

Comparative Characteristic of Toxicity of Nanoparticles using the test of Bacterial Bioluminescence

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This article is devoted to the study of the luminescence intensity of the recombinant strain of *Escherichia coli* with cloned gene *luxCDABE* of the natural marine microorganism *Photobacterium leiognathi* when exposed to 20 equimolar concentrations (4 M – 6×10^{-6} M) of nanoparticles of zinc and copper along with their alloys and mixtures. The levels of toxicity based on the EU50 values decreased in the following order: Zn → Cu-Zn (alloy) → Cu-Zn (mixture) → Cu^b → Cu^a. Small-sized nanoparticles of zinc and copper had the most severe toxic effects. Nanoparticles of Cu-Zn alloy and mixture have intermediate toxicity due to the leveling of the toxic effect of zinc and its potentiation of copper and provide evidence for the possibility of creating innovative antibacterial drugs based on nanoparticles of copper and zinc because of their low toxicity and prolonged periods of antibacterial effect. The combined use of zinc and copper reveals the potential of antimicrobial copper to reduce the toxic effects of zinc. Acting as a functional antagonist of copper, the presence of zinc in the nanoparticle inhibits the peroxidation of lipids and generates antioxidant activity, which seems promising.

Key words: Bacterial bioluminescence, *Escherichia coli* K12 TG1, *Photobacterium. leiognathi* 54D10, toxicities of Zn, Cu nanoparticles.

The intensive development of nanotechnology related to the discovery of the unique properties of nanoparticles indicates the high potential for their wide application in medicine, biology and other fields^{1, 2, 3}. However, one factor that inhibits the application of nanoparticles is the evaluation of their biological safety^{4, 5, 6}. At present, many works are devoted to the analysis of the toxic effects of nanoparticles, particularly metal nanoparticles, and the cytotoxicity of silver and zinc nanoparticles has been established^{7, 8, 9, 10}. Although the safety of certain nanomaterials has already been assessed¹¹⁻¹⁵, information on nanoparticle toxicity, including the effects of size, dose and time interval

of existence, for different nanoparticles is not sufficient. The lack of a detailed evaluation of biological safety in parallel with the proven possibility of their use highlights the need to solve these problems. One universal tool in achieving these goals is a bioluminescent method of analysis using luminescent bacteria¹⁶. This method combines different types of sensitive cellular structures with rapidity, objectivity and quantitative nature of the detected response to the assessed influence¹⁷⁻²⁰. In this context, the objective of this study was to comparatively study the toxicities of copper and zinc nanoparticles and their alloys using inhibition test of bacterial bioluminescence (*Escherichia coli*). It is recommended for medical and biological evaluation of nanomaterials by the current national standard^{21, 22}.

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MATERIALS AND METHODS

The genetically engineered luminescent strain *E. coli* K12 TG1 was used; this strain was engineered to constitutively express the luxCDABE genes of the natural marine microorganism *Photobacterium leiognathi* 54D10 and was produced by Immunotech (Moscow, Russia). In prior studies, the strain *Escherichia coli* K12 TG1 was restored by the addition of chilled distilled water. The suspension of bacteria was maintained at +2-4 °C for 30 min, after which the temperature of the bacterial suspension was brought to 15-25 °C.

The inhibition of bacterial luminescence was tested by placing the cells in 96-well plates containing the test substance and the suspension of luminescent bacteria in a 1:1 ratio. Subsequently, the tray was placed in the measuring unit of an Infinite PROF200 microplate analyzer (TECAN, Austria), which dynamically registered the luminescence intensity for 180 min at intervals of 5 min.

The effects of the nanomaterials on the intensity of bacterial bioluminescence (*I*) were evaluated using the formula:

$$I = \frac{I_{k_{0min}} \times I_{o_{nmin}}}{I_{k_{nmin}} \times I_{o_{0min}}} \quad \dots(1)$$

where *I_k* and *I_o* are the illumination intensities of the control and experimental samples, respectively, from the 0-th and *n*-th minutes of measurement. Three threshold levels of toxicity are taken into account:

1. less than 20 – sample is “non-toxic” (luminescence quenching ≤ 20 %);
2. from 20 to 50 – sample is relatively toxic (luminescence quenching 50 %);
3. equal to or greater than 50 – sample toxic (luminescence quenching ≥ 50 %).

