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Phototoxic effect of conjugates of plasmon-resonance nanoparticles with indocyanine green dye on *Staphylococcus aureus* induced by IR laser radiation

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Abstract. **The effect of IR laser radiation (λ = 805–808 nm) on the bacteria of the strain *Staphylococcus aureus* 209 P, incubated in indocyanine green solutions, is studied, as well as that of colloid gold nanoshells, nanocages and their conjugates with indocyanine green. It is found that the *S. aureus* 209 P cells are equally subjected to the IR laser radiation (λ = 805 nm) after preliminary sensitisation with indocyanine green and gold nanoparticles separately and with conjugates of nanoparticles and indocyanine green. The enhancement of photodynamic and photothermal effects by 5 % is observed after 30 min of laser illumination (λ = 808 nm) of bacteria, treated with conjugates of indocyanine green and nanocages.**

Keywords: *IR lasers, nanoparticles, indocyanine green, aurococcus bacteria.*

1. Introduction

The studies in the field of application of IR laser radiation to the treatment of different acute and chronic infectious diseases, as well as at suppurative–septic complications, have long been known [1, 2]. The therapeutic effect of low-intensity IR laser radiation is commonly associated with the enhancement of activity of the most important enzymes, with the biosynthesis of proteins, DNA and RNA, with the proliferation of cells, regeneration of tissues, enhancement of lymph and blood microcirculation, as well as with the promotion of the immune system activity.

The therapeutic effect can be considerably enhanced by using a combination of laser radiation with different photodynamic and photothermal dyes. In this case, apart from the mentioned biophysical mechanisms of light action, there are additional factors, providing the desired effect, namely, the photodynamic, photothermal and phototoxic action of the dye itself when absorbing the radiation. It is worth noting that the main requirements to the molecular agents are high yield of the singlet oxygen (or the nitrogen monoxide), high molar extinction coefficient, and also low dark toxicity. As to the irradiation parameters, the laser radiation in the region 750–850 nm is also rather promising for the photodynamic and photothermal therapy due to the considerable depth of light penetration into biological tissues.

Indocyanine green is a dye that allows one to obtain both the photodynamic and the photothermal effects at the mentioned wavelengths. This dye is actively used in many fields of medicine (oncology, ophthalmology, cardiology, surgery, dermatology, cosmetology) since it has low toxicity and can be easily and quickly removed from the organism [3, 4].

The impetuous progress in the field of nanotechnologies provided the researchers with a wealth of new materials possessing unique optical and physicochemical properties for the use in biology and medicine. In particular, during the recent years positive results were obtained in the use of gold, silver, silicon and titanium oxide nanoparticles both in protection against radiation and in biosensors, immune analysis, genomics, treatment of oncologic and infectious diseases [5–9].

With a view to biological and medical application the gold nanoparticles with plasmon resonance (PR) are of particular interest. For the majority of applications in biophysics and biomedicine [9] the resonance optical properties of nanostructures should fall within the transparency window of biological tissues [10]. The spectral tuning of PR in nanoparticles is implemented via changing the size, shape, metal and structure of the particles [11–14]. Recent progress in nanoparticle synthesis technology during the last 10–15 years [15, 16] offers a wide range of research objects to choose, from well-known gold nanorods [17–24] and nanoshells [25–28] to exotic structures like 'nanocages' [29]. In the present paper the main attention is paid to two types of nanoparticles, namely, gold nanoshells and nanocages, comprising a dielectric core and a metal shell a few nanometres thick (for nanocages the core is a dispersion medium). The choice of these objects is caused by the possibility to tune the PR in these particles to the region of transparency of biological tissues, and also by their availability and demand for them in the practical and experimental biology and medicine, where such particles are used as a nanobiotechnological platform.

In recent years a number of papers appeared demonstrating the enhancement of the biological activity of different dyes when used together with PR nanoparticles. As a rule, metal nanoparticles are used. Photodynamic dyes or antibiotics are tied to their surface by physical adsorption or covalent bonds.

