Obolensk, Russia).

The diode laser with the radiation maximum at the wavelength $\lambda = 808$ nm and the power density 100 mW cm^{-2} (LAS, St. Petersburg) served as a source of NIR radiation (Fig.1).

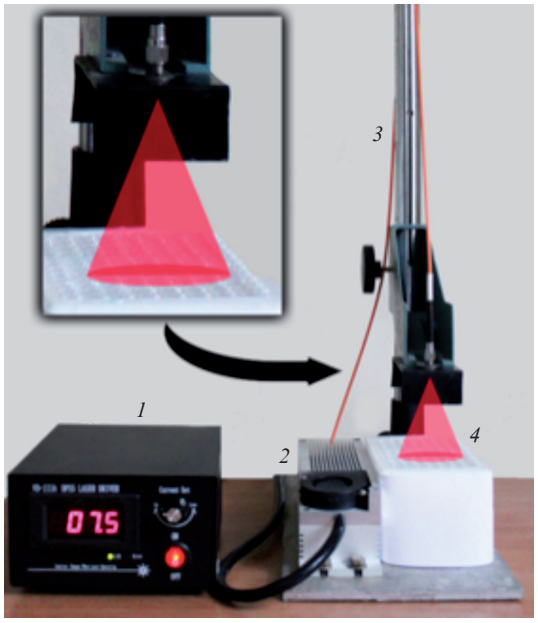

Figure 1. Scheme of the experimental setup on the basis of the diode laser with the wavelength 808 nm:
(*1*) laser power supply unit; (*2*) light source; (*3*) vertical stand with optical fibre; (*4*) culture plate with bacterial suspensions; the inset shows the configuration of irradiation of the culture plate with bacteria in more detail.

All experiments were performed with the cw laser radiation. To measure the solution temperature we used the digital MY62 multimeter (Mastech, China) with the measurement error ±0.5 °C.

The protocol of synthesising gold nanorods comprised two basic parts (Fig. 2). At the first stage the autocatalytic reduction of the tetrachloroauric acid ($HAuCl_4$) with the ascorbic acid in the presence of cetrimonium bromide, silver nitrate and ultra-small gold nanospheres was carried out as described in Refs [24, 25]. The coating of nanoparticles with polyethylene glycol (PEG) was implemented in the acetate buffer with the concentration 100 mM (pH 5.0), containing 50 μM of heterobifunctional PEG fibrils in the form of a mixture of alpha-omega-mercapto-methoxy PEG and alpha-mercapto-omega-carboxy PEG (proportion 9 : 1, molecular mass ~5000 g mol^{-1}). This PEGylation mixture is characterised by colloidal stability, and the resulting coating provides the interaction of the surface of particles with amino groups of the functional molecules [6, 26].

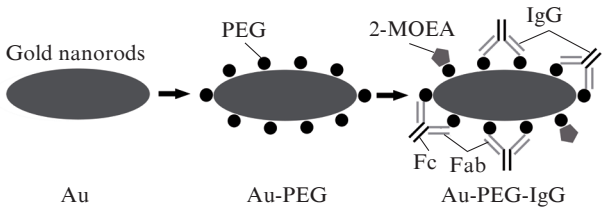

Figure 2. Schematic diagram of synthesising the conjugates of nanorods with antibodies. Gold nanorods (Au) are coated with polyethylene glycol (PEG), and then the antibodies (Ig) are bound to them via the Fab-fragment by amidation. The particles are then stabilised with 2-metoxyelthylamin (2-MOEA).

At the next stage the amidation was carried out by forming the succinimide-carbodiimide bonds in the MES buffer [2-(N-morpholino)ethanesulfonic acid] with the concentration 10 mM (pH 5.5) [27, 28]. The funtionalisation was implemented in the solution of 2-metoxyethylamine under the addition of the human immunoglobulin A (IgA, a ligand with low staphylococcus A protein affinity) or immunoglobulin G (IgG, a ligand with high staphylococcus A protein affinity). The amino groups not bound to Fab-fragments of antibodies were blocked with 2-methoxylamine (Fig. 2). The concentration of the particles amounted to 4 mM.