Commercial samples of metal nanoparticles from Advanced Powder Technology, Russia were used (Table 1).

The metal nanoparticle samples were assessed (particle size, polydispersity, volume, quantitative content of fractions, surface area) by electron scanning, transmission and atomic force microscopy using the following equipment: a LEX T OLS4100, a JSM 7401F and a JEM-2000FX, respectively («JEOL», Tokyo, Japan).

The size distribution of particles was investigated using a Brookhaven 90Plus/BIMAS and ZetaPALS Photocor Compact (Russia) in lysols after dispersing the nanoparticles using an ultrasonic disperser UZDN-2T (Russia) at f-35 kHz, N 300 W, and A-10 μa for 30 min. The toxic effects of the metal nanoparticle samples were evaluated under a wide range of equimolar concentrations (4 - 6×10⁻⁶ M).

All experiments were done in triplicate and processed by variation statistics using the software package Statistika V8 (StatSoft Inc., USA).

RESULTS

The results obtained characterize the dynamics of inhibition of bacterial bioluminescence over time and demonstrate pronounced dependence on the nature of the investigated nanomaterials along with their forms and concentrations.

Characterization of the toxicities of copper nanoparticles with different sizes

The contact of *E. coli* with increasing concentrations of Cu^a nanoparticles in the range of 0.1 to 0.05 M leads to a complete suppression of illumination in the test object in the first 50-70 minutes of contact. This could be interpreted as a manifestation of the severe acute toxicity of the substance (Figure 1A). Subsequent dilutions of suspensions of Cu^a nanoparticles at concentrations ranging from 0.025 to 0.000625 M did not completely suppress bioluminescence. Luminescence inhibited (50-70%) after 130...180 min of contact with the microorganism. As a result, the concentration of 0.003125 M caused only 20-30% luminescence quenching after 175 min, demonstrating a weak toxic effect. Breeding from 0.00156 to 0.000195 M had no effect on the values of bioluminescence in comparison with the control sample, indicating a non-toxic dose.

The development of similar toxic effects for Cu^b nanoparticles required more contact time with the test organisms at the same concentrations (Figure 1B). Thus, suspensions of copper nanoparticles (Cu^b) at 0.1 and 0.05 M (P < 0.05) led to suppressed luminescence after 70 and 110 min of contact. Unlike Cu^a nanoparticles, 0.0125 M Cu^b nanoparticles also resulted in the almost complete suppression of luminescence after 150 min of

contact. Suspensions of 0.00625 M Cu^b nanoparticles caused a 50-70% inhibition of bioluminescence, whereas 0.003125 and 0.001563 M (P <0.05) concentrations resulted in relative inhibitions ranging from 20 to 40% (practically non-toxic). Further dilution from 0.000781 to 0.000195 M had no significant effect on bacterial bioluminescence in comparison with the control. The dynamics of the suppression could be interpreted as a manifestation of severe acute toxicity of this sample. The copper nanoparticles with a pronounced toxic effect had sizes of approximately 55 nm.

Characterization of the toxicities of mixtures and alloys of Cu and Zn nanoparticles

Compared to the control, Cu-Zn (alloy) nanoparticles completely inhibited luminescence at concentrations ranging from 0.1 to 0.003125 M in the first 10 to 60 minutes of contact and at concentrations from 0.00156 to 0.000781 M (P <0.05) after 110 to 150 minutes of contact (Figure 2A).

Cu-Zn (alloy) nanoparticle suspensions diluted to concentrations ranging from 0.000391 to 0.00009 M (P <0.05) produced 50-80% quenching of the luminescence of the bacteria, showing

thorough toxic properties after 130-160 min of contact. The Cu-Zn (alloy) nanoparticles had no toxic effect at a concentration of 0.00004 M throughout the measurement time.

