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Bioluminescent Enzyme Inhibition Based Assay of Metal Nanoparticles

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The bioluminescent enzymatic bioassays for assessment of nanomaterial biotoxicity using the soluble or immobilized coupled enzyme system of luminous bacteria NAD(P)H:FMN-oxidoreductase + luciferase (Red + Luc) as a test system were employed in this study. This method specifically detects the toxic properties of substances based on their effect on the parameters of the bioluminescent enzyme reactions. The commercially available metal nanoparticles (MNPs), including silver nanoparticles (Ag), nanoparticles of silicon dioxide (SiO_2), and titanium dioxide (TiO_2), of different sizes were tested in the study. The inhibitory effects of MNPs on the bioluminescent Red + Luc enzyme system were measured. Results indicated that the soluble Red + Luc coupled enzyme system was more sensitive to the inhibition effect of MNPs than its immobilized form. The inhibitory activity of MNPs decreased in the following order: Ag > TiO_2 > SiO_2. That correlated well with results of other biological methods. Due to substantial advantages such as technical simplicity, short response time and high sensitivity to analysis, this bioluminescent enzymatic bioassay has the potential to be developed as a general bioassay for safety assessment of a wide variety of nanomaterials.

Keywords: enzymatic assay, luciferase, nanostructured materials, nanotoxicity, metal nanoparticles.

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Биолюминесцентный ферментативный ингибиторный анализ наночастиц на основе металлов

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Предложен метод оценки биотоксичности наноматериалов, основанный на использовании в качестве объекта воздействия растворимой и иммобилизованной биолюминесцентной биферментной системы: НАД(Ф)·Н:ФМН-оксидоредуктаза и люцифераза. Принцип метода состоит в обнаружении токсических свойств тестируемых веществ по их влиянию на параметры биолюминесценции используемой биферментной системы. Проведено тестирование коммерчески доступных наночастиц на основе металлов (МНЧ), в том числе наночастиц серебра (Ag), и различающихся по размеру наночастиц диоксидов кремния (SiO_2) и титана (TiO_2). Эти МНЧ оказывают ингибирующий эффект на активность биферментной системы, причем растворимые ферменты в большей степени подвержены ингибирующему воздействию МНЧ по сравнению с иммобилизованными. Степень ингибирующего воздействия уменьшается в ряду Ag > TiO_2 > SiO_2, что согласуется с результатами других биологических методов. Биолюминесцентный ферментативный метод анализа занимает 2-3 мин, отличается высокой чувствительностью, технической простотой и может использоваться для оценки безопасности различных классов наноматериалов.

Ключевые слова: ферментативный анализ, люцифераза, наноматериалы, нанотоксичность, наночастицы на основе металлов.

Introduction

Nanotechnology is a rapidly expanding and advancing field of research. It has provided a variety of commercially available engineered nanomaterials, which are used in medicine, perfume and cosmetics industry, and food industry (Etheridge et al., 2013; Duncan, 2011; Aitken et al., 2006). Carbon, which includes fullerenes and nanotubes, is the most common material mentioned in the product descriptions, silver is the second most referenced, followed by silica and titanium dioxide (Kahru and Dubourguier, 2010). For example, it is estimated that at least 235 products available to the public contain nanosilver, including toothpaste, wound dressings, and hair removal products (Garland, 2009).

The properties of many materials change as their size approaches the nanoscale. For example, the higher surface area versus volume ratio with decreasing size of nanoparticles (NPs) results in increased chemical reactivity compared with the bulk material. There is increasing scientific evidence that physical and chemical properties of manufactured NPs lead to an increase in their bioavailability and toxicity (Nel et al., 2006). Nanoparticles enter the human body through various pathways, reaching different organs and contacting tissues and cells, but all of these interactions are based on nanoparticle-biomacromolecule associations. The driving forces for such interactions are quite complex and include the size, shape, and surface properties (e.g., hydrophobicity, hydrogenbonding capability, π bonds, and stereochemical interactions) of engineered nanomaterials. Nevertheless, nanosized materials have until recently been treated as variations of the technical material or existing formulation and, thus, not requiring a separate registration (Oberdörster et al., 2005). The absence of biosafety regulations relating to the use of nanomaterials has given rise to the concept of nanotoxicity in biology and medicine (Pisanic et al., 2009; Kewal, 2012).