The study of particle morphology was carried out using the scanning electron microscope [CM12(S) TEM, Philips]. As seen from the electron microphotograph (Fig. 3), the synthesised particles have the mean length 44 ± 4 nm and the diameter 10 ± 3 nm. The optical (extinction) characteristics were measured in the cm^{-1} M^{-1}_{Au} units using the V-560 spectrophotometer (Jasco). The particles appeared to possess an expressed plasmon resonance with the peak near 800 nm (Fig. 4), the shape of the spectra being identical for all types of particles produced.

Figure 3. SEM micrograph (25000×) of the synthesised nanorods.

Thus, to carry out the experiments on photothermal impact of NIR laser radiation (808 nm), three types of gold nanorods were used, namely, the gold nanorods coated with PEG (Au-PEG), the gold nanorods coated with PEG and functionalised with IgA (Au-PEG-IgA), and the gold nanorods coated with PEG and functionalised with IgG (Au-PEG-IgG). The concentration of nanoparticles in the used suspensions was 0.4 mM.

In the experiments the laser with the radiation output through an optical fibre was placed above the wells of the sterile polystyrene culture plate (see Fig. 1). Before the measurements the bacterial culture to be used was grown during 24 hours at the temperature 37 °C on the dense nutrient medium. The bacterial suspension was prepared in the sterile saline using the method of sequential tenfold dilution. The final concentra-

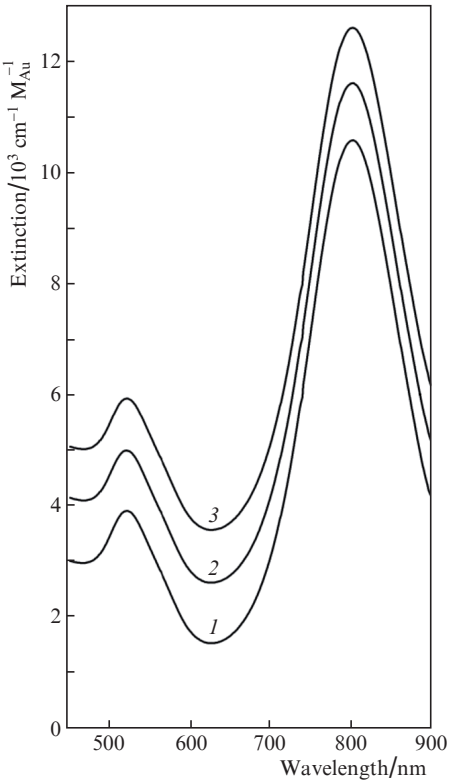

Figure 4. Extinction spectra of nanorods Au-PEG (*1*), Au-PEG-IgA (*2*) and Au-PEG-IgG (*3*). Within the experimental accuracy, the spectra of all samples are similar; in the figure they are artificially shifted by 10^3 cm^{-1} M$_{Au}^{-1}$ for better recognition.

tion amounted to 10^3 microbe cells (m.c.) per millilitre. Then 0.1 mL of the suspension from the 10^4 m.c. mL^{-1} dilution was added to 0.9 mL of suspension of nanoparticles and incubated during 15 min under light-protected conditions.

The bacterial suspension from the final 10^3 m.c. mL^{-1} dilution and from the solutions with nanoparticles was placed into the culture plate wells, the volume of each portion being 0.1 mL. The suspensions were irradiated during 5, 10, 15, and 30 min.

As the exposure time expired, the laser was switched off and the multimeter sensor was immediately placed into the culture plate wells to measure the temperature of the studied solutions. Then the bacterial suspensions were transferred from the cells into Petri dishes with dense nutrient medium and uniformly distributed over its surface with a sterile spatula. The results were determined by counting the number of colony forming units (CFU) in 24 hours after the incubation at 37 °C. The bacterial suspensions neither treated with the sensitizer, nor laser-irradiated served as a control group. Each experiment was repeated ten times. In the use of the laser radiation we followed the All-Russian State Standard R 50723-94 'Laser Safety. General Safety Requirements for Design and Exploitation of Laser Devices' and the Sanitary Regulations and Rules of Laser Design and Exploitation No. 5804-91.

