

TOXICITY OF SiO_2 , TiO_2 AND CeO_2 NANOPARTICLES EVALUATED USING THE BIOLUMINESCENCE ASSAY

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ABSTRACT: The development of technology and industry has made available a variety of different nanomaterials. Particularly popular in practical use have been silicon dioxide nanoparticles and titanium and cerium dioxides. However, it is necessary to evaluate the biological activity of nanoparticles from the point of view of toxicology. Despite the active use of nanoparticles of silicon dioxide and dioxides of titanium and cerium, little is known about their toxic effects on living organisms. This work presents a study of three various nanoparticles of silicon dioxide and dioxides of titanium and cerium. For investigations of nanoparticles morphology and their contact with *E. coli K12 TG1* cells, suspensions alone or previously mixed with nanoparticles, were applied to freshly prepared mica. To assess the activity of the nanoparticles against *E. coli K12 TG1* cells, we used a previously described version of bioluminescent analysis. TiO_2 particles having smaller dimensions were characterized by a higher toxicity compared to SiO_2 . These results characterized TiO_2 (I) as toxic and confirm the statement that size matters. Metal particles of CeO_2 in the studied concentrations of 0.1–0.000195 M did not cause changes in the dynamics of bacterial bioluminescence. The results were confirmed by studies carried out using lux-biosensors: *E. coli K12 MG1655 pkatG::lux* (for the detection of peroxide of hydrogen) and *E. coli K12 MG1655 psoxS::lux* (for the detection of superoxide anion)) for the assessment of oxidative damage.

Keywords: Bacterial bioluminescence, Nanoparticles, Silicon dioxide, Dioxide of titanium, Cerium dioxide.

1. INTRODUCTION

The development of chemical technology and industry has made available a variety of different nanoparticles and nanomaterials. In the nanoscale state, many substances acquire new properties and become very active biologically [1]. This opens up new opportunities for using nanomaterials in the fields of biomedicine, pharmacology, food production, and in solving environmental and agricultural problems. At present, the annual volume of industrial production of various nanoparticles is already hundreds of thousands of tons [2]. Particularly popular in practical use are silicon dioxide nanoparticles and titanium and cerium dioxides [3]. These nanoparticles represent a group of nanomaterials with promising areas. Thus, the bulk of the produced titanium dioxide nanoparticles are used in the paint industry, as a microdispersed food additive known as E171 [4]. Nanoparticles of titanium dioxide are known for their antibacterial and photocatalytic properties. They are used in cosmetics (toothpaste and sunscreens), as well as components of plastic and textile materials [5]. Nano-sized particles of cerium dioxide (CeO_2) are widely used due to their unique physical and chemical properties (mechanical hardness, chemical inertness, heat resistance, high oxygen conductivity,

etc.). These substances are used in catalysis, as components of solar cells [6], fuel cells [7], and also find application in luminescent converters, abrasives, gas sensors, etc. [8].

However, it is necessary to evaluate the biological activity of nanoparticles from the point of view of toxicology. Despite the active use of nanoparticles of silica and dioxides of titanium and cerium, little is known about their toxic effects on living organisms. At present, the use of recombinant luminescent microorganisms is a popular method to evaluate ecological, sanitary and toxicological, and other conditions [9]. The basis of their practical application is the principle of assessing the activity of the luminescence system. This is integrally responsive, showing a decrease of luminescence intensity with the appearance in the test environment of chemical toxicants, or the display of toxic properties of the newly synthesized substances and compounds [10,11]. The advantages of this method are speed, high sensitivity and good correlation with results obtained using more complex methods of biotesting [12].

In addition, taking into account the active use of these particles in industry, it is necessary to develop rapid and sensitive methods for the detection, identification and quantification of these nanomaterials in environmental objects and food

products. The goal of this work was to study the toxic properties of nanoparticles of silicon dioxide and dioxides of titanium and cerium as promising

nanomaterials using a bioluminescent assay that has not previously been involved in studies of these nanoparticles.

2. MATERIAL AND METHODS

2.1 Sample preparation

In this work, three different nanoparticles were examined (Table 1). These nanoparticles were manufactured by Advanced Powder Technologies LLC, Russian Federation).

