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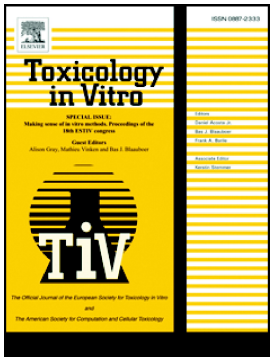

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Bioluminescent enzyme inhibition-based assay to predict the potential toxicity of carbon nanomaterials

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ABSTRACT

A bioluminescent enzyme inhibition-based assay was applied to predict the potential toxicity of carbon nanomaterials (CNM) presented by single- and multi-walled nanotubes (SWCNT and MWCNT) and aqueous solutions of hydrated fullerene C_{60} (C_{60}HyFn). This assay specifically detects the influence of substances on parameters of the soluble or immobilised coupled enzyme system of luminescent bacteria: NAD(P)H:FMN-oxidoreductase + luciferase (Red + Luc). A protocol based on the optical properties of CNM for correcting the results of the bioluminescent assay was also developed. It was shown that the inhibitory activity of CNM on Red + Luc decreased in the following order: MWCNT > SWCNT > C_{60}HyFn. The soluble enzyme system Red + Luc had high sensitivity to MWCNT and SWCNT, with values of the inhibition parameter IC_{50} equal to 0.012 and 0.16 mg/L, respectively. The immobilised enzyme system was more vulnerable to C_{60}HyFn than its soluble form, with an IC_{50} equal to 1.4 mg/L. Due to its technical simplicity, rapid response time and high sensitivity, this bioluminescent method has the potential to be developed as a general enzyme inhibition-based assay for a wide variety of nanomaterials.

Keywords: nanotoxicity; enzyme inhibition-based assay; bioluminescence; luciferase; nanomaterials; nanotubes

1. Introduction

Due to the increasing scale of production and usage of a vast number of new materials in industrial and economic activities, society is faced with problems associated with a lack of materials safety assessment regarding humans, ecosystems and the biosphere as a whole. These materials include engineered nanomaterials, which are actively applied in medicine, perfumes, cosmetics and the food industry (Zhou, 2015). The lack of regulation over the biological safety of such nanomaterials has raised concerns about their toxicity in biology and medicine (Sahu et al., 2009; Jain, 2012; Bottero, 2016). Evaluation of the potential risks of using nanomaterials is complicated due to their physical and chemical properties such as size, distribution, agglomeration state, shape, crystal structure, chemical composition, surface area, surface chemistry, surface charge and porosity (Oberdörster, 2005; Mu, 2014). Moreover, the connection between the size of nanomaterials and their toxicity, in terms of whether there is a specific type of nanotoxicity, remains unknown (Donaldson, 2013). Nowadays, numerous toxicological investigations using living organisms, cell lines, etc. are carried out in laboratories in order to assess the potential risks of using these materials and their biological effects on human health and the environment (Powers et al., 2012; ENRHES, 2009; Maurer-Jones, 2013; Ema et al., 2015; Zhang, 2015). Good results in terms of rapidity and reproducibility have been obtained by using bioluminescent methods based on recombinant or natural strains of luminescent bacteria (Mortimer et al., 2008; Zheng et al., 2010; Deryabin et al., 2012).

The first reports on the toxic effect of carbon nanotubes (CNT) were published in 2003 (Service, 2003); since then, several research studies have proven that CNT are characterised by cyto- and genotoxicity (ENRHES, 2009; Lam et al., 2006; Lanone et al., 2013). Moreover, CNT can amplify the toxic effect of other contaminants (Sanchís et al., 2015). Many authors believe that the toxic effect of CNT is associated with their ability to generate reactive oxygen species (ROS) followed by the development of oxidative stress in cells (Johnston et al., 2010). However, this ROS generation is often explained by the fact that commercial preparations of CNT contain

various contaminants, for example, iron, which enters nanotubes during the process of production (Kagan et al., 2006; Pulskamp et al., 2007). Templeton et al. (2006) have shown that the electrophoretic cleaning of commercial preparations leads to a significant reduction of their toxicity. However, it is critical to evaluate the toxicity of commercial preparations of CNT, because they can have a negative effect on biological objects during their manufacture and use.