In contrast to the Cu-Zn (alloy) nanoparticles, the mixture of Cu-Zn prepared with the same proportion of elements in some doses was significantly more toxic and characterized by complete suppression of luminescence of bacteria in a short period of time (from 10 to 25 minutes) at much lower concentrations from 0.1 to 0.000391 M (P <0.05) compared with control (Figure 2B). For concentration of at 0.000195 M, 50 to 70 % inhibition of bacterial luminescence and an average level of toxicity were observed. However, the subsequent dilution of the mixture of nanoparticles of Cu-Zn to concentrations ranging from 0.00009 to 0.00004 M did not cause significant changes in bioluminescence compared to the control values; these mixtures were not toxic.

Characterization of the toxicity of zinc nanoparticles

Zinc nanoparticles at concentrations ranging from 0.1 to 0.000195 M (P <0.05) completely suppressed the luminescence of bacteria compared

Table 1. Nanoparticle characterization

The name of the nanoparticles	Size, nm	Chemical and phase composition	Method of production
Cu ^a	97	crystal copper: 96,0 ± 4,5 %, copper oxide: 4,0 ± 0,4 %;	High-temperature condensation with modification by oxygen
Cu ^b	55	Cu: 99.7%, O ₂ less 0,3%	Plasma-chemical synthesis
Zn	90	Zn: 97 %, the rest: sorbed gases, ZnO and H ₂ O.	The electric explosion of wire in an argon atmosphere
CuZn (alloy)	65	Cu: 60 %, Zn:40%	The electric explosion of wire in an argon atmosphere
Cu-Zn (mixture)	Cu 55Zn 90	Cu: 60 %, Zn: 40%	Gas-phase

Table 2. Values of EC50 (M) for the test organism *E. coli* K12 TG1 with cloned luxCDABE genes of *P. leiognathi* 54D10 contacting nanoparticles of copper and zinc along with their alloy and mixture

Nanoparticles	Size, nm	The duration of exposure, min		
		60	120	180
Cu ^a	97	0.006±0.003	0.006±0.001	0.006±0.0002
Cu ^b	55	0.003±0.004	0.003±0.002	0.003±0.0001
Zn	90	0.00004±0.000003	0.00004±0.000001	0.00004±0.000001
Cu-Zn (alloy)	65	0.00009±0.000002	0.00009±0.000005	0.00009±0.000001
Cu-Zn (mixture)	Cu 55Zn 90	0.0002±0.00004	0.0002±0.00004	0.00009±0.000002

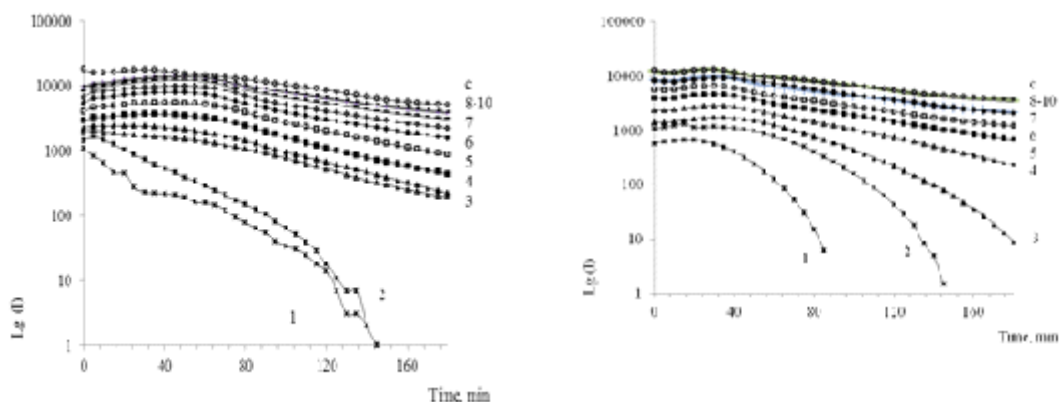


Fig. 1. Dynamics of luminescence of *E. coli* K12 TG1c with cloned luxCDABE genes of *P. leiongnathi* 54D10 in contact with different concentrations of nanoparticles of Cu^a (A) and Cu^b (B): 0.1 M (1); 0.05 M (2); 0.025 M (3); 0.0125 M (4); 0.00625 M (5); 0.003125 M (6); 0.001563 M (7); 0.000781 M (8); 0.000391 M (9); 0.000195 M (10); the control

