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Investigation of Copper Nanoparticles Antibacterial Mechanisms Tested by Luminescent *Escherichia coli* Strains

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Abstract—The electrostatic interaction between positively charged copper nanoparticles aggregates ($\zeta = +15.9 \pm 8.63$ mV) and negatively charged surface of *E. coli* K12 TG1 cells ($\zeta = -50.0 \pm 9.35$ mV) has been established. The time-dependent decline of bacterial cells zeta potential and the coupled inhibition of constitutive bioluminescence level are the results of this interaction. The development of oxidative stress, probably defined by the electron transfer from the cytoplasmic membrane respiratory chains through membrane-integrated copper nanoparticle to the molecular oxygen, is shown as luminescence induction in superoxide- and peroxide-inducible *E. coli* K-12 MG1655 *pSoxS::lux* and *pKatG::lux* reporter strains. The final result of this process, which is responsible for the development of the bactericidal effect of copper nanoparticles, is DNA damage by active oxygen species detected by SOS-inducible *E. coli pRecA::lux* luminescent strain.

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INTRODUCTION

There are two reasons for interest in investigating the biological activity of copper nanoparticles (nanoCu). Firstly, the potential for their application as catalyst components, anticorrosive coatings, and conducting materials suggests that nanoCu will be released in a natural ecosystem and human environment, which requires considerations of the risks involved [1, 2]. Secondly, copper nanoparticles are of interest for the development of a new generation of drugs and composite materials for medicine and agriculture [3, 4], which necessitates the identification of the underlying mechanisms of its biological activity.

Microorganisms can play a significant role in solving this problem, on the one hand, being an important component of aqueous and soil ecosystems potentially contaminated with nanoCu [5, 6] and, on the other hand, as they are considered an important target of copper nanoparticles in the development of antibacterial compounds and surfaces [3, 7, 8]. Currently, however, determining the inter-species differences in the sensitivity of microorganisms to nanoCu with the statement of the bactericidal effect of such treatment is the only result of the conducted research. Separate studies on the mechanisms of copper-nanoparticle biological activity include only an elucidation of its dependence on pH, temperature, and reaction-mixture aeration [9].

Luminescent organisms that have in their structure certain types of sensitive target elements prone to biodamage, optimally combined with the possibility of *lux*-gene cloning under constitutive or inducible promoters [10], can be expected to provide progress in this area. Accordingly, in the first case, the luminescence level inhibition are indicators of the overall toxicity effect of nanoparticles to natural [5, 11] or recombinant microorganisms [6]; in the second case, a characterization of the nature of the damaging effect can be possible based on the luminescence induction due to the expression of gene reporters [12]. Federal recommendations on the application of bacterial luminescence biotests for the assessment of nanoparticles and nanomaterials reflect the information given [13].

The goal of the study to investigate the mechanisms of antibacterial activity of copper nanoparticles with sensor and reporter strains of luminescent *Escherichia coli*.

MATERIALS AND METHODS

Ultradisperse copper particles synthesized with the Gen–Miller method of high-temperature condensation [14] kindly provided by professor N. N. Glushchenko (Doctor of Biology, Institute of Energy Problems of Chemical Physics, Russian Academy of Sciences, Russia) were used in the study. A preliminary investigation with a JSM 7401F (JEOL, Japan) scanning electron microscope characterized this substance as spheres with a diameter of 103 ± 2 nm, which made it possible to classify them as large nanoparticles on

the border between nano- and submicron objects that still have the ability to interact with biological objects at the ultrastructural level. X-ray diffraction and Mossbauer spectroscopy methods identified CuO oxide film on the surface nanoparticles comprising no more than 4% of its mass [15], which allowed relating the registered biological activity with the properties of the nanostructured crystalline copper.

Aliquots of nanoCu (6.4 mg) were placed into glass vials, where 1 mL of chemically pure distilled water was added followed by ultrasonic treatment at 35 kHz and 30 $W \cdot dm^{-3}$ specific sound power in a bath-type source (PKF Sapfir, Russia) for 30 min in order to prepare the initial 0.1 M suspensions. Next, additional glass flasks with 0.5 mL of chemically pure distilled water were placed directly in the ultrasonic bath; they were used to prepare a series of two-fold dilutions up to 1 : 16384 (10^{-4} M) inclusive under continuous sonification.