The published data on the toxicity of the other group of CNM – fullerenes – at first glance seem contradictory. For example, Oberdörster (2004) showed that fullerene suspensions prepared by the method of Colvin et al. (2004) have toxic effects on the brain cells of fish at concentrations of 0.003–5 μM. At the same time, many authors indicated no negative effects, even at much higher concentrations of fullerenes. For instance, Gharbi et al. (2005) showed that using water dispersions of fullerene C_{60} in experiments with rats, even at very high doses (2.5 g/kg and higher), had no toxic effects on their liver. Importantly, Andrievsky et al. (2005) stated that the toxic effect of fullerenes depends on the characteristics of the preparations, primarily from the number and physical-chemical properties of impurities contained in these preparations. The authors of this review hypothesised that the main reason for the inhibitory (toxic) effect of Colvin's dispersions of fullerene on the biological systems is the presence of tetrahydrofuran (THF) molecules and products of their oxidative modification and subsequent polymerisation. This conclusion was further confirmed by Zhu et al. (2006) in a study showing that using THF as a solvent for making C_{60} suspensions leads to a considerable increase in fullerene toxicity determined by the death rate of *Daphnia magna*.

Information about the ability of fullerenes to generate ROS is also contradictory. Some authors stated that C_{60} stimulates ROS generation in solutions (Wang et al., 2011), while others evidenced the antioxidative properties of fullerenes (Gharbi et al., 2005; Andrievsky et al., 2009; Wang et al., 1999). Apparently, the latter statement refers only to aqueous solutions of pristine fullerene and can be explained by an ordered hydration shell surrounding the molecule of C_{60} that prevents the formation of ROS (Andrievsky et al., 2005). Thus, it is clear that the toxic

effects of fullerenes are determined by their structure and physical-chemical properties. Investigations based on human red blood cell haemolysis have also examined the cytotoxic effects of fullerenes (Tramer et al., 2012).

The molecular mechanism behind the effects of nanomaterials consists mainly of 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 this work, the bacterial coupled enzyme system NAD(P)H:FMN-oxidoreductase and luciferase (Red + Luc), which catalyses 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)

$$NAD(P)H + FMN + H^+ \longrightarrow NAD(P)^+ + FMN{\bullet}H_2 \quad (1)$$

Luciferase (Luc)

$$FMN{\bullet}H_2 + RCHO + O_2 \longrightarrow FMN + RCOOH + H_2O + h\nu \quad (2)$$

where FMN and $FMN{\bullet}H_2$ are the oxidised and reduced forms of flavin mononucleotide, $NAD(P)^+$ and NAD(P)H are oxidised and reduced forms of nicotinamide adenine dinucleotide (phosphate), RCHO is myristic aldehyde and RCOOH is the corresponding fatty acid.

The principle of bioluminescent enzymatic bioassay is to detect the toxic properties of substances and mixtures based on their influence on the parameters of these bioluminescent enzymatic reactions (Russian Federal Service for Intellectual Property, 2011; Esimbekova et al., 2013; Esimbekova et al., 2014). This and other similar assays were developed earlier for environmental monitoring and medical diagnostics (Esimbekova et al., 2014; Esimbekova et al., 1999; USSR Federal Service for Intellectual Property, 1991). In addition, this bioassay was used previously to determine the toxicity of biopolymers (Shishatskaya et al., 2002). Moreover, it has been shown that nanodiamonds are able to inhibit the coupled enzyme system Red + Luc (Kudryasheva et al., 1994).

The commercially available carbon-based nanomaterials, including single-walled carbon carboxylated nanotubes (SWCNT), multi-walled carbon nanotubes (MWCNT) and chemically

un-modified C_{60} fullerene water solution (C_{60}FWS), were tested in this study. The effect of these CNM on the activity of the soluble and immobilised coupled enzyme system Red + Luc was verified.

2. Materials and methods

2.1. Chemicals

This work was carried out using the lyophilised 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 lyophilised 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 the *Vibrio fischeri* culture collection IBSO 836. To prepare the enzyme solutions, 5 mL of potassium-phosphate buffer was added to the vial containing the enzymes. The immobilised multicomponent reagents 'Enzymolum' were produced by Prikladnye Biosistemy Ltd. (Krasnoyarsk, Russia). The reagents contain enzymes (Red + Luc) co-immobilised with substrates (NADH and myristic aldehyde) into 3% (w/v) starch gel (Enzymolum^{Starch}) or 1% (w/v) gelatine gel (Enzymolum^{Gelatin}) (Russian Federal Service for Intellectual Property, 2011; Bezrukikh et al., 2014). The reagent Enzymolum was formed as a disk 6–7 mm in diameter and a 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).