The problem is that the currently available toxicity tests have not been designed specifically for NPs. Therefore, new testing paradigms need to be invented for the evaluation and assessment especially of the inhalation toxicity of NPs (Aschberger et al., 2010). Also the risk assessment methodology as currently used for the evaluation of chemicals needs adaptation to account for the specific properties of NPs.

As all changes in living organisms induced by toxic substances originally occur at the lowest, molecular, level of organization, enzyme inhibition based assays have great potential to assess safety of nanoparticles. Really, there are data indicating that the molecular mechanism of nanomaterials effect consists in DNA degradation or enzyme inhibition (Wang et al., 2009; Wang et al., 2010; Zhang et al., 2012; Chang et al., 2014; Käkinen et al., 2013; Vale et al., 2015). *In vitro* techniques allow specific biological and mechanistic pathways to be isolated and tested under controlled conditions, in ways that are not feasible using *in vivo* tests.

Metal (and metal oxide) nanoparticles (MNPs) are of great scientific interest, as they often exhibit different properties to those of the bulk material. In this work, we have sought to assess the potential risks of applying metal NPs according to their effects on enzymatic activities. Bacterial coupled enzyme system NAD(P)H:FMN-oxidoreductase and luciferase (Red + Luc) catalyzing the following reactions (1 and 2) was used as a test system in our attempt to replace luminescent bacteria:

NAD(P)H:FMN – oxidoreductase (Red)

$$\mathrm{NAD(P)H + FMN + H^+ \rightarrow}$$
$$\mathrm{\rightarrow NAD(P)^+ + FMN{\cdot}H_2} \quad (1)$$

Luciferase (Luc)

$$\mathrm{FMN{\cdot}H_2 + RCHO + O_2 \rightarrow}$$
$$\mathrm{\rightarrow FMN + RCOOH + H_2O} + h\nu \quad (2)$$

The principle of bioluminescent enzyme inhibition based assay is to detect the toxic properties of the substances and mixtures based on their influence on the parameters of these bioluminescent enzymatic reactions (Esimbekova et al., 2013; Esimbekova et al., 2014). Such bioassays were developed earlier for environmental monitoring and medical diagnostics (Esimbekova et al., 2014; Esimbekova et al., 1999). This approach combines the rapidity and reproducibility of the *in vivo* bioluminescent methods (Zheng et al., 2010; Mortimer et al., 2008; Deryabin et al., 2012) with the indication of the molecular effect produced by the materials analyzed, using *in vitro* methods (Kratasyuk and Esimbekova, 2015).

The commercially available MNPs, including silver nanoparticles (Ag NPs), nanoparticles of silicon dioxide (SiO_2), and titanium dioxide (TiO_2), were tested in the study. We compared the effects of these nanoparticles on the activity of the soluble and immobilized forms of the Red + Luc coupled enzyme system and obtained relationships between the strength of the inhibitory effect exerted by nanoparticles on enzyme activity and the size of nanoparticles.

Materials and methods

Chemicals

This work was done using the lyophilized preparations of highly purified enzymes produced in the Laboratory of Nanobiotechnology and Bioluminescence of the Institute of Biophysics SB RAS (Krasnoyarsk, Russia). Each vial of the lyophilized preparation of enzymes contained 0.5 mg luciferase EC 1.14.14.3 from the recombinant strain *E. coli* and 0.15 units of NAD(P)H:FMN-oxidoreductase EC 1.5.1.29 from *Vibrio fischeri* culture collection IBSO 836. To prepare the enzyme solutions, 5 mL of potassium-phosphate buffer was added to the vial with enzymes. The immobilized multi-component reagents Enzymolum were manufactured at Prikladnye Biosistemy Ltd. (Krasnoyarsk, Russia). The reagents contained enzymes (Red + Luc) co-immobilized with substrates (NADH and myristic aldehyde) into 3 % (w/v) starch gel (Bezrukikh et al., 2014). The reagent Enzymolum was formed as a disk, 6–7 mm in diameter and having dry weight of 1.5 ± 0.2 mg.