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Such nanostructures are referred as conjugates, and the procedure of attaching a biological macromolecule to the nanoparticle surface is often called functionalisation [30].

Practically, each type of nanoparticle functionalisation has its highs and lows. Covalent attachment provides strong chemical bond between the dye and the particle surface and eliminates desorption. Electrostatic attachment, in turn, does not require modification of the dye molecules and chemically is much simpler.

The mechanism of enhancement of photodynamic or phototoxic effect of molecular dyes at conjugation with PR-nanoparticles is now a subject of discussions. Three possible mechanisms can be assumed: (i) enhancement of interaction between the molecule and the light wave due to the enhancement of the field near the particle surface (in analogy with the giant Raman scattering near a coarse metal surface); (ii) mutual photodynamic action of the dye and photothermal action of the particle; (iii) increase in the local dye concentration due to the conjugate adsorption on the surface of the target cell.

In favour of the first mechanism are the studies carried out by Russell's group [31, 32], in which the authors managed to increase the quantum yield of singlet oxygen or nitrogen monoxide in photodynamic therapy. Particularly, they demonstrated [31] doubling of singlet oxygen quantum yield due to photosensitisation by phthalocyanine conjugated with gold nanoparticles. Moreover, the conjugation allowed stabilisation of the agent in the course of its transfer from the organic phase (toluene) into the aqueous one.

The efficiency of using the mutual photodynamic and phototoxic properties of conjugates was demonstrated by the example of gold nanorods conjugated with methylene blue [7] and indocyanine green [33].

Finally, the reported enhancement of the antibiotic activity in mixture with gold nanoparticles [34, 35] also evidences in favour of the concentration effects.

The goal of this study is to estimate the influence of IR laser radiation (805–808 nm) on the cells of *Staphylococcus aureus* 209 P, incubated in solutions of indocyanine green, colloid gold nanoshells, nanocages and their conjugates with indocyanine green.

2. Materials and methods

The bacterial strain *Staphylococcus aureus* 209 P (Tarasevich State Institute of Standardization and Control of Biomedical Preparations, Moscow) was taken as a model. The staphylococci were grown at the temperature 37 °C on the universal dense nutritional medium (GRM-agar, Obolensk). Diode lasers with the peak-power wavelength λ = 805 nm and the power density 46 mW cm^{-2} (Acculaser, Photothera Inc., USA) and with λ = 808 nm and the radiation power density 60 mW cm^{-2} (LAS, Saint-Petersburg) served as radiation sources. All experiments were carried out in the cw oscillation regime.

The temperature of solutions was measured with the digital multimeter (MY62, Mastech, China) having the measurement error of $\pm$0.5 °C. The aqueous solution of indocyanine green (ICG, Sigma-Aldich Co., USA), having the maximum of the absorption spectrum at λ = 775 nm, with the concentration 2.5 g mL^{-1} served as a photosensitiser.

The gold nanoshells (Au–S) were obtained following the technique of Ref. [36], based on the directional recovery of gold on the surface of silicate particles produced by means of Stöber's method [37]. The suspensions of nanoparticles consisting of a silicate core with the diameter 120$\pm$15 nm and a golden shell 20$\pm$7 nm thick were prepared. The concentration of nanoparticles was $\sim$10^9 mL^{-1}. Nanoparticles with such geometric parameters possess a PR in the IR region (the extinction maximum at 850 nm). The optical density of the suspension at this wavelength was $\sim$2.

For gold nanocages (Au–C) the protocol of synthesis included two basic stages [38]. At the first stage silver nanoparticles, mainly having a cubic shape, were produced as a result of the polyol synthesis induced by silver sulphide. At the second stage the silver nanocubes were used as templates for transformation into golden nanocages via the galvanic replacement of silver with gold. The hollow particles obtained had the mean size 53$\pm$5 nm, the wall thickness 7$\pm$2 nm, the diagonal size 78 nm and the shape, commonly referred as 'nanocage' in the literature. The concentration of particles was $\sim$10^{10} mL^{-1}, the PR was localised near 750 nm, and the optical density of the suspension at this wavelength was about 2.