3. Results

It was found that the NIR laser radiation with the wavelength 808 nm and power density 100 mW cm^{-2} suppresses the growth of the cells *S. aureus* MS. With the exposure time up to 30 min the decrease in the CFU number by 25%–56% was observed in comparison with the control group (Fig. 5a). The data obtained with the action of NIR laser radiation on the cells of *S. aureus* MR demonstrate a similar tendency. However, this strain was more resistant to the radiation, since the CFU number was reduced only by 15%–44% (Fig. 5b). The mean temperature growth in the irradiated bacterial suspensions amounted to nearly 2.7 °C (Table 1).

The addition of PEGylated gold nanorods to the cell suspension slightly suppresses the growth of *S. aureus* bacteria in the absence of irradiation, while the NIR exposure leads to an essential reduction of their number by 27% (5 min of exposure), 61% (10 min), 72% (16 min), and 82% (30 min) (Fig. 5a). When the laser radiation was acting on the *S. aureus*,

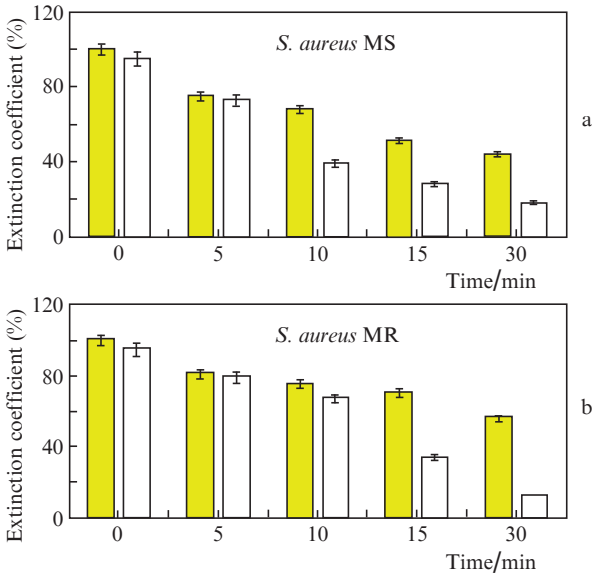

Figure 5. Effect of NIR radiation (808 nm) on the bacteria without nanoparticles (□) and on the bacteria in the suspension with gold nanorods coated with PEG (Au-PEG) (□).

Table 1. The mean temperature change in bacterial suspensions under the action of NIR laser radiation (λ = 808 nm).

Impact type	Mean temperature (°C) for different time of NIR laser radiation exposure					ΔT/°C	$\Delta T_{sol} - \Delta T_{NIR}$/°C
	0	5 min	10 min	15 min	30 min		
NIR radiation	20.0 ± 0.0	20.0 ± 0.2	20.7 ± 0.2	21.2 ± 0.2	22.7 ± 0.2	2.7 ± 0.2	–
NIR + Au-PEG	20.0 ± 0.0	34.5 ± 0.2	36.5 ± 0.2	39.7 ± 0.2	41.5 ± 0.2	11.5 ± 0.2	8.7 ± 0.2
NIR + Au-PEG-IgA	20.0 ± 0.0	36.7 ± 0.2	38.2 ± 0.2	41.5 ± 0.2	45.5 ± 0.2	15.5 ± 1.0	12.7 ± 0.7
NIR + Au-PEG-IgG	20.0 ± 0.0	37.5 ± 0.2	41.2 ± 0.2	43.5 ± 0.2	48.0 ± 0.2	18.0 ± 1.0	15.2 ± 1.2

Note: NIR + Au-PEG stands for NIR radiation and gold nanorods; NIR + Au-PEG-IgA – NIR radiation and conjugates of gold nanorods with immunoglobulin A; NIR + Au-PEG-IgG – NIR radiation and conjugates of gold nanorods with immunoglobulin G.

incubated with gold nanorods, the CFU number decreased by 20%–87% (Fig. 5b). During the entire time of exposure the mean temperature of the solution, containing the staphylococcus cells and gold nanorods, increased by 8.7 °C (Table 1) with respect to the case of irradiation of pure suspensions.