FMN (Serva), NADH (Gerbu), and tetradecanal (Merck) were used as the substrates of Red and Luc.

A 0.0025 % (v/v) solution of myristic aldehyde was prepared by mixing 50 μL of 0.25 % (v/v) ethanol solution of aldehyde and 5 mL of 0.05 M potassium-phosphate buffer (pH 6.9). NADH solution was prepared in 0.05 M potassium-phosphate buffer (pH 6.9).

The following MNPs were chosen for testing: nanoparticles of SiO_2, 120–150 nm, 100–120 nm and 10–15 nm in diameter (Plasmotherm, Russia); nanoparticles of TiO_2, 100–190 nm and 50–70 nm in diameter (Plasmotherm, Russia) and silver nanoparticles, 20 nm in diameter (SintekNano, Russia).

Assay based on inhibition of the soluble and immobilized Red + Luc coupled enzyme system

Preparation of MNPs suspensions for bioluminescent enzymatic assay was carried out using 0.01 M potassium-phosphate buffer pH 7.0. Then nanoparticles were dispersed by sonication with 35 kHz frequency and power level of 300 W in the sonication bath (Sapfir, Russia) for 25 min.

The activity of the soluble coupled enzyme system Red + Luc was measured in the reaction mixture containing: 300 μL of 0.05 M potassium-phosphate buffer pH 6.9, 2–5 μL of enzyme solution, 50 μL of 0.0025 % (v/v) aldehyde solution, 50 μL of 0.4 mM NADH solution, and 10 μL of 0.5 mM FMN solution. At the beginning, we registered the control luminescence intensity of the enzyme system (I_c). For I_c registration, all components of the reaction mixture and 50 μL of the control solution were sequentially added to the luminometer tube, quickly mixed, and the maximum intensity of the luminescence was measured. For registration of the luminescence intensity in the presence of the nanoparticles (I_{exp}), 50 μL of the control solution was replaced with 50 μL of the nanoparticle solutions.

The activity of the immobilized reagent Enzymolum was measured in the reaction mixture containing 1 disk of the reagent Enzymolum, 300 μL of distilled water, 50 μL of the nanoparticle solutions (or control solution), and 10 μL of 0.5 mM FMN solution.

The residual luminescence was calculated according to the formula (I_{exp}/I_c)•100 %. It shows the inhibitory effect of the nanomaterials on the soluble and immobilized Red + Luc coupled enzyme systems. The values of the inhibition parameters IC_{20} and IC_{50} (concentrations of nanomaterials causing the system inhibition by 20 % and 50 %, respectively) were determined. The luminescence intensity of the soluble Red + Luc coupled enzyme system and the reagent Enzymolum was measured using a Lumat LB 9507 luminometer (Berthold Technologies, Germany).

The optical correction coefficients of bioluminescent signal

If the value of optical density of the nanomaterial solutions was more than 0.1 in the range of 400–600 nm, the light emission intensity was multiplied by the correction factors *k*, which were calculated according to the following equation (Aleshina et al., 2010):

$$k = \frac{1}{\sum_{i=1}^{n} \frac{g(\lambda_i)}{D_i(\lambda_i)\left(\frac{L}{l}\right)}\left[1-\exp\left(-D_i(\lambda_i)\left(\frac{L}{l}\right)\right)\right]}, \quad (3)$$

where $g(\lambda_i)$ is the proportion of the intensity of the luminescence at the wavelength of λ_i from the total bioluminescence intensity for the optical path L and $D_i(\lambda_i)$ is the value of absorption of nanomaterial solution at the wavelength λ_i for the optical path l.

The absorption spectra of the fresh solutions were measured with an Uvicon 943 spectrophotometer (Kontron instruments, Italy). The bioluminescence spectrum was measured with an Aminco Bowman Series 2 fluorescent spectrometer (Thermo Spectronics, U.S.).