To conjugate the nanoparticles with the fluorescent dye we used the technique [39], illustrated schematically in Fig.1. The golden nanoshells were precoated with a layer of silicon dioxide having a controlled nanometre thickness. Then 3-aminopropyltrimethoxysilane was added to the suspension of particles, coated with SiO_2, to functionalise the particles with amine groups. The produced amine-functionalised particles were precipitated on the bottom of the reaction flack. The sediment was dissolved in an aqueous solution of indocyanine green. As a result, the indocyanine green was adsorbed on the particle surface due to the electrostatic interaction with positively charged amine groups. The obtained conjugate was centrifuged and redissolved in water or buffer. The separation between the fluorophore and the gold surface in this case was determined by the thickness of the SiO_2 layer and was equal to 10–15 nm. The concentration of the adsorbed indocyanine green was evaluated by the difference between the conjugate

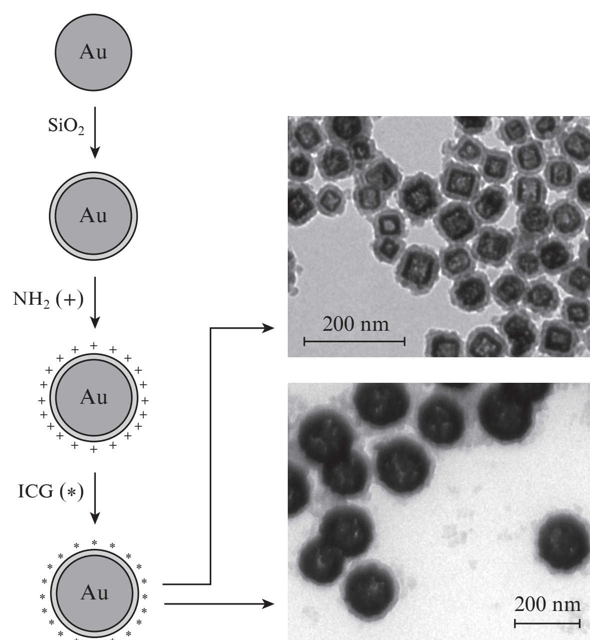

Figure 1. Schematic diagram of the conjugate synthesis of PR-nanoparticles (nanocages – top; nanoshells – bottom) with indocyanine green (ICG).

Table 1. Geometric, optical and physicochemical parameters of the samples.

Sample	ICG concentration/mg mL^{-1}	Concentration of particles/mL^{-1}	Wavelength of maximal extinction/nm	Optical density	Surface modification
ICG aqueous solution	2.5	—	775	0.35	—
Conjugate of nanoshells with ICG	2.5	10^9	850	2	ICG
Conjugate of nanocages with ICG	2.5	10^{10}	775	2	ICG
Suspension of nanoshells	—	10^9	850	2	PEG
Suspension of nanocages	—	10^{10}	750	2	PEG