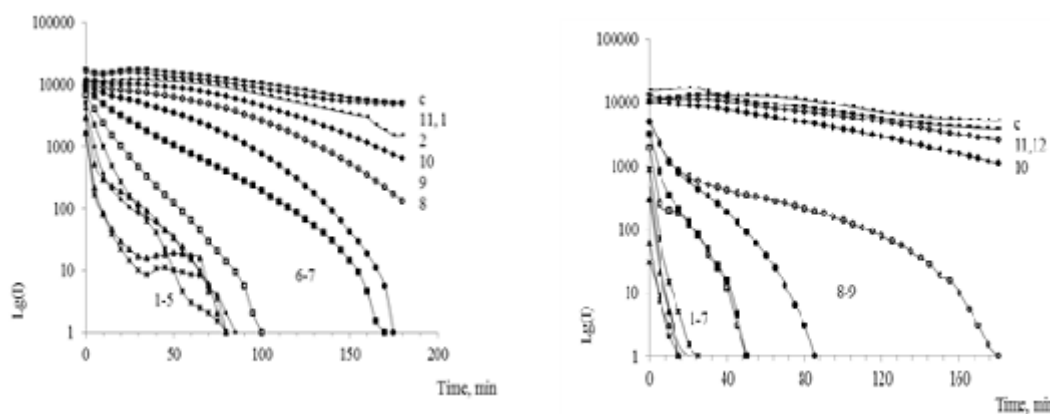


Fig. 2. Dynamics of luminescence of *E. coli* K12 TG1c with cloned luxCDABE genes of *P. leiongnathi* 54D10 in contact with Cu-Zn (alloy) nanoparticles (A) and a mixture of Cu-Zn nanoparticles (B) at concentrations of 0.1M (1); 0.05 M (2); 0.025 M (3); 0.0125 M (4); 0.00625 M (5); 0.003125 M (6); 0.001563 M (7); 0.000781 M (8); 0.000391 M (9); 0.000195 M (10); 0.00009 M (11); 0.00004 M (12); and C - the control

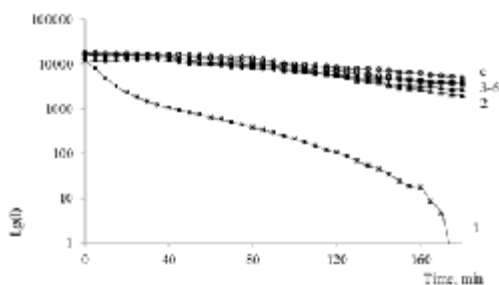


Fig. 3. Dynamics of luminescence of *E. coli* K12 TG1c cloned luxCDABE genes of *P. leiongnathi* 54D10 in contact with zinc nanoparticles with concentrations of 0.000195 M (1); 0.00009 M (2); 0.00004 M (3); 0.00002 M (4); 0.00001 M (5); 0.000006 M (6); and C - the control

to the control sample. It was necessary to prepare additional dilutions with concentrations ranging from 0.000195 to 0.000006 M. The contact of zinc nanoparticles in the concentration range of 0.00009 to 0.00004 M with *E. coli* K12 TG1 with cloned luxCDABE genes of *P. leiongnathi* 54D10 results in 50-70% inhibition of luminescence after 70 min compared to the control; thus, these solutions are classified as toxic (Figure 3). Nanoparticle suspensions with concentrations of 0.00002 and 0.00001 M ($P < 0.05$) cause 20-50% quenching of luminescence and are classified as relatively or weakly toxic; further dilutions are not toxic.

Dependence of dose-response

The above results were used to construct dose-response curves (Figure 4) for each of the tested nanoparticle samples. The EU50 values corresponding to the molar concentrations causing 50% inhibition of bacterial bioluminescence compared with the control at different durations of exposure were also determined (Table 2).

The results show that copper nanoparticles with different sizes clearly have different toxicities. Cu^a nanoparticles are one order of magnitude less toxic than Cu^b nanoparticles (Table 2). A 50% inhibition of bioluminescence requires a two-fold higher concentration of Cu^a nanoparticles compared to Cu^b nanoparticles.