For performing the investigation, the nanoparticles were dispersed for 30 minutes in isotonic or distilled water using an ultrasonic disperser (f-35 kHz, N-300 W, A-10 μ A).

Table 1. Characteristics of the metal nanoparticles according to the manufacturer.

Nanoparticles	Diameter, nm	Chemical and phase composition	Method of synthesis	Specific surface area (S_{sp} , M^2/g)
SiO ₂	40.9	SiO ₂ : 99.8 %; Cl ₂ : <0.2%	Gas-phase	55.4
TiO ₂	90	TiO ₂ : 99.8% mass. Cl ₂ : <0.2%	Plasma-chemical synthesis	16.5
CeO ₂	15.8	CeO ₂ : 99.8%	Gas-phase synthesis	49.6

2.2 Atomic-force microscopy investigation

For the investigations of the nanoparticles morphology and their contact with bacterial cells, aliquots (20 μ L) of nanoparticle aqueous suspensions were applied to freshly prepared mica. The samples were incubated at 93% relative humidity and 20–22°C and scanned with the atomic force microscope Certus V Light (Nanoscan technology, Russian Federation) equipped with cantilevers NSG 10 (nominal spring constant 37.6 N/m; curvature radius < 10 nm) (Tips Nano, Estonia). The samples were scanned in air using the tapping mode.

2.3 Zeta-potential measurements

The size and zeta-potential of nanoparticles dispersed in aqueous suspensions were assessed with a laser autocorrelation analyzer Photocor (Photocor, Russian Federation). The samples were placed in 10 × 10 × 45 mm polystyrene cuvettes and were illuminated by a 633 nm helium-neon laser. The light scattering was measured at an angle of 90° and the particles size distribution was calculated from the diffusion coefficient according to the Smoluchovski equation. The average diameter ± width (nm) of the nanoparticle aggregates in aqueous suspensions was calculated according to the volume size distribution data by using the software of the instrument.

2.4 Bioluminescent toxicological assay

The biological activity of the nanomaterials was tested involving lux-biosensors with constitutive and inducible characteristics of bacterial luminescence. *Escherichia coli* strain *K12 TG1 pF1* (Ecolum, Russia) was used in the constitutive method of fluorescence.

To assess the activity of the nanoparticles against *E. coli K12 TG1 pF1* cells, we used a previously described version of bioluminescent analysis for carbon-based nanomaterials [13]. Doing similar biotesting, we rehydrated commercially available freeze-dried products lux-biosensors *E. coli K12 TG1 pF1* (Ecolum, Russia) by adding 1.5% solution of NaCl or distilled water chilled up to 8°C, after which we kept them at a temperature of 2–4°C for 30 minutes. Briefly, aqueous suspensions of nanoparticles (μ g/ μ L) were added to the wells of a Microplate 2+ microplate with non-transparent side walls (Thermo, USA), wherein they were further doubly diluted in sterile deionized water, from 1:1 to 1:1024, up to a final volume of 50 μ L. To the filled wells were then added 50 μ L of a previously prepared suspension of constitutively luminescent *E. coli K12 TG1 lac::luxCDABE* cells. Wells filled with sterile deionized water and containing an appropriate amount of bacterial biosensor were used as negative controls. *E. coli K12 MG1655 pKatG::lux* (for detection of peroxide of hydrogen) and *E. coli K12 MG1655 psoxS::lux* (for detection of superoxide anion) strains were used in the inducible method of bacterial luminescence. These strains were generously provided by Doctor of Biology I.V. Manukhov (GOSNIIGenetika, Russia).

The work used strains grown from LB-broth with 20 µg/ µL of ampicillin at 37° for 16–18 hours. Directly before the experiment the culture was additionally grown in the same fresh substratum at 1:20 and was incubated for 3–5 hours, then it was suspended in 0.5% solution of NaCl to reach OD 450=0.05. The received suspensions at the amount of 50 µL were put into the tablet pits which contained nanomaterials prepared in advance at the amount of 50 µL; the suspensions were kept for 15 min, after which 100 µL of LB-broth were additionally put in each pit.