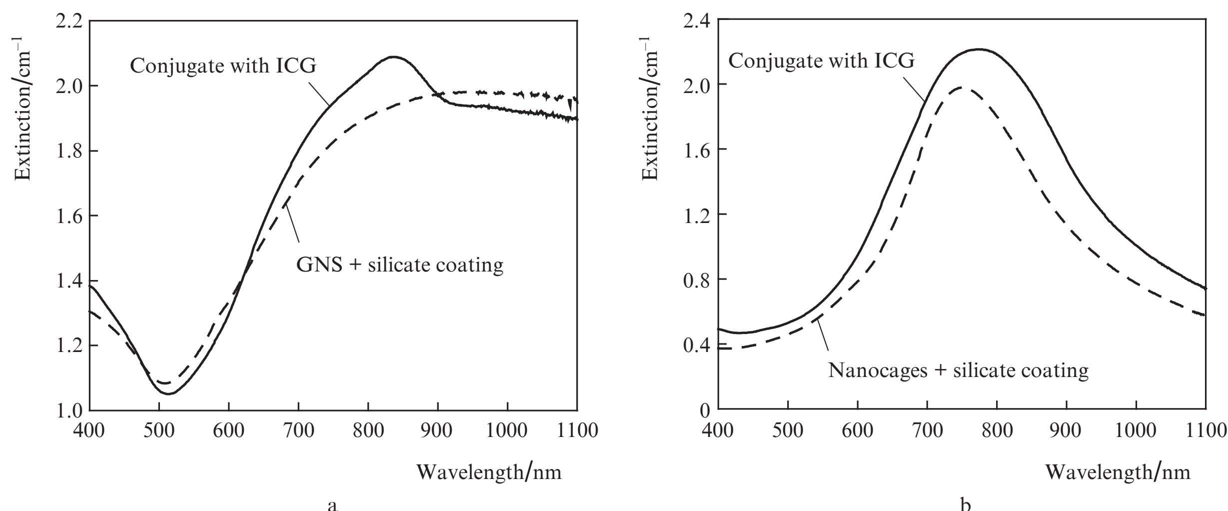

Figure 2. Extinction spectra of gold nanoshells (GNS) (a) and nanocages (b) before the conjugation with indocyanine green (dashed lines) and after the conjugation (solid lines).

optical density values before and after the adsorption procedure. For the two kinds of particles it was nearly 2.5 mg mL^{-1}.

Figure 2 shows the extinction coefficient spectra of gold nanoshells and nanocages. Dashed lines present the spectra of nanoparticles coated with silicon dioxide, solid lines refer to the conjugates with indocyanine green. It is seen, that in the case of nanoshells the conjugation leads to the appearance of extra extinction peaks at nearly 800 nm, which correspond to the molecular absorption of the adsorbed dye. For nanocages having a pronounced PR peak at 750 nm the dye adsorption leads to the shift of the total extinction maximum towards the long-wavelength region.

Besides the conjugates of nanoparticles with indocyanine green, we studied the photothermal activity of nanoparticle suspensions with the same concentrations, but without dye. To increase the colloid stability in the buffer, the nanoparticles were conjugated with the molecules of polyethylenglycole (PEG), as described in [40].

The geometric, optical and physicochemical parameters of the studied samples are summarised in Table 1.

To provide aseptic conditions in the course of the experiment, we used a sterile disposable 96-well plate; the source of radiation was placed above the wells of the plate. We used the *S. aureus* 209 P culture previously grown during 24 hours at the temperature 37 °C on the dense nutritional medium. The bacterial suspension was prepared in the sterile saline solution using the method of tenfold serial dilution; the final concentration was 10^3 microbe cells (m.c.) in 1 mL. Then 0.1 mL of the suspension from the 10^4 m.c. mL^{-1} dilution was introduced into 0.9 mL of the solution of photosensitiser, nanoparticles or conjugates, and incubated during 15 min without light access. To estimate the interaction of nanoshells with the

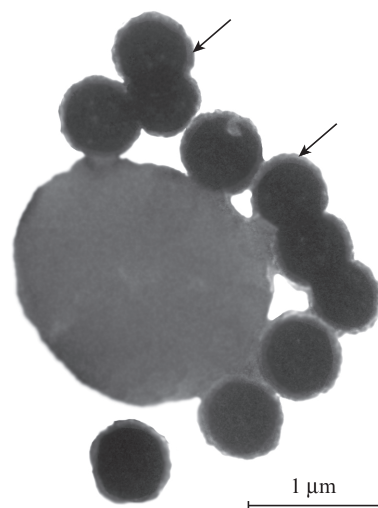

Figure 3. *S. aureus* 209 P cell surrounded by gold nanospheres; the arrows indicate the outer silicon layer, containing indocyanine green (electron microphotograph, magnification 600 000×).

S.aureus 209 P cells we used the electron microscope Libra 120 (Carl Zeiss, Germany) (Fig. 3).

The bacterial suspensions from the 10^3 m.c. mL^{-1} dilution, as well as from the solutions of the photosensitiser and nanoparticles, were placed into the wells of the plate in the volume of 0.1 mL. The irradiation of the suspension was performed during 5, 10, 15, or 30 min.