2.2. Characteristics of analysed CNM

The following CNM were chosen for testing: single-walled carbon carboxylated nanotubes c-SWCNT-90A (OOO 'Carbon Chg', Russia), multi-walled carbon nanofiber 'Activated

nanocarbon material' (OOO 'PRANKOR', Russia), C_{60} fullerene water solution (C_{60}FWS) and the commercial product 'Water with hydrated fullerene C_{60}' based on the C_{60}FWS, trademark 'C_{60} Water of Life' (Institute of Physiological Active Compounds, Ukraine).

The analysed single-walled carbon carboxylated nanotubes c-SWCNT-90A were obtained by arc discharge synthesis and are characterised by a narrow diameter distribution in the range of 1.2–1.6 nm, a SWCNT content not less than 90% and low aggregation in the form of nanotube cords with a length of 2–10 μm and a cross-sectional diameter of up to 10 nm.

The analysed activated multi-walled carbon nanofiber contained multi-walled nanotubes with variable internal (2–29 nm) and external (10–90 nm) diameters, as well as large particles of amorphous carbon and graphitised soot of varying size from 500 nm to 3 μm and carbon fibres of diameter 60–330 nm. The content of structured carbon in the material was 50%–80%, the average diameter of tubes and fibres was 10–200 nm, the average length of tubes and fibres was 4–20 μm and the mass fraction of graphite-like and amorphous carbon was 10%–30% and 20%–40%, respectively.

Stock C_{60}FWS contained hydrated fullerene C_{60} (C_{60}HyFn) at a concentration of 105 μM (75 mg/L). The commercial product 'Water with hydrated fullerene C_{60}' contained highly purified water and C_{60}HyFn at a concentration of 2.8 nM (2 μg/L). The method of preparation of C_{60}FWS consists of transferring of C_{60} molecules (MER Corp., USA) from their solution in an organic solvent to an aqueous phase by ultrasound treatment without using any solubilisers (Andrievsky et al., 1995). In terms of the chemical structure, C_{60}HyFn is a highly hydrophilic and stable donor-acceptor complex of C_{60} with molecules of water: $C_{60}\{H_2O\}_n$, where n = 22–24. So, analysed solutions contain both C_{60} single molecules and their labile nanoscale clusters (3–36 nm).

2.3. Assay based on inhibition of soluble and immobilised coupled enzyme system Red + Luc

Preparation of SWCNT and MWCNT suspensions for bioluminescent enzymatic assay was carried out using the method described earlier (Deryabin et al., 2012). Briefly, 2 mg of SWCNT and MWCNT were diluted by 20 mL of 1 mg/L aqueous solution of sodium lauryl sulphate (SLS). Then, nanomaterials were dispersed by sonication at a frequency of 35 kHz and a power level of 300 W in the sonication bath (Sapfir, Russia) for 30 min. A similar preparation of a 1 mg/L solution of SLS was used as a control. After dispersion, the nanomaterial solutions were diluted with 1 mg/L SLS solution. The FWS samples were prepared by diluting the previously obtained preparations in distilled water.

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), 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, the control luminescence intensity of the enzyme system (I_c) was registered. For I_c registration, all components of the reaction mixture and 50 µL of the control solution were subsequently added to the tube of a Glomax 20/20 luminometer (Promega, USA) and quickly mixed, and the maximum intensity of luminescence was measured. For registration of the luminescence intensity in the presence of the nanomaterial (I_{exp}), 50 µL of the control solution was placed on 50 µL of the nanomaterial solutions. The activity of the immobilised reagent 'Enzymolum' was measured in the reaction mixture containing the following: 1 disc of the reagent Enzymolum^{Starch} or Enzymolum^{Gelatin}, 300 µL of distilled water, 50 µL of the nanomaterial solution (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 immobilised coupled enzyme systems Red + Luc. The values of the inhibition parameters IC_{20} and IC_{50} (concentrations of nanomaterials inhibiting the system by 20% and 50%, respectively) were determined.

2.4. Optical correction coefficients of the bioluminescent signal

If the value of optical density of the nanomaterial solution was greater 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. $D_i(\lambda_i)$ is the value of absorption of a nanomaterial solution at the wavelength λ_i for the optical path *l*. The absorption spectra of the fresh solutions were measured with a Cary 5000i spectrophotometer (Agilent Technologies, USA). The bioluminescence spectrum was measured with an Aminco Bowman Series 2 fluorescent spectrometer (Thermo Spectronics, USA).

2.5. Statistical analyses

Data are presented as the mean ± SE. Statistical analyses of the differences in means between the two groups were conducted using a two-tailed Student's *t*-test. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Absorption characteristics of nanomaterials

In the bioluminescent assays, the signal (intensity of light emission) is registered optically. Therefore, the absorption characteristics of the nanomaterial solutions could affect bioassay results. Results were corrected using the absorption characteristics of the CNM to minimise bioluminescent signal distortion due to optical effects of CNM solutions (scattering, absorption).