A pure analysis grade $CuSO_4 \cdot 5H_2O$ salt (Lenreactiv, Russia) and a commercial sample of spherical 40 μm copper microparticles (Alfa Aesar, Germany) were used as standard compounds for comparison. The suspension preparation method was identical to the one used for nanoparticles.

An *E. coli* K12 TG1 strain sensor constitutively expressed *luxCDABE*-genes of the natural marine microorganism *Photobacterium leiognathi* 54D10 [16], and commercially available as Ekolum (NVO Immunotekh, Russia) was the first test object. It was reconstituted from the lyophilized state immediately before the investigation by the addition of 10 mL of cooled distilled water and was allowed to stand at 2–4°C for 30 min, followed by the adjustment of the bacterial suspension temperature to 25°C.

Reporter *E. coli* K12 MG1655 strains carrying plasmids with gene fusions *soxS::lux*, *katG::lux,* and *recA::lux* generously provided by Doctor of Biology I.V. Manukhov (GOSNIIgenetika, Russia) served as a second group of objects. As a result of genetic organization, these constructs were characterized with a low level of initial luminescence, but responded with its induction in a relatively specific manner to the exposure with superoxide anion, hydrogen peroxide, or DNA damaging factors [12]. These strains were grown before the experiment for 18–24 h on LB-agar supplemented with 100 μg/mL of ampicillin–selective factor of the hybrid plasmids, followed by the transfer of the obtained biomass in the fresh LB broth and additional growth to the early exponential phase ($OD_{540} = 0.35$).

A measurement of the size and zeta potential of the copper nanoparticles as well as *E. coli* K12 TG1 cells before and after contact with nanoCu was conducted in distilled water at 25°C with a laser autocorrelation analyzer Zetasizer Nano (Malvern Instruments, United Kingdom). In the latter case, microorganisms contacted copper nanoparticles at a final concentration 0.05 M, and prior to investigation aliquots of the incubated mixture were taken at 0, 30, 60, and 90 min and diluted 1 : 10 in distilled water directly in the measuring cell of the instrument.

NanoCu particles, *E. coli* K12 TG1 cells, and their mixtures were applied on the freshly cleaved mica surface and dried for 24 h at 95% relative humidity and 22–24°C temperature. The samples were investigated with atomic force microscopy in a contact mode with a SMM-2000 (ZAO KPD, Russia) multimicroscope using cantilevers MSCT-AUNM (Park Scientific Instruments, United States) with a 0.01 N/m spring constant and 15–20 nm curvature radius of the tip.

A method similar to that suggested earlier for the analysis of carbon nanomaterial biotoxicity [17] was used for bacterial bioluminescence inhibition biotest. One hundred microliters of 2-fold dilutions of the investigated copper compounds from 0.1 to 10^{-4} M were placed in the wells of the 96-well plate made from nontransparent plastic. Wells filled with 100 μL of distilled water were used as a control. Next, 50 μL of the *E. coli* K12 TG1 sensor strain suspension was added to each well, the plate was placed in the measuring chamber of a LM-01T luminometer (Immunotech, Czech Republic), and the luminescence intensity was recorded dynamically for 90 min. The algorithm previously suggested for the bioluminescence assessment in turbid (colored) suspensions of nanoparticles was used to obviate the optical effects of the tested compounds [11]. The essence of it is in the measurement of luminescence of the bacterial biosensor immediately after contact with the tested compound, as well as after 30, 60, and 90 min of incubation. The gradual development of the biological activity in the process allows one to assess an optical effect at the initial moment, in comparison with which the development of an inhibitory effect can be revealed. The equation used was as follows: $I_{\text{inh}} = \dfrac{Ik_o \times It_n}{Ik_n \times It_o}$, where *Ik* and *It* are the luminescence intensity of the control and test samples at time zero as well as after 30, 60, and 90 min of measurement.

The results were used to calculate the EC_{50} toxicological parameter corresponding to the molar concentrations of the tested copper compounds causing the 50% inhibition of the bacterial luminescence in comparison with control.