Statistical analysis

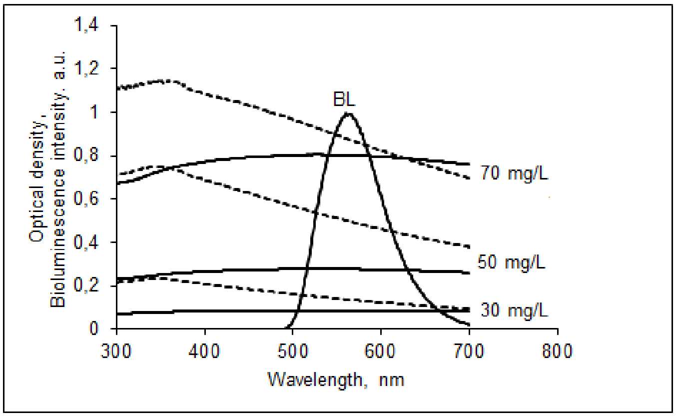
The data are presented as the mean ± SE. The statistical analysis between the two groups was conducted using a two-tailed Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

Results and discussion

To avoid distortion of the bioluminescent signal by optical effects (scattering, absorption), we studied absorption properties of nanoparticles and made the necessary correction of results obtained in the *in vitro* bioassays. The filter effect in an optically opaque medium should be taken into account in the analysis of the emitted light intensity if the optical density of the medium is greater than 0.1 (Lacowicz, 2006). Therefore, when the optical density of the sample was more than 0.1 in the 400–600 nm range, the values of bioluminescence intensity obtained in the presence of nanoparticles were multiplied by the correction coefficients of the optical properties of the solutions, *k*. Correction coefficients were calculated from formula (3). Absorption spectra of nanoparticle suspensions prepared for bioluminescent bioassay are shown in Fig. 1.

Bioluminescent bioassays of 50–70 nm and 100–190 nm TiO_2 nanoparticles showed that optical properties of nanoparticles did not produce any significant effect on results of bioassays if their concentration in the sample was no more than 30 mg/L. Then, for nanoparticle concentrations higher than 30 mg/L, correction coefficients were calculated from formula (3), based on the measured absorption spectra. For SiO_2 nanoparticles of the sizes investigated in this study, the optical density in the 400–600 nm range was no more than 0.1 for all samples; therefore, we did not calculate correction coefficient while analyzing results of bioassay of silica NPs. For Ag NPs, correction coefficient was calculated for the samples with NPs concentrations higher

(A)



(B)

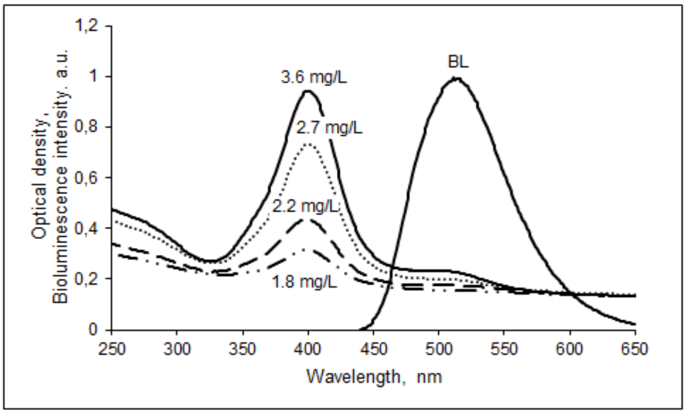


Fig. 1. The absorption spectra of TiO_2 nanoparticles (*A*) and Ag nanoparticles (*B*) at different concentrations. In (*A*), spectra of TiO_2 nanoparticles with sizes of 50–70 nm are shown as solid lines, and TiO_2 nanoparticles with sizes of 100–190 nm as dash-dot lines. The emission spectrum of the bacterial bioluminescence *in vitro* (BL) is shown as a solid line

than 1.5 mg/L. At a lower NPs concentration, the optical density of the samples was no more than 0.1.

The inhibition analysis of nanoparticles yielded dose-effect dependences (Fig. 2–4). The MNPs inhibited the activities of both the soluble and immobilized Red + Luc coupled enzyme systems. The concentration dependences were used to calculate the values of the inhibition parameters IC_{20} and IC_{50} (Table 1). In accordance with the document "Criteria for classifying dangerous wastes by their hazard to the environment" (approved by the order of the Russian Ministry for Natural Resources of June 15, 2001, No. 511), the results of bioassays showed that the nanomaterials tested in this study belonged to Hazard Class 4 (low-hazard substances), as the values of IC_{20} were higher than 1 mg/L.