In the course of experiments it was found that the use of conjugates of nanoparticles with Fc-fragments of immunoglobulins leads to the enhancement of the NIR radiation effect. When the NIR radiation was applied together with the nanorods labelled with anti-staphylococcus IgA, the CFU number for *S. aureus* MS decreased by 33%–87% (Fig. 6a). The incubation of *S. aureus* MR with IgA-nanorods conjugates with subsequent laser irradiation lead to the reduction of the CFU number by 26%–94% (Fig 6b). In this case the mean temperature of the exposed solutions increased by 12.7 °C in comparison with the temperature of laser-irradiated solutions, containing no gold nanoparticles (see Table 1).

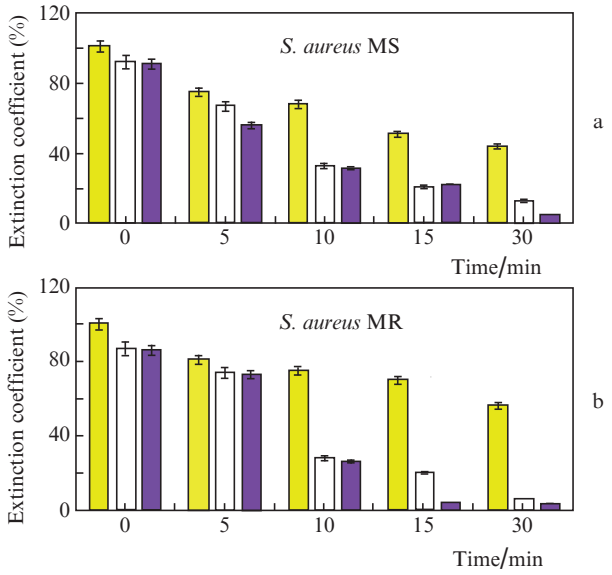

Figure 6. Effect of NIR radiation (808 nm) on the bacteria, treated with PEG-coated gold nanorods (▣), conjugated with antibodies IgA (Au-PEG-IgA) (□) and IgG (Au-PEG-IgG) (■).

The use of Fc-fragments of G-class immunoglobulins as label molecules increased the efficiency of complex effect of the nanoparticles and the NIR laser radiation. The number of *S. aureus* MS cells in suspension with gold nanorods decreased by 44% after 5 min exposure, by 69% after 10 min exposure, by 78% after 15 min exposure, and by 95% after 30 min exposure (Fig. 6a). For the *S. aureus* MR strain a pronounced suppression in comparison with the previous version of the experiment was detected after 15 min exposure. The number of cells incubated with IgG-conjugates of gold nanorods decreased by 96% after 15 min of exposure and by 97% after 30 min (Fig. 6b). The mean temperature of the bacterial suspensions increased by 15.2 °C, when the exposure time was increased from 0 to 30 min (see Table 1).

4. Discussion

The complex photodynamic/photothermal action of laser radiation with the wavelengths in the red and infrared spectral regions in combination with gold nanoparticles is known to possess pronounced antimicrobial properties [1, 29–35]. As shown by the analysis of the literature data, the main attention of the researchers is focused on the growth suppression of such clinically significant bacteria as *Staphylococcus aureus* and *Pseudomonas aeruginosa* [29–31], characterised by resistivity to many present-day antibiotic preparations [36–38]. Immunoglobulins are used for targeted delivery of such photosensitizers, as chlorine e6 [29], gold nanoparticles, containing vancomycin [30–32], gold nanorods coated with toluidine blue [33].

In our earlier studies the analogous schemes were used [8, 9 35]. It was found that the NIR laser radiation (805, 808 nm; 46–60 mW cm^{-2}) in combination with gold plasmon nanoparticles, conjugated with the indocyanine green dye, leads to the death of 75% of the population of the methicillin-sensitive strain of *S. aureus* [8, 35]. In spite of the synergetic effect of the combined action of photodynamic/phototoxic dye and plasmon nanoshells with close absorption bands in the region 800 nm under their laser irradiation, discovered in these papers, the suppression of the bacterial flora appeared to be only slightly expressed. In this connection, the creation of antibody-labelled nanoparticles for targeted interaction with the surface of bacterial cells became a logical continuation of the preceding studies.