The biomass of the *E. coli pSoxS::lux*, *pKatG::lux, pRecA::lux* strains was washed with centrifugation and resuspended in 0.85% NaCl solutions; next, 50 μL aliquots were added to the wells of 96-well plates made from nontransparent plastic, where equal volumes of solutions or suspension of the tested copper compounds (see above) were placed before the start of the experiment. The mixture was incubated for 15 min followed by the addition of 150 μL of LB broth. The plate was placed in the measuring chamber of the luminom-

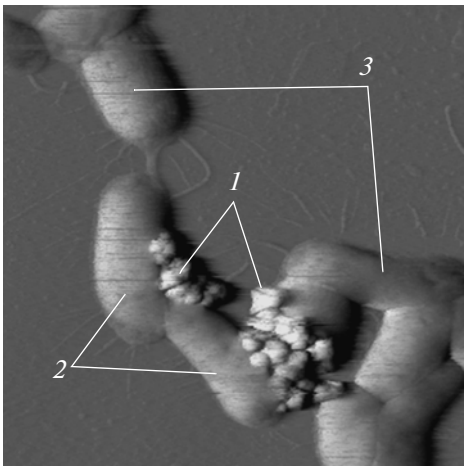

Fig. 1. SEM image of copper nanoparticles in contact with *E. coli* K12 TG1 cells. Notes: (*1*) copper nanoparticle aggregates, (*2*) bacterial cells in contact with them, and (*3*) intact bacterial cells

eter, and the growth of bacterial luminescence was dynamically recorded for 120 min. The expression level reflecting the induction of the respective promoters (I_{ind}) was estimated according to the equation presented above.

Ten-microliter aliquots were taken from the test and control samples of *E. coli* K12 TG1 + nanoCu mixtures at 30, 60, and 90 min and their dilutions were plated onto BCP agar (bioMérieux, France) supplemented with 100 μg/mL ampicillin. A number of colonies were enumerated after additional 18- to 24-h incubation at +37°C followed by a calculation of the fraction of cells that lost their viability due to contact with nanoCu particles, as well as LD_{50} values, which characterized the concentration of the compound-casing bactericidal effect in 50% of microorganisms at different exposure times

RESULTS

An investigation into the zeta potential (ζ) and size characteristics of copper nanoparticles at neutral pH made it possible to elucidate a number of their features in the formed colloid systems. The implementation of M3-PALS technology (an alternating electrical field with phase and frequency analysis of the scattered light) revealed the availability of the positive zeta potential in 90.3% of the tested nanoCu particles characterized with $\zeta = +15.9 \pm 8.63$ mV. The moderate value of this parameter, which was almost 2-fold lower than the dispersion stability threshold level ±30 mV, defined the tendency of copper nanoparticles to form larger aggregates. The application of noninvasive backscatter technology supplemented with atomic force microscopy data showed that the characteristic size of 93.5% of these aggregates is 432.0 ± 202.7 nm, with the polydispersity coefficient of the formed suspension 0.322.

A similar investigation into the sensor *E. coli* K12 TG1 strain characterized them as sufficiently homogenous 1069.0 ± 162.5 nm objects with $\zeta = -50.0 \pm 9.35$ mV.

The difference in sign of the zeta potential of copper nanoparticles and *E. coli* K12 TG1 cells is the most likely reason for their interaction recorded with atomic force microscopy (Fig. 1), which involves the formation of electrostatic contact of the nanoCu aggregates with the bacterial surface. The formation of the tight physical contact of nanoparticles with the surface membrane structures of bacterial cells was a result of that.