Having compared the strength of the effects of NPs on the soluble and immobilized coupled enzyme systems, we found that the NPs inhibited the activity of immobilized enzymes to a lesser degree. The reason for that could be the enhanced stability of the enzymes immobilized in starch gel towards the chemical factors of the environment (Bezrukikh et al., 2014).

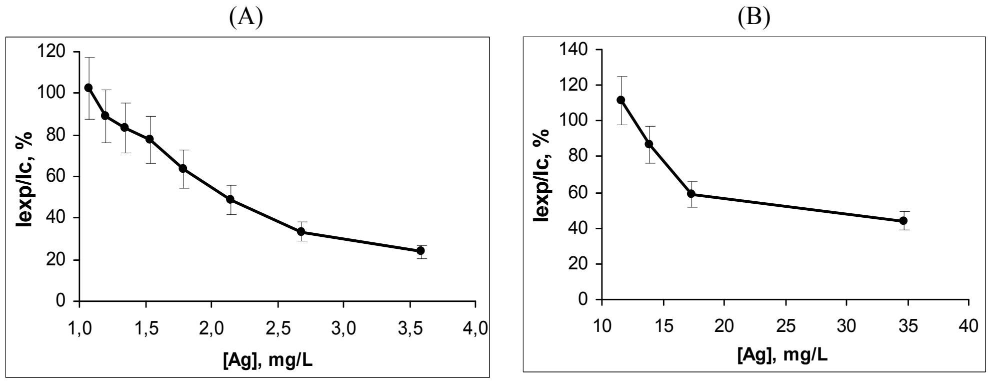


Fig. 2. Residual intensity of luminescence of the Red + Luc coupled enzyme system in the presence of silver nanoparticles; (A) – soluble Red + Luc, (B) – immobilized Red + Luc

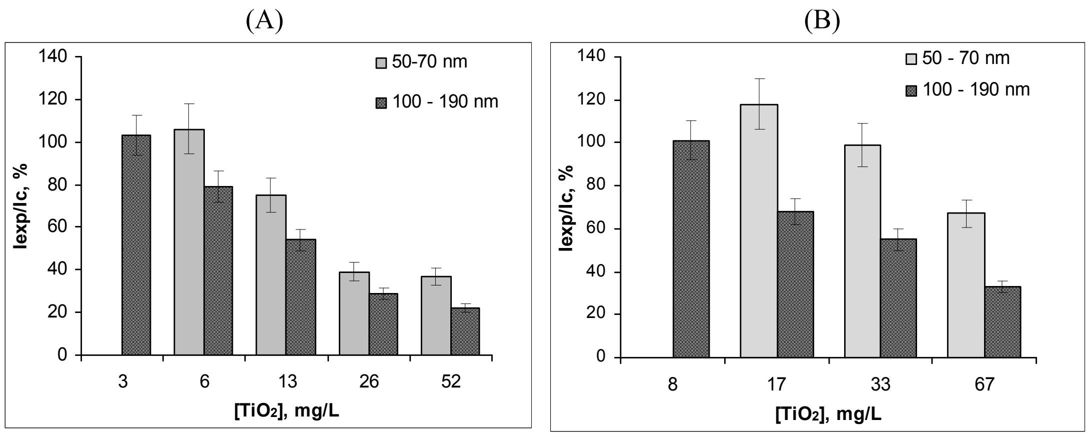


Fig. 3. Residual intensity of luminescence of the Red + Luc coupled enzyme system in the presence of TiO_2 NPs of different sizes; (A) – soluble Red + Luc, (B) – immobilized Red + Luc