The rate and quality of fighting the infectious agents depend, first of all, on the condition of the immune system of the macroorganism, and only next on the efficiency of pharmaceutical or other treatment. In the normal condition the phagocytes recognise the general microbial antigens by means of their own receptors, or use the antibodies, synthesised by the organism, as opsonins – the molecules activating the recognition. (Fig. 7a). However, a variety of adaptation mechanisms is known that allow the microorganisms to neutralise the effect of immune factors [37, 39–41]. A typical example is the presence of trap proteins in bacteria (protein A in staphylococcus, protein G in streptococcus) that possess a high degree of affinity to certain effector molecules of the immune system. The staphylococcus protein A is a small protein (42 kDa), covalently bound to the peptidoglycan of the cell wall and capable of high-affinity binding the Fc-fragment of immunoglobulin molecules (IgG and IgA), making the cell 'invisible' for phagocytes (Fig. 7b).

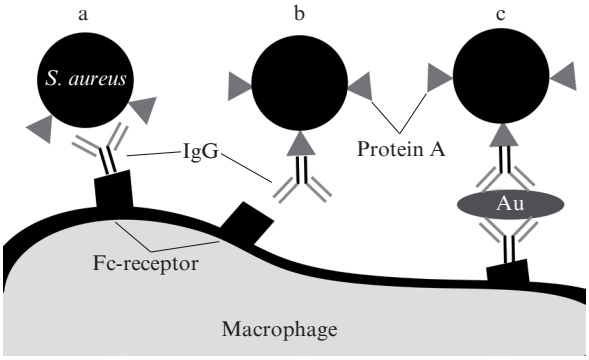

Figure 7. Interaction of macrophage membrane receptors with *S. aureus* cells: (a) immunoglobulin (IgG) acts as an opsonin, stimulating the phagocytosis via the Fc-receptor; (b) the protein A binds the Fc-fragment of IgG, which makes the binding to macrophage impossible; (c) gold nanorods are bound to the protein A and bring additional IgG for interacting with the macrophage.

In the present paper we propose to use the affinity of protein A of staphylococcus and the Fc-fragment of the antibody molecules in order to increase the efficiency of binding nanoparticles to microorganisms and, therefore, the efficiency of the laser radiation impact on the bacteria, labelled with gold nanoparticles. In this case the advantage is not only the selectivity of interaction between the nanoparticles and the bacterial cells, but also the improvement of the bacteria opsonisation and the phagocytosis stimulation (Fig. 7c), which would have a positive effect on the functioning of the immune system as a whole.

In the course of the study it was shown that without light irradiation the PEGylated nanorods demonstrate no expressed toxic effect. The slight reduction of the number of staphylococci (by 7%–13%) was found using the conjugates of nanorods and immunoglobulins. Probably, this is related to the agglutination effect, i.e., the formation of large-size macromolecular complexes of antibodies and antigens, which leads to inactivation of the bacteria.

The basic damaging effect of the NIR laser radiation and gold nanoparticles is associated with the local heating of the medium in the vicinity of cell membranes. The absorption of radiation by nanoparticles at the wavelengths within their plasmon resonance leads to significant temperature increase near nanoparticles and, therefore, to damaging the cell structures [1, 3, 5, 6]. The averaged control of temperature effects in the present work was implemented by measuring the temperature of cell suspension immediately after the irradiation. The increase in the bacterial suspension temperature depended on the exposure, the maximal values being recorded after 30 min of NIR irradiation. The temperature of suspensions containing no nanoparticles, increased insignificantly after the irradiation (see Table 1).