This is critical in order to avoid false-positive results in the bioluminescent assays (Deryabin et al., 2012). The absorption spectra of the solutions of carbon nanotubes (CNT) and hydrated fullerenes prepared for bioluminescent bioassay were measured (Fig. 1).

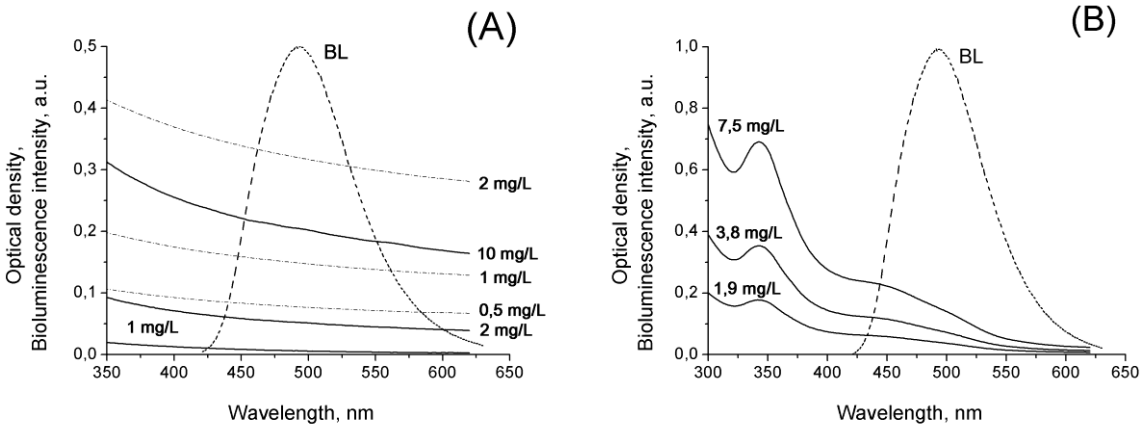

Fig. 1. The absorption spectra of nanotubes (*A*): and C_{60}HyFn (*B*) at different concentrations. On (*A*) SWCNT spectra are shown as solid lines and MWCNT as dash-dot lines. The emission spectrum of the bacterial bioluminescence *in vitro* (BL) is shown as a dashed line.

The optical density (OD) of the nanotubes in the range of 400–600 nm decreased monotonically without noticeable peaks and could be fitted to a power function (Fig. 1A). This result indicates that turbidity (or light scattering) is responsible for the optical characteristics of these samples due to low solubility of the nanotubes in water. Thus, absorption bands of the nanotubes do not disturb the signal of bioluminescent bioassays. In general, it was found that MWCNT suspensions are characterised by higher OD than those of SWCNT at the same concentration (i.e. for 2 mg/L, 0.32 and 0.05 at 500 nm, respectively), which could be due to the heterogeneity of the tested MWCNT samples. It was also concluded that the optical effect of the samples can be neglected if the concentration of SWCNT and MWCNT in the samples is not higher than 5 and 0.5 mg/L, respectively (OD < 0.1). For higher concentrations, the correction

factors were calculated according to equation (3). The largest value of the correction factor (1.16) was obtained for the sample containing 2 mg/L of MWCNT.

Absorption spectra obtained for the solutions of hydrated fullerenes C_{60}HyFn were found to have two bands with maxima at approximately 344 and 450 nm (Fig. 1B). This result is in accordance with published data (Kato et al., 2009). A small overlap of absorption with the emission spectrum of the bacterial bioluminescence *in vitro* led to the assessment that at fullerene concentrations lower than 7.5 mg/L, the optical effect of the samples is negligible (in this case $k < 1.05$).

Thus, the absorption properties of CNM allowed us to estimate the highest concentrations that do not require signal correction in bioluminescent bioassays as 5, 0.5 and 7.5 mg/L for SWCNT, MWCNT and C_{60}HyFn, respectively. After more concentrated samples were tested, all measured intensities were multiplied by the corresponding coefficients *k* during further experiments.

3.2. The effect of carbon nanotubes on the bioluminescent coupled enzyme system Red + Luc

The effect of commercial SWCNT and MWCNT on the coupled enzyme system Red + Luc was determined using both soluble and immobilised Red + Luc in starch or gelatin gels (Enzymolum^{Starch} or Enzymolum^{Gelatin}). To achieve better dispersion of CNT suspensions, a 1 mg/L solution of SLS for primary suspension of CNT and a procedure of suspension sonification was used. The 1 mg/L solution of SLS had no effect on the activity of the soluble and immobilised coupled enzyme system Red + Luc. Accordingly, the 1 mg/L solution of SLS was used for further dilutions of CNT suspensions.