Bioluminescence measurements were carried out using the Infinite PRO200 (TECAN, Austria) microplate reader, which dynamically registered the luminescence intensity of the samples for 180 min,

3. RESULTS AND DISCUSSION

3.1 Assessment of the silica nanoparticles toxicity

The nanoparticles manufacturer indicates the size of the silicon dioxide in the range of 40.9 nm (Table 1). However, according to dynamic light scattering data, the SiO₂ particles in aqueous suspensions form three fractions with different dimensions (Table 2). In particular, only 23% of the particles dispersed in the aqueous suspension have a diameter 57 ± 8.5 nm, while most have a diameter between 100–200 nm. In turn, the zeta potential of the SiO₂ particles is characterized by a negative value.

Visualization using atomic force microscopy made it possible to characterize the silica particles as

estimated in relative light units (RLU). The data were analyzed using the software provided with the instrument. Quantification of the bioluminescence inhibition index (I) due to nanoparticle toxicity was calculated by the algorithm (1).

$$I = \text{RLU}_{c0} \times \text{RLU}_{tn} / \text{RLU}_{cn} \times \text{RLU}_{t0}, \quad (1)$$

where c and t are the RLU values of the control and test samples at the 0-th and n-th minute of measurement.

All experiments were performed in 3 replicates. The data obtained was processed using a variational statistics method with Microsoft Excel software (Microsoft Corporation, USA) and Statistica V8 (StatSoft Inc., USA).

Table 2. The main physical characteristics of nanoparticle preparations

Samples	Particle distribution, %	Diameter, nm	Zeta-Potential, mV
CeO ₂	45	192 ± 62	-15,98 ± 0,19
SiO ₂	23	57 ± 8.5	-25.13 ± 0.33
TiO ₂	22	105 ± 10,3	-19.40 ± 0.27

large aggregates with an average diameter of about 1.2 ± 0.5 µm (Fig. 1a).

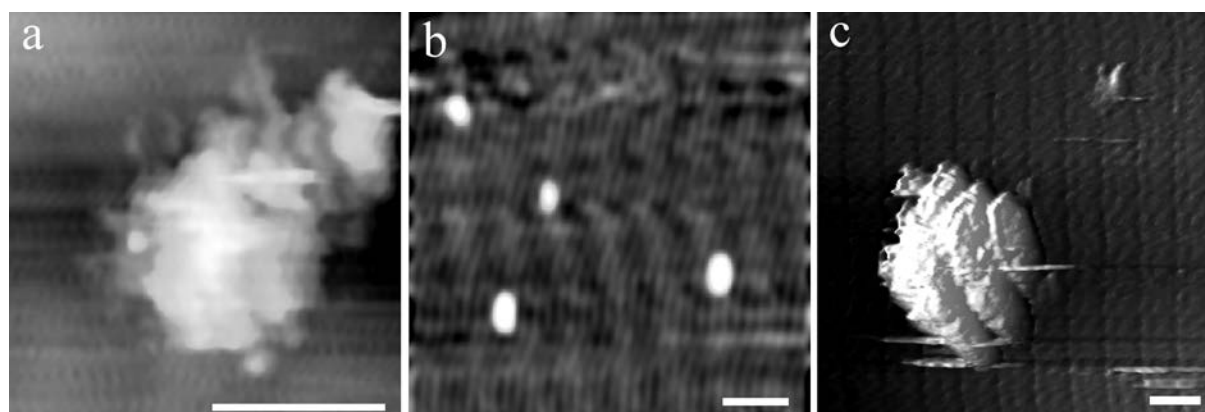


Fig. 1. Microscopy visualization of metal particles dispersed in water and dried on mica. AFM-images of silicon dioxide (a), titanium dioxide (b) and cerium dioxide (c). Bar scale – 500 nm.

The bioluminescence assay performed showed that silicon dioxide in concentrations of 0.1–0.000195 M does not affect the dynamics of bacterial bioluminescence compared to the control.