Let us discuss our results based on the concept that the local heating in the contact region between the nanoparticle and the microorganism wall is essentially stronger than the mean heating of the entire cell suspension. As a result of the local heating by several nanoparticles at once, the cell wall and membrane are damaged and the cell dies. The appropriate calculations of the local heating were presented in Ref. [42]. In Refs [8, 9] it was shown that the death of the microorganisms occurs under the heating of the suspension by a few degrees only. This fact means that the local temperature near the nanoparticles, attached to the cell wall, is essentially higher than the mean value, since the threshold of biological material destruction by the cw radiation amounts to 50–60 °C [43]. For microorganisms with sufficiently thick (100 nm) wall the destruction thresholds appear to be even somewhat higher [41].

All types of the synthesised particles had identical extinction spectra (see Fig. 4). The observed increase of the mean temperature in the series of suspensions with PEGylated nanoparticles (control) (8.7 ± 0.2 °C) and with nanoparticles functionalised with immunoglobulins A (12.7 ± 0.7 °C) and G (15.2 ± 1.2 °C) with respect to the laser heating of the suspension in the absence of nanoparticles can be associated with the formation of clusters of nanoparticles as a result of their efficient accumulation on the cell surface during the functionalisation. This, probably, leads both to a more efficient photothermal effect at the local level and to a certain increase in the mean temperature [3, 44].

Though the increase in the mean temperature of the medium under heating with NIR radiation did not exceed 15–16 °C, the local heating of nanorods and the vicinity of cell walls could attain a few tens or hundreds of degrees. Special control studies of the survival of the studied bacteria under the heating of suspension up to 50 °C during 30 min have shown that the staphylococci remain viable under such conditions. This fact completely agrees with the literature data on the tolerance of staphylococci to the environmental factors, including the temperature as high as 65–70 °C [41]. Moreover, the temperature increase by 15–16 °C was observed only at the end of 30 min light exposure. Since in the experiments on the impact of NIR radiation in combination with gold nanorods the number of microorganisms of two studied strains decreased proportionally to the increase in the mean temperature, one can assume that the major contribution to the damage of bacterial cells is made by the local photothermal effect, associated with the activation of plasmon resonance at the surface of nanoparticles with cluster formation, rather than by the total heating of the suspension.

The G-class immunoglobulins are known to possess a more expressed affinity to the protein A of the staphylococcus than the A-class immunoglobulins [45, 46]. Possibly, the insufficient strength of binding nanoparticles to the bacterial surface is just the explanation of the smaller sensitivity of staphylococci to the action of radiation and conjugates on the base of IgA (reduction of CFU number by 33%–87% for *S. aureus* MS and by 36%–94% for *S. aureus* MR) in comparison with the conjugates on the base of IgG (reduction of CFU number by 44%–95% for *S. aureus* MS and by 37%–97% for *S. aureus* MR).

An interesting fact is that the kinetics of the population number reduction under the action of NIR radiation and nanorods labelled with antibodies was similar for both studied strains of *S. aureus*. Under short-time exposure (5 min) the greater susceptibility was demonstrated by the methicillin-sensitive strain, while the longer exposures (10 min and greater) were more efficient in suppressing the methicillin-resistant strain, and at 30 min the reduction of CFU number by 97% was recorded (Fig. 6b). Thus, the developed method can be used for efficient eradication of bacteria possessing some degrees of resistance to antibiotics.

5. Conclusions

In this work the inverted binding of immunoglobulin molecules to the PEGylated surface of gold nanorods was used for the first time to bind the protein A of the staphylococcus with the aim to use it in the photothermal impact of the NIR (808 nm) laser radiation on the bacteria. The conjugates of nanoparticles with IgG appeared to be more efficient than those with IgA. The sufficiently high efficiency of the novel technology for the selective suppression of the growth of aurococcus bacteria, both resistant and non-resistant to antibiotics is demonstrated. However, in order to provide the optimal local heating of nanoparticles and the biological structure surrounding them without essential heating of the environment of the microorganisms, the present method should be optimised with respect to the concentrations of all components, including the nanoparticles, and the power density of laser radiation, its dose and, probably, the transition to pulsed laser mode.

Further study of the influence of nanoparticle conjugates with immunoglobulins on the human immune system should be carried out using the laboratory animal models. These studies will allow for determination of new regularities in laser deactivation of the staphylococcus infection in the living

organism with the influence of immune response taken into account, under the real conditions of illumination and distribution of microorganisms.

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