The samples of SWCNT inhibited the activity of the soluble and immobilised coupled enzyme system Red + Luc (Fig. 2). SWCNT had the least impact on the activity of the coupled enzyme system Red + Luc immobilised to the gelatine gel. This result is probably related to the high stability of the coupled enzyme system in gelatine-containing media in the presence of

external compounds (Bezrukikh et al., 2014). The MWCNT samples inhibited the activity of the coupled enzyme system Red + Luc more strongly than the SWCNT samples (Fig. 2). The soluble system Red + Luc was found to be the most sensitive to the effect of MWCNT samples. The sensitivity of the immobilised multicomponent reagent Enzymolum^{Starch} to MWCNT was 3-fold higher than that of the reagent Enzymolum^{Gelatin}.

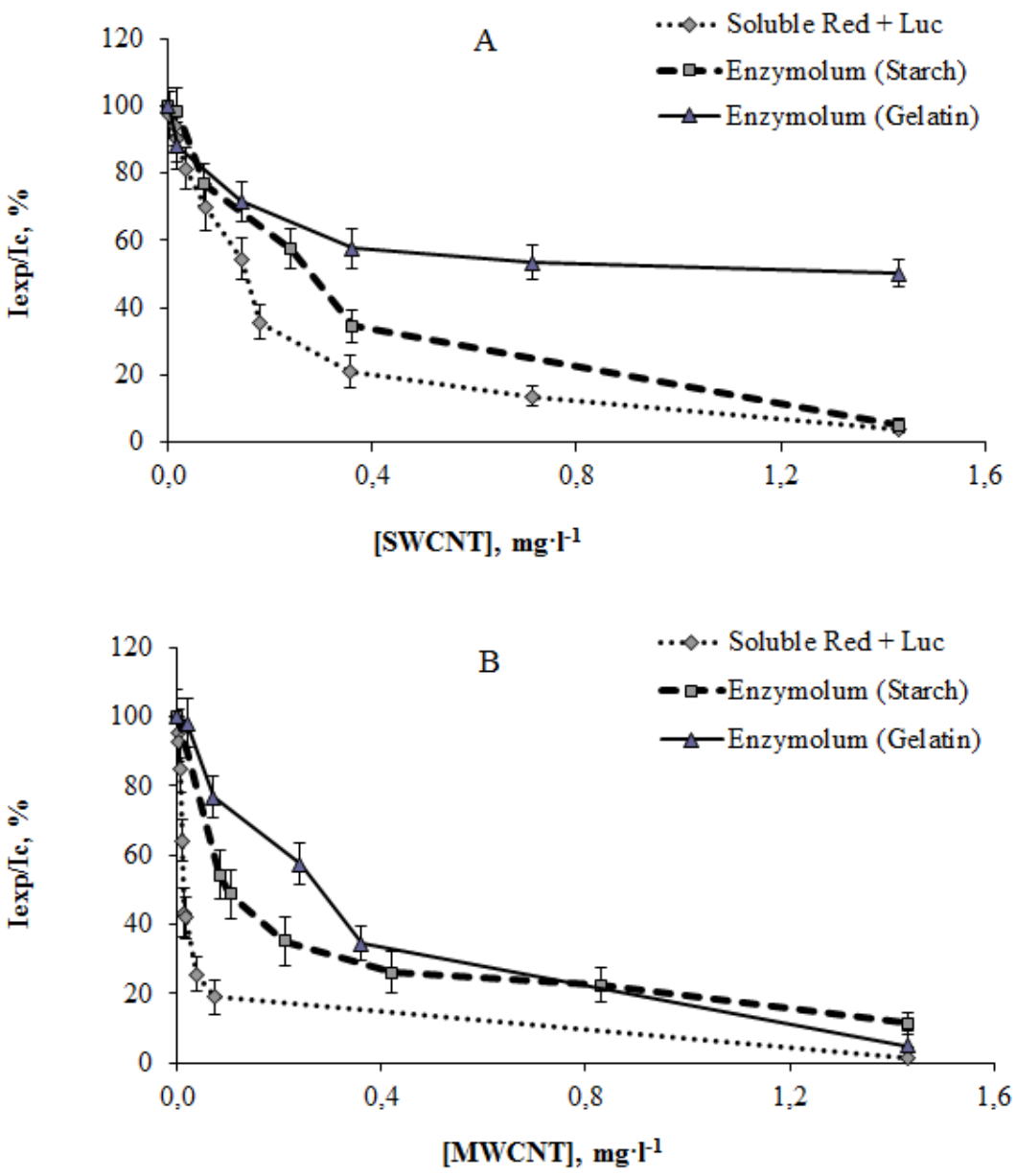

Fig. 2. Residual luminescence intensity of the coupled enzyme system Red + Luc in the presence of SWCNT (*A*) and MWCNT (*B*).