On the expiry of the treatment time the laser was switched off and the measuring probe of the multimeter was placed

into the wells of the plate to measure the temperature of the studied solutions. Then the bacterial suspensions were taken from these wells, placed onto Petri dishes with dense nutritional medium and evenly distributed over the surface with a sterile spatula. After 24 hours of incubation at 37 °C the number of colony forming units (CFU) was counted. The bacterial suspensions not treated with the sensitiser and not subjected to irradiation were used for control. Each experiment was repeated ten times.

3. Results

It was found that the laser radiation with the wavelength 805 nm and the intensity 46 mW cm^{-2} insignificantly suppresses the growth of the *S. aureus* 209 P cells. The number of CFU decreased by 5%–21% as compared to the control data (Fig. 4). At increasing the exposure time from 0 to 30 min the temperature of the saline solution, containing the bacteria, increased only by 1 °C (Table 2). Similar results were obtained acting on the bacterial cells with the laser radiation having the wavelength 808 nm and the intensity 60 mW cm^{-2}, namely, the number of CFU decreased by 5%–24% (Fig. 5), and the temperature of the irradiated solution increased also by nearly 1 °C (Table 2).

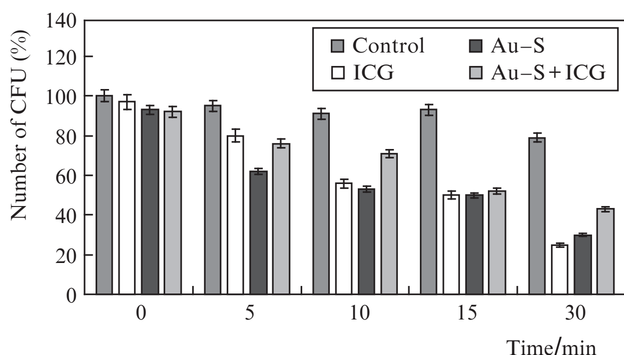

Figure 4. The effect of IR laser radiation (805 nm) on the bacteria, treated with indocyanine green (ICG), gold nanoshells (Au–S) and conjugates of the gold nanoshells with indocyanine (Au–S + ICG).

It is known that indocyanine green possesses strong phototoxic effect, which can be explained by its photodynamic effect [4, 41–43] or by the formation of cytotoxic final products under the action of light [44]. Under the action of laser radiation (805 nm, 46 mW cm^{-2}) the number of *S. aureus* 209 P cells decreased by 20%–75% depending on the exposure time. The temperature of the solution, containing the microorganism cells and indocyanine green, increased by 1.5 °C (Table 2). The laser irradiation of the bacterial suspension at 808 nm and 60mW cm^{-2} reduced the number of CFU by 5%–65% (Fig. 5); the temperature in this case increased by no more than 1 °C (Table 2).

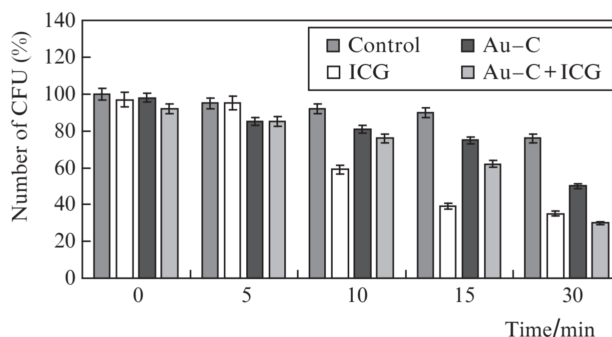

Figure 5. The effect of IR laser radiation (808 nm) on the bacteria, treated with indocyanine green (ICG), gold nanocages (Au–C) and conjugates of the gold nanocages with indocyanine (Au–C + ICG).