Another consequence of the interaction was the change in the nominal value of the bacterial cells zeta potential developing with time in the *E. coli* K12 TG1 + nanoCu mixtures (Fig. 2). The average value of this parameter at a time of 30 min was $\zeta = -23.7 \pm 12.2$ mV with the value in the control remaining constant. The asymmetrical distribution of the zeta-potential value in the *E. coli* K12 TG1 population was recorded at the same time, which remained at the level −43.1 ± 5.78 mV for 20.3% of the tested cells and decreased to −19.3 ± 7.78 mV for the residual 79.7% of the tested objects. The probabilistic nature of the electrostatic interaction in the *E. coli* K12 TG1 + nanoCu system that keeps a certain fraction of bacterial cells in the intact state can be an explanation of this fact (Fig. 1). Against this background, the increase in contact time to 90 min resulted in a further decrease in the average zeta potential level to −10.1 ± 24.6 mV due to the decrease in the fraction of the cell with the initial value reaching 6.18%, as well as the emergence of 15.9% of objects with $\zeta = +27.4 \pm 6.75$ mV, which were probably microorganisms that lost their viability after contact with copper nanoparticles (see below).

A study of the bioluminescence of the *E. coli* K12 TG1 constitutively expressing *P.leiognathi luxCDABE*-genes allowed an estimation of the quantitative and dynamic parameters of nanoCu biotoxicity, as well as a comparison of those with copper ions and microparticles. Thus, contact between the mentioned strain with the increasing nanoCu concentrations resulted in luminescence inhibition time-dependent that was characterized with $EC_{50} = 160 \pm 15$ μM value at 30-min exposure, which progressed to 77 ± 11 μM and 51 ± 9 μM with an increase in contact time to 60 and 90 min, respectively (table). At the same time, one feature of the Cu^{2+} copper ions was a much faster and more pronounced toxic effect at early stages, which was characterized with $EC_{50} = 20 \pm 4$ μM increasing only 2-fold upon further exposure. On the other hand, the use of copper microparticles decreased the rate of luminescence inhibition and made it less pronounced, becoming more significant only at the end of the

experiment, and over 1500-fold (EC_{50} = 80000 ± 250 μM) less that the analogous activity of nanoCu.

Hence, our results made it possible to establish the existence of a toxic effect of nanoparticles towards the sensor *E. coli* K12 TG1 strain similar to the one in the inhibition test with a natural isolate *Vibrio fischeri* NRRL B-11177 [5] or recombinant *Pseudomonas putida* KT 2440 strain [6]. The decline in the biotoxicity level of the copper compounds in a series ions → nanoparticles → microparticles was observed in good agreement with the results of animal studies [18] estimating nanoCu toxicity (LD_{50} = 413 mg/kg) as being below the one of $CuCl_2$, but exceeding the one of microparticles: LD_{50} = 110 mg/kg and LD_{50} > 5000 mg/kg, respectively. At the same time, while the general tendency coincides, the absolute toxicity values of the compared compounds were 40- to 120-fold higher for mice than for microorganisms, indicating the latter as biological objects that are much more sensitive to the effect of copper.

Further investigation into the impact of the *E. coli* K12 TG1 contact with nanoCu particles indicated that it was not limited by the inhibition of bacterial luminescence but was accompanied by the death of target bacterial cells. Hence, the application of these compounds in a concentration range 6.25–100 mM irreversibly inactivated no less than 99% of cells in the sample after only 30 min, which correlated with the concept of the distinct bactericidal potential of high doses of copper nanoparticles towards a wide range of Gram-positive and Gram-negative microorganisms [6–8]. On the other hand, the dependence of the nanoCu bactericidal effect manifestation on time was demonstrated for lower doses, which was exhibited by the change in LD_{50} from 550 ± 74 μM at 30-min exposure to 260 ± 30 μM and 165 ± 12 μM at 60 and 90 min of contact, respectively (table). While this result reproduced the tendency revealed earlier on the dynamic recording of bioluminescence, the nominal LD_{50} values were more than 3-fold lower than EC_{50} values. These data indicated a certain delay of the bactericidal effect relative to the luminescence inhibition and required for the search of additional causes of death of model organisms upon contact with nanoCu.

In this context, the possibility of oxidative stress development in the target bacterial cells, which can be triggered by the integration of electrically conductive copper nanoparticles into electron transport (respiratory) chains localized in cytoplasmic membranes, was analyzed at the next stage of the study. Reporter luminescent *E. coli* K12 MG1655 strains carrying plasmids with gene fusions *pSoxS::lux* or *pKatG::lux* that served earlier for the identification of similar mechanisms of biological activity of TiO_2 nanoparticles [12] were used as bacterial biosensors in this experimental series.