The inhibition parameters IC_{20} and IC_{50} were calculated (Table 1). According to EC Directive 93/67/EEC for aquatic organisms, chemicals are classified by their degree of toxicity based on EC_{50} values. We hypothesised that this classification was correlated with IC_{50} values and revealed that MWCNT and SWCNT samples might be characterised as extremely toxic and very toxic, respectively.

Table 1

Values of the inhibition parameters IC_{50} and IC_{20} (mg/L) determined during the assessment of the effect of CNM on the luminescence intensity of the soluble and immobilised coupled enzyme system Red + Luc

Analysed CNM	Soluble Red + Luc		Enzymolum^{Starch}		Enzymolum^{Gelatin}		Classification$^{\#}$
	IC_{50}	IC_{20}	IC_{50}	IC_{20}	IC_{50}	IC_{20}	
SWCNT	0.16* ±0.03	0.04* ±0.01	0.28±0.04	0.08±0.01	1.4±0.2	0.08±0.01	Very toxic
MWCNT	0.012* ±0.003	0.004* ±0.001	0.12±0.02	0.08±0.01	0.28±0.04	0.06±0.01	Extremely toxic
C_{60}HyFn	*nd*	3.7* ±0.7	1.4±0.2	0.5±0.1	5.4±0.8	0.4±0.1	Toxic

Data are represented as M ± m, n = 5; $*p < 0.05$ compared with IC_{50} and IC_{20} parameters for Enzymolum^{Starch} and Enzymolum^{Gelatin}. $^{\#}$this classification is hypothetical. *nd* = not determined, low inhibition (%) at the maximum concentration tested.

3.3. The effect of hydrated fullerene C_{60} on the bioluminescence of the coupled enzyme system Red + Luc

The inhibitory effect of the fullerene C_{60}HyFn solutions (at concentrations up to of 7.5 mg/L) was determined on the soluble and immobilised coupled enzyme system Red + Luc. Distilled water was used as a control sample. Results showed that C_{60}FWS has little inhibitory effect on the bioluminescence parameters of the soluble coupled enzyme system Red + Luc (Fig.

3). IC_{20} was found to be 3.7 mg/L, but the value of IC_{50} was indeterminable because it exceeded the available maximum concentration in the sample (7.5 mg/L).