The use of gold nanoparticles (independent of their shape) causes nearly the same suppression of the growth of *S. aureus* 209 P bacteria as the use of the ICG dye solely. The treatment of the bacterial cells with gold nanoshells in combination with the laser radiation (805 nm, 46 mW cm^{-2}) caused the reduction of the *S. aureus* 209 P cell number by 38% after 5 min of exposure, 47% after 10 min, 50% after 15 min, and 70% after 30 min (Fig .4). During the whole exposure time the temperature of the solution, containing bacterial cells and gold nanoshells, has increased by 3 °C (Table 2). Under the action of radiation (808 nm, 60 mW cm^{-2}) on the *S. aureus* 209 P, incubated with golden nanocages, the reduction of CFU by 15%–50% in comparison with the control data was registered (Fig. 5). The temperature of the solution, containing *S. aureus* 209 P and golden nanocages, in this case has increased by 5 °C (Table 2).

It was found in the experiments that the use of conjugates of nanoparticles with indocyanine green as photosensitisers does not give rise to essential synergetic effects in suppressing the bacteria under study. The reduction of the CFU number by 24%–57% in comparison with the control one is recorded (Fig. 4) under the action of laser radiation (805 nm, 46 mW cm^{-2}) and the conjugates of indocyanine green with gold nanoshells.

Table 2. Measurement of the temperature of the suspension, containing the *S. aureus* 209 P bacteria, under the action of IR laser radiation.

Time/min	Temperature/°C					
	λ = 805 nm			λ = 808 nm		
	IR	IR + Au–S	IR + ICG + Au–S	IR	IR + Au–C	IR + ICG + Au–C
0	22	22	22	22	22	22
5	23	24	23.5	23	23.5	25
10	23	25	24	23	24.5	25.5
15	23	25	25.5	23	25	26
30	23	26	27	23	28	26
	(1), [0]	(4), [3]	(5), [4]	(1), [0]	(6), [5]	(4), [3]

Note: IR + Au-S – IR radiation and gold nanoshells; IR + ICG+Au–S – IR radiation and conjugates of gold nanoshells with indocyanine green; IR+Au–C – IR radiation and gold nanocages; IR + ICG + Au–C – IR radiation and conjugates of gold nanocages with indocyanine green. The figures in parentheses indicate the temperature growth after 30 min of heating, in square brackets is the solution temperature change without the IR radiation contribution taken into account.

The temperature of such a solution increased by 4 °C as the irradiation time increased from 0 to 30 min (Table 2). The incubation of the *S. aureus* 209 P bacteria with the conjugates of indocyanine green and nanocages followed by laser irradiation (808 nm, 60 mW cm^{-2}) lead to the reduction of the CFU number by 15%–70% (Fig. 5). In this case the temperature of the irradiated solution increased by 3 °C (Table 2).

4. Discussion

It was shown in some papers that the combined use of gold nanoparticles, photodynamic dyes and laser radiation in the red (600–660 nm) [5, 8] or infrared (700–900 nm) [6, 7, 33] range is very efficient against such bacteria as *S. aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, and *Escherichia coli*. In comparison with the common methods of therapy, the photodynamic and photothermal technologies offer essential advantages in the treatment of pyoinflammatory diseases, particularly caused by the antibiotic-resistant bacterial strains, because in these strains no stability to the used methods of external action is formed. Using the sources of near-IR radiation allows sufficiently deep and noninvasive action on the lesion focus. It should be noted, that in the papers cited above and in Ref. [45] the concentrations of dye used in combination with IR laser radiation (810 nm, 48 mW cm^{-2}, 30 min) were relatively high (25–250 μg mL^{-1}). Under these conditions the growth suppression by 90%–99% was observed in *S aureus*, *P. aeruginosa*, and *Streptococcus pyogenes*. In our experiments the dye concentration was 1–2 orders of magnitude lower (2.5 μg mL^{-1}). This is related to the maximal sorptive capacity of the gold nanoparticle colloids. The simplest estimates of the particles molar concentration yield the number of the dye molecules (~2000), adsorbed on one particle, which corresponds to a molecular monolayer. Even at such low mass concentration of indocyanine green we observed pronounced suppressing action (by 75%) of the radiation on the *S. aureus* 209 P.