Our results made it possible to establish that the contact of bacterial cells with nanoCu particles in the concentration range 0.625–5 mM resulted in the formation of reactive oxygen species (ROS) that were detected with the specifically induced transcription of the reporter *luxCDABE*-gene cassette under PsoxS and

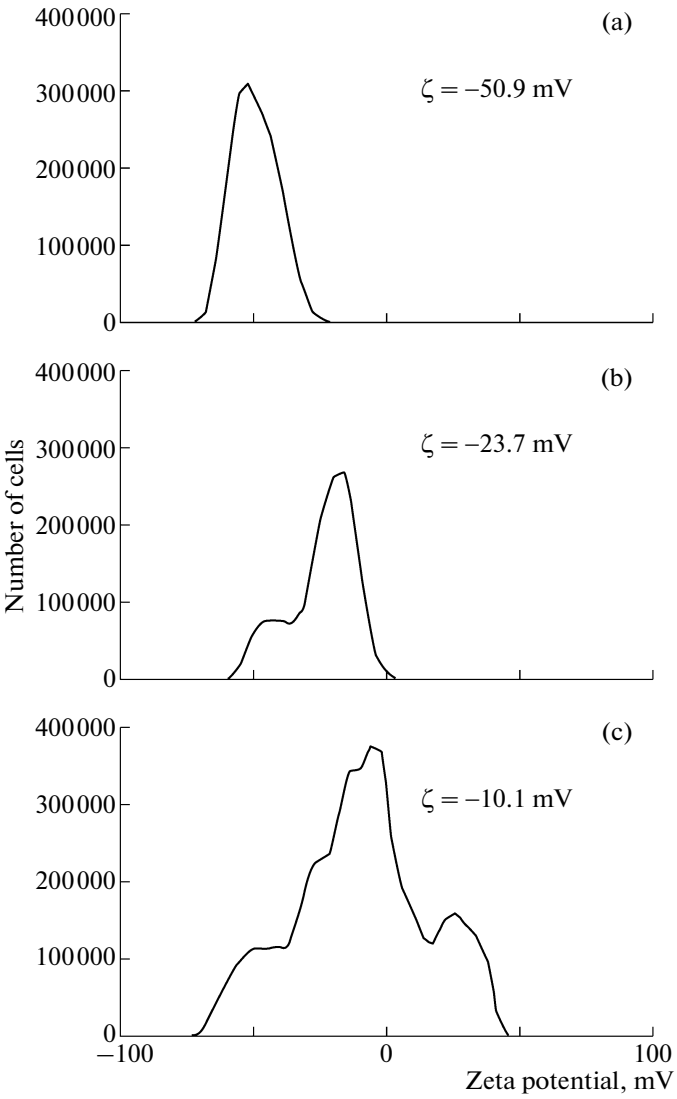

Fig. 2. *E. coli* K12 TG1 cells zeta-potential after (a) 0 min, (b) 30 min, and (c) 90 min of contact with copper nanoparticles.

Effect of copper nanoparticles on the bioluminescence level (EC_{50}) and growth (LD_{50}) of the *E. coli* K12 TG1 sensor strain cells with cloned *P.leiognathi luxCDABE* genes

Parameter	Exposure time		
	30 min	60 min	90 min
Bioluminescence inhibition (EC_{50}, μM)	160 ± 25	77 ± 11	51 ± 9
Bactericidal effect (LD_{50}, μM)	550 ± 74	260 ± 30	165 ± 12