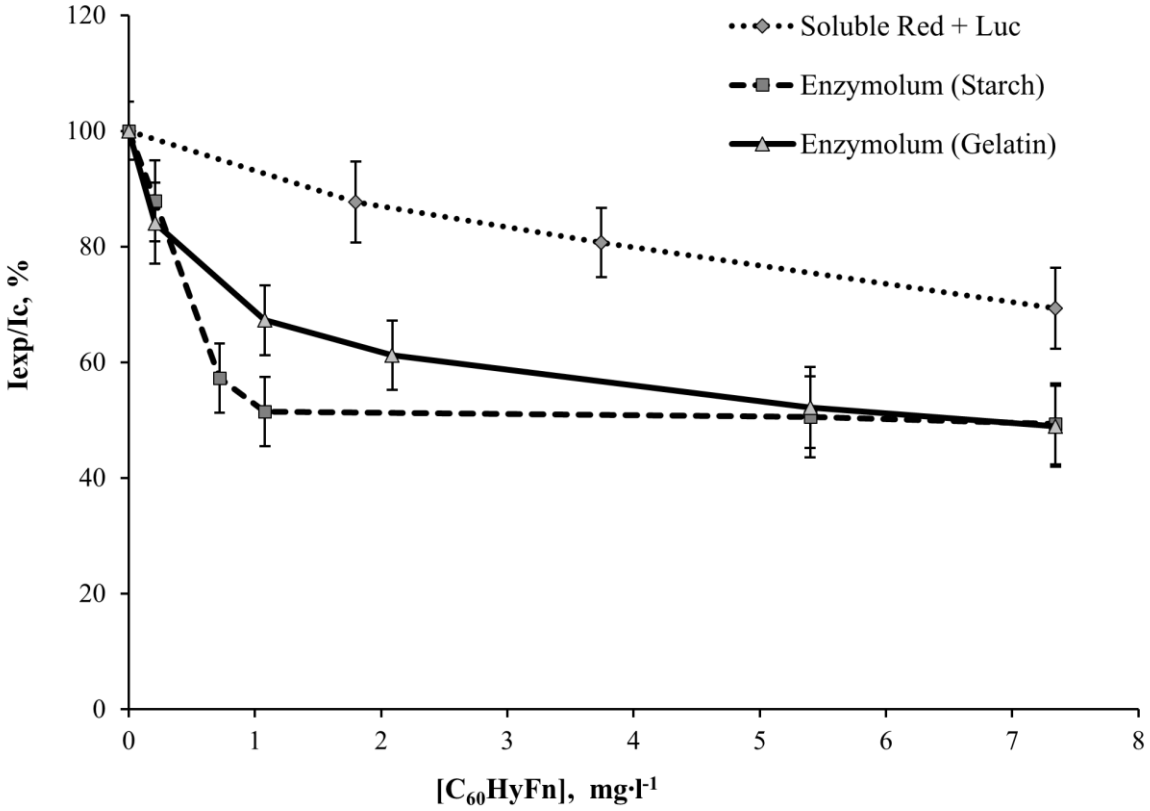

Fig. 3. Residual intensity of luminescence of the coupled enzyme system Red + Luc in the presence of C_{60}HyFn.

In contrast to CNT, the fullerene solutions had an inhibitory effect on the enzymes within the starch and gelatine Enzymolum reagents (Fig. 3). The values of IC_{50} and IC_{20} for the fullerene solutions are shown in Table 1. Among the immobilised reagents, the multicomponent reagent Enzymolum^{Starch} had the highest sensitivity to the effects of hydrated fullerenes. The soluble enzyme system Red + Luc showed the lowest sensitivity to the effects of the hydrated fullerene C_{60}FWS.

Analysis of the commercial product 'Water with hydrated fullerene C_{60}' revealed no significant inhibition of the activity of the coupled enzyme system by this sample: the residual activity was not less than 90% of the control activity. The concentration of C_{60}HyFn in this commercial product is 2 μg/L, which is considerably less than the IC_{50} and IC_{20}; therefore, this product can be used as a standard (control) sample in studying the aqueous solutions of nanomaterials.

4. Discussion

4.1. Effect of carbon nanotubes on bioluminescence of the coupled enzyme system Red + Luc

An inhibitory effect of CNT on the coupled enzyme system of luminescent bacteria Red + Luc was detected (Fig. 2). Deryabin et al. (2012) found a stimulatory effect of CNT on the luminescent bacteria after 30–45 min of incubation, which is associated with the adaptation of metabolic processes in bacteria that reduce the toxic effects of CNT. Indeed, the increase in contact time between the bacteria and CNT of up to 180 min and longer led to a significant inhibitory effect instead of stimulation (Deryabin et al., 2012; Zarubina et al., 2009). Also, when the luminescent bacteria were used as indicators (Zheng et al., 2010; Deryabin et al., 2012), the EC_{50} for SWCNT and MWCNT were found to be 2–3 orders of magnitude higher in comparison with the IC_{50} values obtained with Red + Luc. Thus, the bioluminescent method based on the enzyme system Red + Luc showed higher sensitivity to the effect of CNT than the *in vivo* test based on luminescent bacteria.

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 carbon nanotubes are 0.001–0.8 ng/L and 3.69–32.66 ng/L for surface waters and wastewater treatment plant effluents, respectively. Although the obtained values of IC_{50} for CNT are considerably higher than the PECs of those calculated

by Gottschalk and co-authors, when the sharp increase in the production and use of CNT is considered, a conclusion can be made about the potential toxicity of these materials to living organisms (primarily at the molecular level).

4.2. Effect of hydrated fullerene C_{60} on bioluminescence of the coupled enzyme system Red + Luc

In contrast to the nanomaterials based on carbon nanotubes, aqueous solutions of C_{60}HyFn are of greater interest because manufacturers suggest using them as a standard (reference) sample during testing of different nanostructures and nanomaterials for their toxicological characteristics and environmental safety (Andrievsky et al., 2005). Moreover, the Ministry of Health of Ukraine government has approved the industrial use of solutions of the 'Fullerene C_{60} hydrated concentrate' as one of the antioxidant supplements in the food, perfume, cosmetics, biotechnological and microbiological industries (Safety and Health Certificate of the Ministry of Health of Ukraine No 05.03.02-04/89993 of 19 November 2010). The recommended range of C_{60}HyFn concentration in the final product by the latter protocol is 0.1 μg/L–1 mg/L. However, we found a C_{60}HyFn concentration higher than 0.5 mg/L to have an inhibitory effect on the reagents Enzymolum^{Starch} and Enzymolum^{Gelatin}.