An increase of the concentration to 4 M led to a 30% inhibition of the bioluminescence; however, such a level characterized this substance as slightly toxic or non-toxic (Fig. 2).

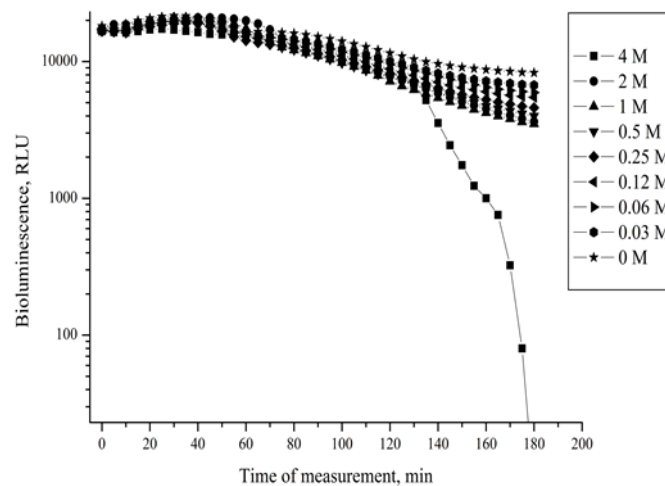


Fig. 2. Dynamics of bioluminescence of *E. coli* K12 TG1 pF1 (Ecolum) treated with SiO₂ nanoparticles taken at various concentrations

The results obtained by using lux-biosensors with the inducible characteristic of bacterial luminescence proved the effects obtained earlier and let us define the nature of the oxidative activity. It was identified that the induction of luminescence

was conditioned by the influence of superoxide anion which was formed as a result of contact of nanoparticles with bacterial cells and it led to the death of the test-organisms at high concentrations of the analyzed material (Fig. 3a, b).

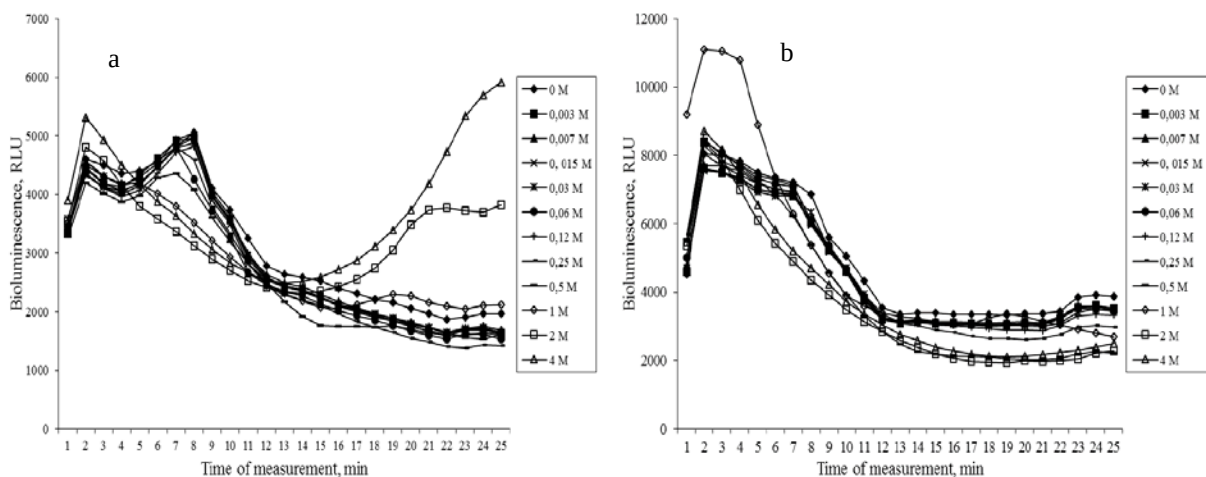


Fig. 3. Dynamics of bioluminescence of lux-biosensors (a – *E. coli* K12 MG1655 psoxS':lux; b – *E. coli* K12 MG1655 pkatG':lux) treated with SiO₂ nanoparticles taken at various concentrations

3.2 Assessment of the titanium dioxide nanoparticles toxicity

The manufacturer of these particles stated a parameter of about 90 nm (Table 1). The measured zeta-potential of titanium dioxide has a low negative value -19.40 ± 0.27 that suggested the tendency of the particles to aggregate [15]. This was confirmed by dynamic light scattering measurements showing three fractions of titanium dioxide in water ranging in size from 105 ± 10.3 nm to more than $1.5 \mu\text{m}$ (Table 2); it was also confirmed by AFM, revealing

the presence of particles of titanium dioxide with a diameter less than 200 nm.