According to the results of the EU50 calculation (after the contact with Cu-Zn mixture) the minimum concentrations causing 50% inhibition of luminescence were determined after

60 min (0.0002 M). It is 2.2 times less in relation to the alloy. If the period of contact is longer (up to 180 min), indicators EU50 match. A peculiar biological activity of the Cu-Zn nanoparticles alloy (alloy) was characterized by EC50 values after 60 min of contact; they were 0.00009±of 0.00002 M. These values do not change after the increase of contact time. So, the activity of this alloy is less toxic compared to the mixture of Cu-Z nanoparticles.

The analysis of the EU50 values showed that the biological activity of zinc nanoparticles manifests at a minimal concentration of 0.00004 M to obtain a 50% quenching of luminescence at all stages of contact.

Based on similar calculations of EU50, the levels of toxicity of the different nanoparticle samples to the genetically engineered luminescent strain of *E. coli* decrease in the following order: Zn → Cu-Zn (alloy) → Cu-Zn (mixture) → Cu^b → Cu^a.

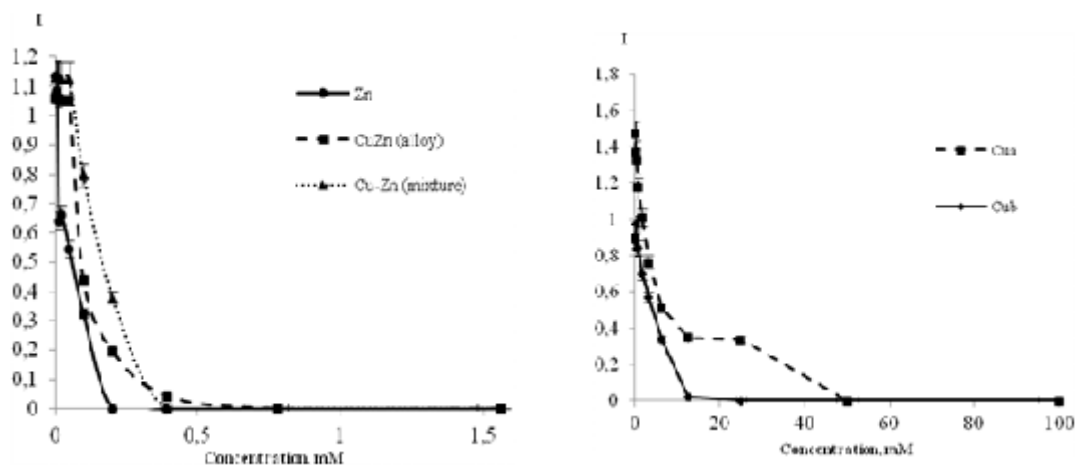


Fig. 4. The relative values of luminous intensity for a luminescent strain of *E. coli* in contact with nanoparticles. The ordinate is the relative value of luminescence intensity in comparison with the control

DISCUSSION

The main parameters that determine the toxicities of nanoparticles include chemical composition, size and shape²³. In this study, nanoparticles with dimensions ranging from 55 to 97 nm were studied.

By comparing two different samples of copper nanoparticles, the smaller-sized nanoparticles were found to be more toxic. The biological activity of the nanoparticles increased with decreasing particle size. According to literature

data, these changes are explained by differences in the properties of individual particles and their clusters, the degree of correlation of the geometric structure and the electronic structural shell in the interaction with the biological object²⁴. A similar dependence of biological activity on nanoparticle size has been described for a number of nanoparticles²⁵⁻²⁸.

Thus, the 55-nm Cu^b nanoparticles caused 100% suppression of luminescence in a series of dilutions to concentrations of 0.0125 M, 50 and 20% suppression in concentrations of 0.003

and 0.002 M; in contrast, 97-nm Cu^a nanoparticles exhibited lower toxicity, resulting in 100% inhibition of bioluminescence at concentrations ranging from 4 to 0.025 M. Dilutions that had no significant impact on the luminescence of bacteria and can be characterized as biotic doses began with 0.00078 M and below. When the study of the copper nanoparticles toxicity on mammals²⁹, it was found out that it exceeds EC50 values for microorganisms, that makes it possible to use them as antibacterial drugs.