We showed that the immobilised forms of the enzymes Red + Luc in the reagent Enzymolum are more sensitive to the effect of the aqueous solutions of C_{60}HyFn compared with the soluble forms (Fig. 3). It is known that fullerenes can cause biological effects via exclusive adsorption to biomolecules (Nielsen et al., 2008). Probably, C_{60}HyFn is adsorbed on the enzyme molecules located on the surface of the discs, and the high sensitivity of the reagents Enzymolum^{Starch} and Enzymolum^{Gelatin} to C_{60}HyFn could be explained by this adsorption preventing the activities of the enzyme molecules, which are immobilised in the deeper layers of starch and gelatine gels. Moreover, some authors suggest that the immobilised enzyme systems in natural gels (gelatine and starch) could be used as a model system for natural enzymes within

the cells (Hastings et al., 1985; Kratasyuk et al., 1994). Such models simulate the viscous intracellular microenvironment of the enzymes. Indeed, most intracellular enzymes are normally located in a complex, heterogeneous environment and not in a diluted solution (Trevan, 1980). From this point of view, the results of high sensitivity of immobilised enzymes to the effect of fullerenes should be regarded as an enhanced biological effect on patterns that are more related to the cell structure.

5. Conclusion

CNM analysed in the study show an inhibitory effect on the enzymes of luminescent bacteria, which suggests that there is a negative effect of CNM on the molecular level of biological systems. The soluble coupled enzyme system Red + Luc has high sensitivity to MWCNT and SWCNT, and the immobilised enzyme system Red + Luc is more sensitive to C_{60}HyFn in comparison with soluble enzymes. The inhibitory activity of CNM decreases in the following order: MWCNT > SWCNT > C60HyFn, which correlates with known toxicities for luminescent bacteria (Deryabin et al., 2012). Thus, the bioluminescent enzymatic method is suitable to reveal the potential toxicity of CNM and can be used as a basis for new methods of screening various nanomaterials. The analysis is simple, takes only 2–3 minutes and has sensitivity competitive with other methods of toxicology (ENRHES, 2009).

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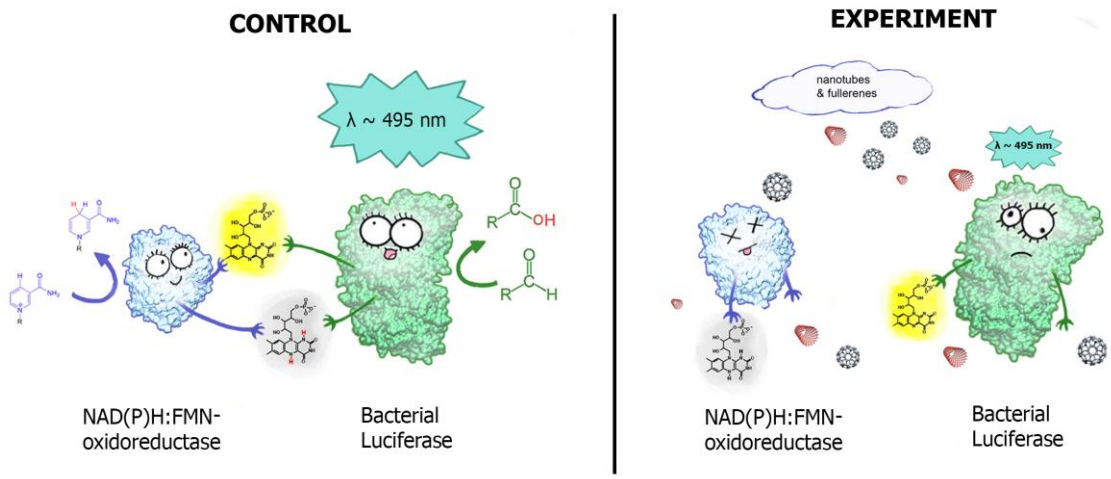

Graphical abstract

Highlights

- Bioluminescent enzymatic assay for CNM potential toxicity was proposed
- The assay predicts toxicity decreasing in the following order: MWCNT>SWCNT>C_{60}HyFn
- Soluble and immobilized enzymes differ qualitatively in CNM toxicity prediction