The bioluminescence assay performed showed that titanium dioxide did not have a toxic effect on bacteria at $0.1 \dots 0.000195$ M (Fig. 2b). In turn, increasing the concentrations to 4 M exerted an inhibitory effect on the *E. coli* biosensor (Fig. 4). Thus, the toxicity of the aqueous suspension of TiO₂ particles was manifested at 0.5 M. EC 50 value was reached after 175 minutes of contact with bacteria. But complete suppression of bioluminescence was obtained at 4 M of concentration and after 155 min of co-incubation with the biosensor.

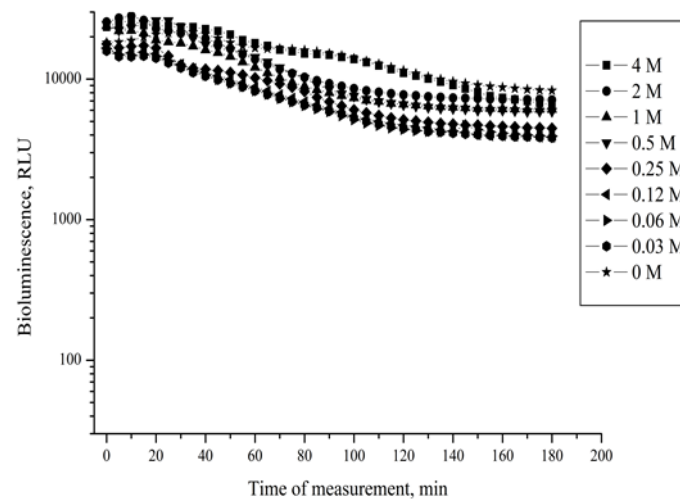


Fig. 4. Dynamics of luminescence of *E. coli* K12 TG1 *pF1* (Ecolum) treated with TiO_2 nanoparticles taken at various concentrations

The experimental study involving *Escherichia coli* K12 MG1655 *pkatG'::lux*, and *Escherichia coli* K12 MG1655 *psoxS'::lux* also did not show the oxidative activity of the analyzed nanoparticles (Fig

5a, b). TiO_2 particles having smaller dimensions were characterized by a higher toxicity compared to SiO_2 . These results characterized TiO_2 (I) as toxic and confirm the statement that size matters.