Depending on the photosensitiser used, the total change in the temperature as a result of irradiation was in the range of 1–6 °C. Therefore, the influence of medium heating on the suppression of the microorganism growth at the power densities of laser radiation mentioned above may be neglected. Nevertheless, the incubation with gold nanoparticles of both classes (nanoshells and nanocages) followed by IR laser irradiation lead to the death of 50%–70% of the *S aureus* 209 P cells. In the absence of irradiation the nanoparticles exerted no suppressive influence on the cells. In this case the possible mechanisms of the effect are associated with the accumulation and migration of energy in the process of absorption of light by nanoparticles at frequencies close to that of plasmon resonance, which may cause photothermal damage of membranes. In other words, the local heating of a nanoparticle, adsorbed on the surface of a bacterial cell, may increase its temperature to values, high enough to damage the cell wall.

In the course of the present investigation it was shown that the studied strain *S. aureus* 209 P is more affected by the action of IR laser radiation in the presence of gold nanoshells than in the case when the gold nanocages are involved. Nanoshells have larger dimensions (130 nm) and, therefore, essentially larger absorption cross section, than the smaller (50 nm) nanocages with a hollow structure. This fact also evidences in favour of the possibility of local nanoparticle heating.

It is known that the complexes of indocyanine green with proteins possess higher photostability in the presence of gold colloids [46]. In this connection the use of the complexes of indocyanine green with nanoparticles of gold for phototherapy seems quite reasonable. However, in our experiments it was shown that the efficiency of using such complexes in not sufficient. In the cases of using the conjugates of golden nanoshells with the adsorbed indocyanine green the reduction of the *S. aureus* 209 P CFU number did not exceed 43%. Possibly, the use of conjugates impedes the production of singlet oxygen and active radicals by the dye molecules. The increase of survivability in this case, in comparison with the CFU indicators at separate use of indocyanine green or gold nanospheres solely, is probably related to the small (from 22 to 28 °C) increase in the solution temperature. The optimal solution temperature for the growth and development of *S. aureus* 209 P bacteria is 37 °C. Some part of the bacteria population dies as a result of the joint action of nanoparticles and laser radiation. The survived cells continue to multiply as the experimental conditions approach the physiological ones, which finally manifests itself in a slower decrease in the cell number.

Another obstacle for enhancing the efficiency of photodynamic suppression of pathogenic microorganisms is the insufficient degree of nonspecific binding of conjugates with cells in the process of incubation. In the recent paper [47] it was shown that the efficiency of the specific labelling of the pig embryo kidney cells, carrying an oncovirus, was 17 times higher than that of nonspecific adsorption.

5. Conclusions

It is found that the cells of *S. aureus* 209 P are equally subjected to the action of IR laser radiation (805 nm, 46 mW cm^{-2}) following the sensitisation with indocyanine green, gold nanoparticles solely and conjugates of nanoparticles with indocyanine green. Insignificant (by 5%) mutual enhancement of photodynamic and photothermal effects is indicated after 30-min laser irradiation (808 nm, 60 mW cm^{-2}) of bacteria, incubated with conjugates of indocyanine green with nanocages.

Let us formulate the basic directions of activating the suppressor action of IR laser radiation on the *S. aureus* 209 P bacteria, sensitised by complexes of a photodynamic dye and gold nanoparticles. First, one should expect better efficiency when increasing the radiation power density, which was relatively low (46–60 mW cm^{-2}) in our experiments. The value of power density should be limited only by the level of physiological temperature, i.e., the heating of the bacterial suspension from the room temperature to 37–40 °C (nearly by 20 °C) in the course of light exposure. It is important that in this case an additional effect of photothermal damage of the cell wall in microorganisms will be induced by the nanoparticles. Second, the use of nanosecond pulse regimes allows a substantial increase in the local heating without strong heating of the bulk suspension, since during the pulse action (10–20 ns) the heat transfer between the particle and the environment has no time to occur. And, finally, specific labelling of the suspension with conjugates of nanoparticles with dyes (e.g., using phage antibodies) will allow an essential increase in the degree of adsorption of nanoparticles on the cell wall and the local concentration of the dye.

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