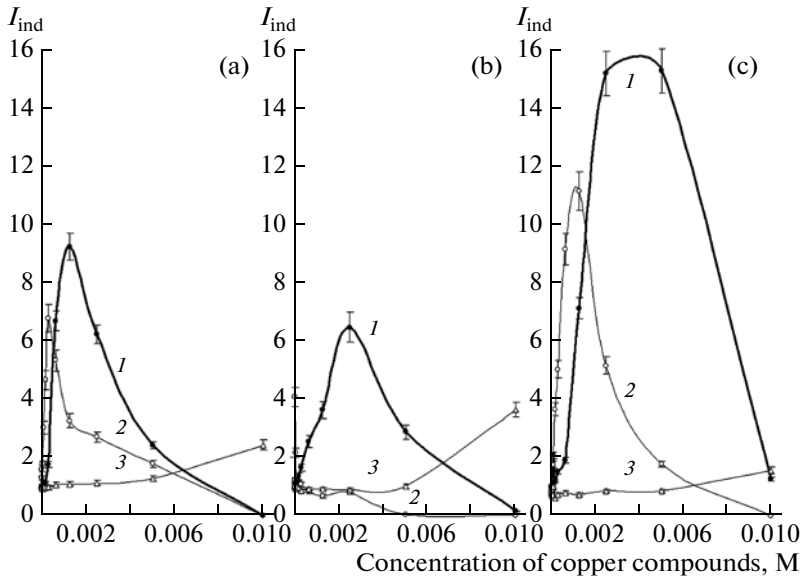

Fig. 3. Ratio of the luminescence induction (I_{ind}) of the *E. coli* K12 MG1655 reporter strains carrying (a) *pSoxS::lux*, (b) *pKatG::lux*, and (c) *pRecA::lux* plasmids upon contact with copper (*1*) nanoparticles, (*2*) ions, and (*3*) microparticles.

PkatG promoters as a response to superoxide anion and hydrogen peroxide, respectively (Figs. 3a, b). The more pronounced induction of the s*oxS::lux* construct ($I_{ind} = 9.23 \pm 0.46$) in comparison with *katG::lux* ($I_{ind} = 6.49 \pm 0.33$) in the process can be used as evidence of the primary formation of the superoxide anion potentially emerging on the electron transfer from the electrically conductive copper nanoparticle to the molecular oxygen followed by the spontaneous dismutation to hydrogen peroxide. On the other hand, the data presented in Fig. 3 indicate that the ability for oxidative stress induction in microorganisms is not exclusive for nanoCu; it is typical of other tested copper compounds. The significant features of this activity are that, in the presence of Cu^{2+} ions, the transcription of gene reporters under PsoxS and PkatG promoters was induced at lower concentrations of this metal (0.02–1.25 mM) and in the presence of copper microparticles it was introduced at higher ones (5–10 mM); but the maximum luminescence induction was 1.5- to 3-fold lower than in the case of nanoCu.

In the conclusion of this series of experiments, it was established using the *E. coli pRecA::lux* reporter strain, which reacts with the luminescence induction to the development of the SOS-response, that the copper nanoparticles produced this effect on the target bacterial cells as a result of DNA damage by the reactive oxygen species that formed during oxidative stress (Fig. 3c). The luminescence development was recorded in a wide concentration range in the process (1.25–5 mM) and it was characterized with the highest induction levels ($I_{ind} = 15.54 \pm 0.77$), which made it possible to consider DNA damage as a major mechanism of nanoCu antibacterial activity. The comparable induction level of the *recA::lux* reporter construct upon exposure to Cu^{2+} ions but at lower level of oxidative stress can be due to the development of Fenton and Haber–Weiss reactions that effectively generate hydroxyl radicals with the pronounced ability to intensely damage the genetic apparatus of the target bacterial cells. On the other hand, the reaction was minimal upon contact with copper microparticles, which defined the weak activity of these compounds in the used system of bacterial tests.

DISCUSSION

The antibacterial effect of copper nanoparticles is a well-documented fact [3, 5–8]. This statement fully applies to the ultradisperse copper particles produced with the Gen–Miller method [14] and, hence, suggested as a wound-healing treatment [4]. However, the mechanism of its development that potentially defines the selective or, on the contrary, nonselective effect of nanoCu on the prokaryotic cells to a great extent remains unclear.

The results obtained in this work allow us to consider the electrostatic interaction between the negatively charged bacterial surface of the *E. coli* K12 TG1 cells with $\zeta = -50.0 \pm 9.35$ mV and the positively charged nanoCu particles with the moderate zeta potential value ($\zeta = +15.9 \pm 8.63$ mV), which in an aqueous solution are present in an aggregated form of several tens of particles, as a "triggering" moment of the development of the antibacterial effect. A similar interaction could be possible for other metallic nanoparticles that carry a positive surface charge in neutral water solutions [19] and for both prokaryotic and eukaryotic cells characterized with negative zeta potentials that are different in value but identical in sign.