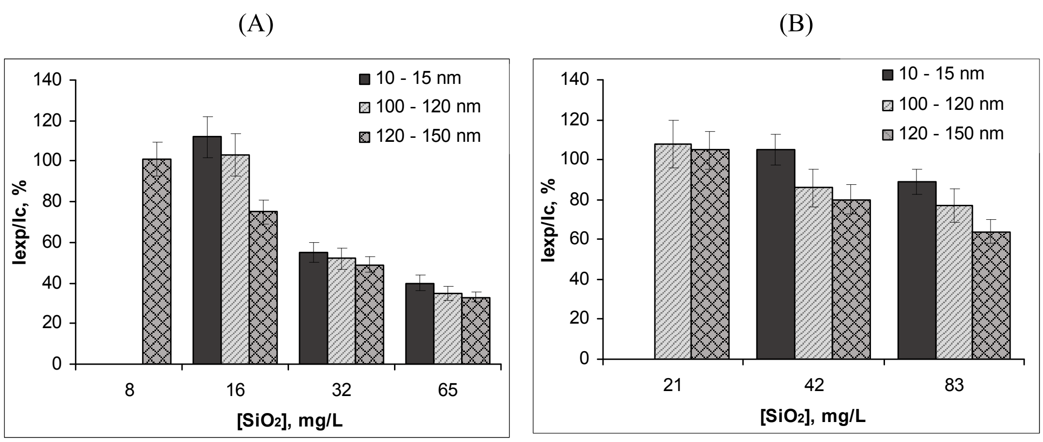


Fig. 4. Residual intensity of luminescence of the Red + Luc coupled enzyme system in the presence of silica nanoparticles of different sizes; (A) – soluble Red + Luc, (B) – immobilized Red + Luc

Table 1. Values of IC_{50} and IC_{20} (mg/L) determined in the assessment of the impact of NPs on the level of bioluminescence of the soluble and immobilized Red + Luc coupled system

NPs	Size, nm	Soluble Red + Luc		Immobilized Red + Luc	
		IC_{20}	IC_{50}	IC_{20}	IC_{50}
SiO_2	10–15	26.2±2.4	42.1±3.8	-	-
	100–120	23.9±2.4	35.8±3.6	57.1±6.3	-
	120–150	15.1±1.2	31.0±2.5	43.7±3.9	-
TiO_2	50–70	13.3±1.2	22.9±2.5	53.5±5.3	-
	100–190	6.7±0.7	15.3±1.7	13.2±1.2	36.6±3.3
Ag	20	1.3±0.2	2.2 ±0.3	14.8±1.8	21.2±6.1

"-" – In the studied concentration range, the parameter value is not determined.

Ag nanoparticles produced the greatest inhibitory effect on the coupled enzyme system (Fig. 2). Taking into account correction coefficients, the IC_{50} values were 2.2 and 21.2 mg/L for the soluble and immobilized Red + Luc coupled systems, respectively (Table 1).

The toxic effects of Ag NPs have been studied quite well at the level of the organism (Gaillet and Rouanet, 2015; Ivask et al., 2014). Recent studies suggest that Ag NPs exhibit toxic effects on the key organisms of the aquatic environment (Blinova et al., 2013; Fabrega et al., 2011). These effects have mostly been explained by a combination of dissolved Ag^+ ions and specific nanoeffects (Zhao and Wang, 2012). The mechanism of Ag NPs toxicity to microbes is associated with the interaction of Ag ions with thiol groups of vital enzymes and proteins, affecting cellular respiration and transport of ions across membranes, with ultimate cell death (Li et al., 2010; Rahmanin et al., 2014). A possible reason for the high sensitivity of the Red + Luc coupled enzyme system to Ag NPs may be a considerable inhibitory effect of Ag NPs on the activity of NAD(P)H:FMN-oxidoreductase, which is one of the key enzymes of the respiratory chain.

TiO_2 NPs inhibits the activity of the Red + Luc coupled enzyme system to a greater extent than SiO_2 NPs (Fig. 3, 4). In addition, the degree of inhibition is determined by the size of the particles: larger-diameter TiO_2 nanoparticles exert a stronger inhibitory effect than the smaller-diameter particles of the same concentration (Fig. 3). A similar effect has been noted with SiO_2 NPs (Fig. 4).

Titanium dioxide is considered to be biologically inert in the micro-size state. However, the degree of its biological inertness in the nano-size state is debatable. Numerous studies describe various effects of TiO_2 NPs on vital parameters of different organisms and parameters of *in vitro* bioassays (Tomankova et al., 2015).