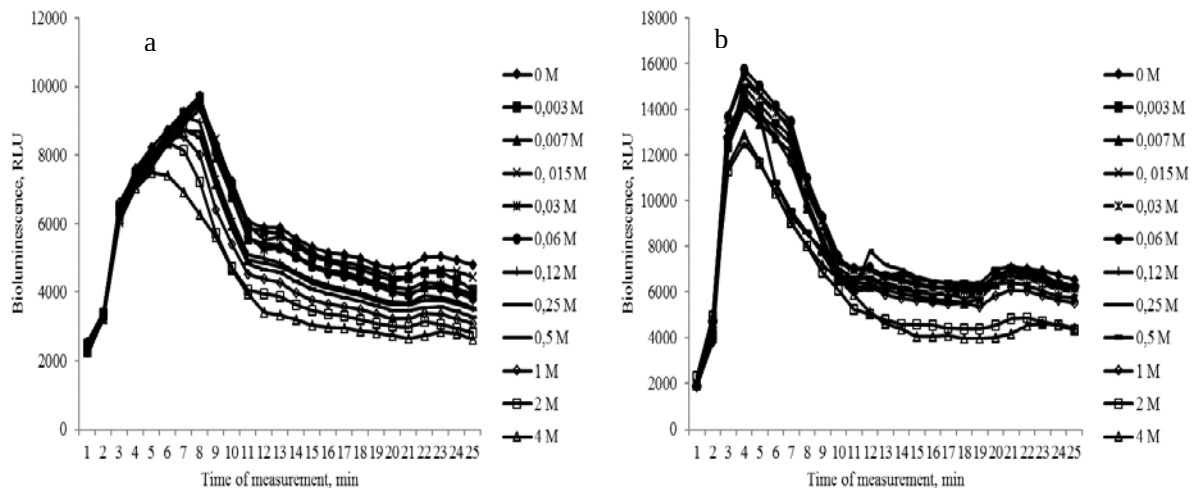


Fig. 5. Dynamics of bioluminescence of lux-biosensors (a - *Escherichia coli* K12 MG1655 *psoxS'::lux*; b - *Escherichia coli* K12 MG1655 *pkatG'::lux*) treated with TiO_2 nanoparticles taken at various concentrations

3.3 Assessment of the cerium dioxide nanoparticles toxicity

The diameter of 15 nm that was declared by the manufacturer could not be fixed either by the DLS-method (Table 2) or by atomic force microscopy (Fig. 1 c). The particles of cerium oxide

dispersed in dH_2O were characterized by aggregates ranging from 192 ± 62 nm to more than $1.5 \mu\text{m}$ in diameter (Table 2). Metal particles of CeO_2 in the studied concentrations of 0.1–0.000195 M did not cause changes in the dynamics of bacterial bioluminescence (Fig. 6).

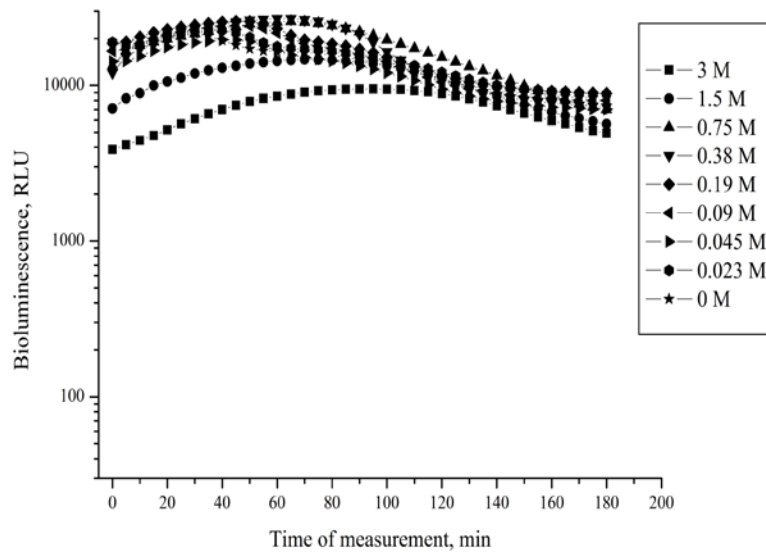


Fig. 6. Dynamics of luminescence of *E. coli* K12 TG1 pF1 (Ecolum) treated with CeO₂ nanoparticles taken at various concentrations

An increase of concentrations up to 4 M did not cause a toxic effect, which characterized cerium dioxide particles as a conventionally safe substance for living organisms.

The use of lux-biosensors with the inducible characteristic of bacterial luminescence proved the data received earlier. Nanoparticles of cerium dioxide did not have the oxidative activity and were neutral to the bacterial cell (Fig. 7 a, b).