The first manifestation of nanoCu biological activity in this system could be the disruption of energy metabolism in the target bacterial cells, which causes the fast-developing inhibition of the *E. coli* K12 TG1 luminescence as well as of other biosensors with a constitutive character of luminescence [5, 6]. The direct cause of this effect could be the decrease in transmembrane electrochemical potential of the cytoplasmic membranes contacted with nanoCu particles (Fig. 4), followed by the disruption of energy and substrate supply of the bacterial luminescence reaction. At the same time, the results indicate the similarity of this mechanism for various copper compounds with nanoparticles being less effective than Cu^{2+} ions but superior to microparticles of this metal.

The bacteriostatic effect could be an obvious consequence of energy-metabolitic disruption; however, a deeper lethal damage of the target bacterial cells as a result of the biological activity of copper nanoparticles was observed in the current and previous studies [6–8]. The results indicated a certain delay in the bactericidal effect relative to the disruption of the cell-energy pathways, which necessitated the suggestion of the existence of additional factors leading to the mentioned final manifestation of nanoCu biological activity.

Luminescent reporter *E. coli* strains carrying inducible by the specific damaging actions genetic constructs were the tools that offered a solution to this problem. The use of *E. coli pSoxS::lux* and *pKatG::lux* in the specified experimental conditions made it possible to detect the oxidative stress in the target bacterial cells that was supposedly defined by the electron transfer from the integrated with the cytoplasmic membrane copper nanoparticles to molecular oxygen, followed by the dismutation of the superoxide anion into hydrogen peroxide (Fig. 4). At the same time, this mechanism could aggravate the deterioration of the energy metabolism in bacterial cells upon contact with nanoCu by interfering with the electron transport (respiratory) chains.

Finally, using the *E. coli pRecA::lux*, the DNA damage by the ROS was identified as a direct cause of death for the model organism. The results obtained in the process testified in favor of the existence of the same mechanism for all the compared copper compounds, which was the most pronounced for nanoCu particles.

Hence, the results necessitate a consideration of the observed biological activity of copper nanoparticles not as much as a demonstration of their antibacterial properties, but more as a manifestation of the general toxic properties achieved with regards to the biological objects of different organization [2, 5]. As a consequence of this, recommendations for the practical application of nanoCu in medicine and veterinary medicine [4, 8] seem premature even considering the differences in the toxicity levels for microorganisms and mammals [18] determined with chemotherapeutic indexes. The nature of the nanoCu biotoxicity characterized simultaneously was proven to be similar to that of TiO_2 and ZnO nanoparticles [12, 20], which favors a significant similarity of the mechanisms of biological activity of various metal nanoparticles and their oxides and allows for an explanation of the biological effects of nanosized metal admixtures in the composition of other nanomaterials, including carbon-based nanomaterials [21].

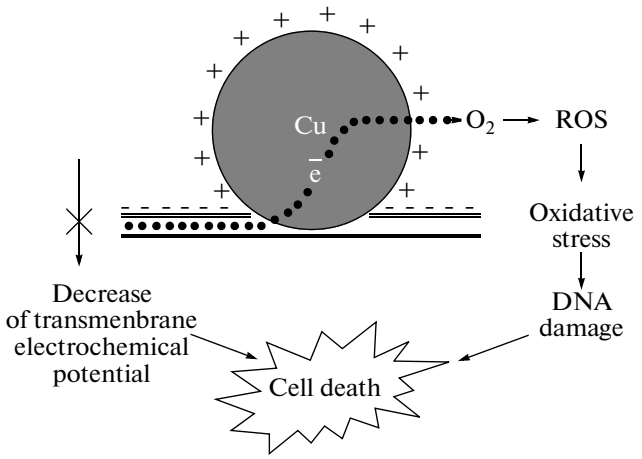

Fig. 4. Predicted mechanisms of antibacterial activity of copper nanoparticles (explanations in text).

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