It is a proven fact that specific physicochemical properties of nanoparticles determine the way they cross the barriers in the organism and their distribution and accumulation in the organism and removal from it (Kahru et al., 2008). For example, in the case of bacteria, high concentrations of TiO_2 nanoparticles – 5,000 mg/L – were needed to inhibit the growth of *Escherichia coli* by 72 % (66 nm particle size), and 1,000 mg/L of TiO_2 nanoparticles resulted in 75 % growth inhibition of *Bacillus subtilis* (Adams et al., 2006). Moreover, no toxic effect was observed for the marine bacterium *Vibrio fischeri* (30-min EC_{50} > 20,000 mg/L) (Heinlaan et al., 2008). According to Warheit et al. (2007), the *Daphnia magna* 48h EC_{50} values and rainbow trout *Oncorhynchus mykiss* 96 h LC_{50} values for TiO_2 nanoparticles were > 100 mg/L. In another study, titanium dioxide nanoparticles showed some sublethal toxic effects (including oxidative stress) in rainbow trout when exposed to low levels of TiO_2 nanoparticles, 0.1–1.0 mg/L, for up to 14 days (Federici et al., 2007).

According to Kahru et al. (2008), in addition to target organism and exposure time, two major abiotic parameters such as particle size/aggregation and illumination seem to be involved in the actual (eco)toxicity of TiO_2. In our research, the value IC_{50} for 50–70 nm TiO_2 nanoparticles was higher than that for 100–190 nm TiO_2 nanoparticles (22.9 and 15.3 mg/L, respectively).

Amorphous silicon dioxide (silica) is now commonly used as a food additive (E551) and a component of various medicinal tablets (Abaeva et al., 2010) and cosmetic preparations. The absence of any toxic effect of amorphous SiO_2 on the human organism is considered proven. At the same time, safety evaluations of different samples of nanostructured SiO_2 gave contradictory results. Shumakova et al. (2014a, b; 2015a, b) reported an integrated study of toxicity of SiO_2 nanoparticles in experiments with animals, which involved analysis of integrated, enzymatic, biochemical, microecological, hematological, immunological, allergological and other parameters. The authors of that study revealed changes in a number of markers, including lower activity of the CYP2B1 isoform and decreased crude protein, albumin, and glucose in blood serum. They concluded that the maximum orally administered non-effective dose of nanostructured SiO_2 was below 100 mg per 1 kg body mass a day if taken daily for 1 to 3 months. Zaitseva (2014) reported an evaluation of acute toxicity of the aqueous suspension of nanostructured SiO_2 under single intragastric administration by gavage to male BALM/C mice; the value of LD_{50} was higher than 10 000 mg/kg, suggesting no toxic effect of the nanoparticles.

Gottschalk et al. (2009) studied the predicted environmental concentrations (PECs) of nanoparticles with the current understanding of nanoparticle transformations and fate. In particular, the calculated values of PECs for TiO_2 nanoparticles are 2.5–10.8 µg/L and 12–57 ng/L for wastewater treatment plant effluents and the surface waters, respectively. The values of PECs for Ag NPs are 32.8–111 ng/L and 0.588–2.16 ng/L for wastewater treatment plant effluents and the surface waters, respectively. Although the values of IC_{50} for TiO_2 and Ag nanoparticles are considerably higher than the PECs of those that Gottschalk and co-authors calculated, by considering the dramatic increase in the production and use of NPs, a conclusion can be made about the potential risk of these NPs on living organisms.

Conclusion

Toxicological analysis of metal-based nanoparticles was performed using the *in vitro* bioluminescent method. The nanoparticles were ranked according to the strength of their inhibitory effects on the Red + Luc coupled enzyme system. The bioluminescent enzyme assay showed that Ag NPs produced the strongest inhibitory effect. Within the range of NPs concentrations tested in this study, the degree of their inhibitory effect on the activity of the Red + Luc coupled enzyme system decreased as follows: Ag > TiO_2 > SiO_2. That was consistent with the results obtained by other biological methods. The bioluminescent enzyme test systems used in this study have good potential for developing methods for analyzing toxicity of different types of nanomaterials. The analysis takes 2 or 3 minutes and is technically simple, being no less sensitive than other conventional methods of toxicology.

Acknowledgments

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