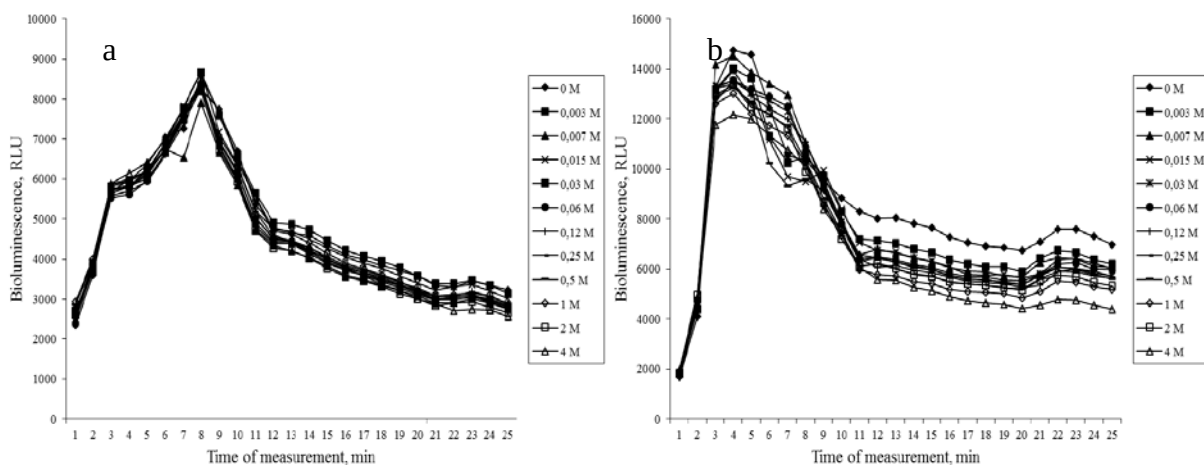


Fig. 7. Dynamics of bioluminescence of lux-biosensors (a – *E. coli* K12 MG1655 psoxS':lux; b – *E. coli* K12 MG1655 pkatG':lux) treated with CeO₂ nanoparticles taken at various concentrations

All nanoparticles have different activity. The results obtained are in agreement with data which revealed the toxicity of silica nanoparticles only in the presence of additional factors such as an auxiliary substance (metatitanic acid) [14], photodynamic inactivation [15] or the simultaneous presence of more toxic nanoparticles (Ag-SiO₂) [16, 17]. The use of silicon dioxide nanoparticles without additional factors does not lead to toxic effects on

cells, and in some cases, in particular when in contact with a bacterial cell, contribute to an increase in microbial activity [18].

The obtained bacterial luminescence data, in particular the values of the toxicological parameter, allowed us to conclude that metal particles of titanium dioxide are a substance of low toxicity. The results are consistent with those of Allard et al. [19,20].

However, exciting data describe titanium nanoparticles as toxic in microdoses. The toxic properties of titanium nanoparticles are manifested with their preliminary photoactivation by ultraviolet. In studies by Kumari et al. [21], Dworniczek et al. [22], Zhukova [23] and other authors, the manifestation of the bactericidal activity of UV-irradiated titanium nanoparticles was demonstrated against gram-positive and gram-negative microorganisms. It is assumed that the main mechanism of action of titanium nanoparticles on the cell is realized through the formation of reactive oxygen species [24].

The toxic properties of cerium compounds depend on particle size and are related to the surface charge density. Direct contact of cerium oxide particles with the bacteria is mediated by electrostatic attraction between the cell wall and positively charged with cerium particles, which will lead to external destabilization of the bacterial membrane and the development of a cytotoxic effect [25-28]. As revealed by DLS, the zeta potential of the sample of CeO₂ used was a negative low value (Table 2) which determines their aggregation and the absence of toxicity for bacteria.

4. CONCLUSIONS

The presented data show that an increase of the concentration of silica led to a 30% inhibition of the bioluminescence. It was identified that the induction of luminescence was conditioned by the influence of superoxide anion which was formed as a result of contact of nanoparticles with bacterial cells. Such a level characterized these nanoparticles as slightly toxic. The bioluminescence assay performed showed that titanium dioxide did not have a toxic effect on the bacteria and its nanoparticles could be described as non-toxic. The presented data from the bioluminescence assay of cerium dioxide did not show a toxic effect, but characterized its particles as a conventionally safe substance for living organisms. Thus, the negative impact of nanoparticles is determined by a number of physical and chemical factors, and the use of reporter microorganisms as models for the implementation of the action of nanoparticles represents a promising direction for creating a generic test system for the evaluation of the biological activity of a number of substances.

5. ACKNOWLEDGMENTS

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