Possible approaches for understanding the mechanism of toxic action of copper nanoparticles on *E. coli* are associated with increasing electron charge density in the outer membrane of *E. coli* in contact with copper nanoparticles. This is correlated with their ability to inhibit the growth of bacteria and lower the activation energy of electron transfer at the site of nanoparticle-*E. coli* contact. The analysis of these data allows us to adjust conditions for obtaining samples of nanoparticles with desired properties of oxide film.

In its turn, electrostatic contact between the positively charged aggregates of copper nanoparticles ($\alpha = +15.9 \pm 8.63$ MB) with the negatively charged surface of *E. coli* K12 MG1655 pSoxS::lux and pKatG::lux with the inducible nature of the illumination ($\alpha = -50.0 \pm 9.35$ mV) showed the development of oxidative stress in model microorganisms. It was presumably determined by the transport of electrons through copper nanoparticles integrated with cytoplasmic membrane to molecular oxygen. The final result of this process was damage to DNA by reactive oxygen species. It was detected using the reporter strain *E. coli* pRecA::lux leading to the development of bactericidal effect³⁰.

Zinc nanoparticles also inhibited bioluminescence, although several orders of magnitude lower concentration were required to achieve an inhibitor effect similar to that of copper. In the range of concentrations with the highest toxicity against *Escherichia coli* K12 TG1 were observed in concentrations from 4 to 2×10^{-5} . This toxicity can be characterized as acute and able to cause complete suppression of luminescence of the microorganisms even being in very small doses. Our results are consistent with those of other authors who studied the inhibition of

bioluminescence by zinc nanoparticles in comparison with copper. Ko *et al.* (2014)³¹ showed that the toxicity of zinc oxide nanoparticles is greater than that of copper oxide nanoparticles. Mortimer *et al.* (2008)²⁰ also showed the high toxicity of zinc oxide nanoparticles compared to copper oxide nanoparticles in terms of bioluminescence inhibition.

The combined use of copper and zinc nanoparticles leads to the potentiation of the toxic effect of copper nanoparticles. In the presence of zinc and copper nanoparticles, EU50 increases from 15 times (when compared with Cu (a, b, Cu-Zn mixture, alloy) to 66 times at all stages of contact (i.e., toxicity is characterized by prolonged manifestation). Similar results were obtained when evaluating the effects of copper oxide and zinc nanoparticles on the other test objects such as infusoria and rats. Thus, introducing zinc oxide nanoparticles into the environment of cell line HepG2 modulates the cytotoxicity of copper nanoparticles. The accumulation of a large number of zinc nanoparticles in cells alters cell membranes, and the cytotoxicity of copper nanoparticles increases³². The mechanism of toxic action is associated with an increased intracellular Zn²⁺ concentration, leading to the excessive generation of intracellular ROS, plasma membrane leakage, mitochondrial dysfunction, and cell death along with the increased production of ROS, damage to lysosomal membranes, and activation of caspase-3 and caspase-7, eventually leading to apoptosis³³.

Alloy and mixture of metal antagonists of Zn and Cu show less toxicity compared to individual testing Zn.

CONCLUSION

The combined use of zinc and copper reveals the potential of antimicrobial copper to reduce the toxic effects of zinc. Acting as a functional antagonist of copper, the presence of zinc in the nanoparticle inhibits the peroxidation of lipids and generates antioxidant activity, which seems promising.

Thus, the level of toxicity of the nanoparticle samples that was characterized by EU50 values using genetically engineered luminescent strain of *E. coli* decreased in the series: Zn → Cu-Zn (alloy) → Cu-Zn (mixture) → Cu^b →

Cu^a. The toxicity of copper nanoparticles is higher in particles with smaller sizes and has a prolonged effect.

Nanoparticles of Cu-Zn alloy and mixture have intermediate toxicity due to the leveling of the toxic effect of zinc and its potentiation of copper and provide evidence for the possibility of creating innovative antibacterial drugs based on nanoparticles of copper and zinc because of their low toxicity and prolonged periods of antibacterial effect.

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