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EXPERIMENTAL ARTICLES

Application of the Inhibition of Bacterial Bioluminescence Test for Assessment of Toxicity of Carbon-Based Nanomaterials

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Abstract—A method for assessment of integral biological toxicity of carbon-based nanomaterials (CBN) using a bacterial luminescent biosensor was developed, which accounted for the properties of the objects under study. The proposed approach includes a special procedure for the preparation of the analyzed CBN samples aimed at obtaining highly dispersed suspensions, an extended period of dynamic monitoring of the luminescence of the contacting sensor microorganism, and the special algorithm for quantification of the results of the bioluminescence analysis, in order to exclude the effect of the optical properties of CBN on the results of investigation. This method was used for assessment of the biological toxicity of a broad spectrum of CBN, such as single- and multi-walled nanotubes, nanofibres, and C60- and C70-fullerenes, in order to compare them in this respect to amorphous (nonstructured) carbon.

Keywords: carbon-based nanomaterials, toxicity, biosensors, bioluminescence, *E. coli*

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atural and recombinant luminescent microorganisms are currently an accepted tool for environmental, sanitary, toxicological, and other investigations [1]. Their practical application is based on assessment of activity of the luminescent system, which reacts by an integral decrease in intensity to the presence of chemical pollutants in the environment [2] or to the toxic properties of the newly synthesized compounds [3]. An important advantage of the test of inhibition of bacterial bioluminescence, apart from its sensitivity and speed of response, is its good correlation with the results obtained by more complex procedures of biotesting [4].

Investigation of the biological toxicity of carbon-based nanomaterials (CBN), which are presently considered among important potential contaminants of natural ecosystems and human habitats, is a promising field for the application of bioluminescence analysis [5]. Thus, the actual national guidelines [6] specify the testing of nanomaterials using the recombinant luminescent strain *Escherichia coli* K12 TG1 with the cloned *luxCDABE* genes of *Photobacterium leiognathi*. The rate of suppression of the luminescence of the test organism is considered a measure of integral biological toxicity.

However, the first instance of such biotesting [7], as well as the analogous attempts to use the Microtox test system based on the marine luminescent bacterium *Vibrio fischeri* NRRL B-11177 [8, 9], revealed a number of limitations of bioluminescent analysis, which depended on the physicochemical and optical characteristics of CBN.

Thus, the goal of the present work was to develop the methodical basis for assessment of integral biological toxicity of carbon-based nanomaterials by luminescent analysis, as well as to carry out comparative investigation of a representative sample of commercially available and laboratory CBN.

MATERIALS AND METHODS

The recombinant strain *E. coli* K12 TG1 with constructive expression of the *luxCDABE* genes of the marine luminescent microorganism *P. leiognathi* [10] was used as an object of treatment. The strain is produced by Immunotekh (Russia) under the commercial name Ecolum and is recommended for the proclaimed purposes by the actual national guideline [6].

Immediately before the work, the lyophilized preparation was hydrated with 10 mL of cooled distilled water and incubated for 30 min at 2–4°C; the temperature was then increased to 15–25°C. The subsequent biotesting procedure included preparation of suspensions of the analyzed compounds, their mixing and incubation with the sensor microorganism, and quantitative assessment of bioluminescence in experimental and control samples in order to calculate the toxicity indices of the tested CBN.

The investigated CBN included four commercial preparations of single-walled carbon nanotubes (SWNT) produced by NanoCarbLab (Russia) [11], namely, the samples with 2–5% of the terminal

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COOH-groups (SWNT-1) or NH_2-groups (SWNT-2), the shortened variants with 5–10% of the terminal COOH-groups (SWNT-3), and single-walled carbon nanotubes with COOH-groups annealed under vacuum (SWNT-4). Laboratory CBN preparations included nanofibres (NF) synthesized in the Mendeleev Russian University of Chemistry and Technology, multi-walled carbon nanotubes (MWNT) developed in the Institute of the Problems of Chemical Physics, Russian Academy of Sciences, C60- and C70-fullerenes, and amorphous carbon (AC). Investigation of these CBN samples on an Elan-6100 mass spectrometer (PerkinElmer, United States) confirmed that most of them were at least 99% pure from attached foreign material, with the SWNT-4 sample 97.64% pure [12]. Morphological characterization of the CBN confirming their compliance with the declared parameters was previously carried out using atomic force microscopy [13].

To prepare CBN suspensions, a weighted portion (4 mg for nanotubes, NF, and AC and 4×10^{-3} mM for fullerenes) was placed into a glass vessel and supplemented with 1 mL of distilled water, dimethyl sulfoxide (DMSO), or ethanol. Half of the suspension was mixed by intensive pipetting, while the other half was sonicated for 5–30 min at 35 kHz in a Sapfir TTTs bath sonicator (Sapfir, Russia). The degree of dispersion for the CBN suspensions formed with different solvents and sonicated or not sonicated was assessed by their sedimentation stability at 100, 1000, and 10000 *g* (MiniSpin centrifuge, Eppendorf, Germany) with subsequent testing of the supernatants on a Flyuorat-02 Panorama spectrofluorimeter (Lyumeks, Russia).

The CBN samples (10 μL) were injected into the wells of 96-well plates containing 190 μL of distilled water. From this mixture, a series of twofold dilutions up to 1 : 1024 was prepared. Depending on the initial character of the CBN suspensions, distilled water or 2.5% DMSO aqueous solution was used. The control samples contained the solvents alone.

At the next stage, the wells were supplemented with 100 μL of the bacterial biosensor and placed into the measuring block of an LM-01T luminometer (Immunotech, Czech Republic). The luminescence intensity was monitored for 180 min with 2-min intervals. The toxicity indices of the samples were determined from their effect on the intensity of bioluminescence according to the equation $\frac{Ik_{0\min} \times Io_{n\min}}{Ik_{n\min} \times Io_{0\min}}$, where *Ik* and *Io* are the luminescence intensities in the control and experimental samples at 0, 60, 120, and 180 min of incubation. Based on these data, the values of the toxicological parameters EC_{20} and EC_{50} were calculated, which corresponded to the CBN concentrations resulting in 20 and 50% inhibition (compared to the control) of the luminescence of the test microorganism.

All experiments were carried out in at least three repeats and treated by the variation statistical methods using the Statistics V8 software package (StatSoft, United States), including the modules of multifactor dispersion and correlation analyses.

RESULTS AND DISCUSSION

Bioluminescence analysis in the variant recommended for assessment of the biological toxicity of nanoparticles and nanomaterials [6] did not reveal this activity in any of the studied CBN samples. This was somewhat contradictory to the results of CBN testing by other microbiological techniques [14, 15]. The lyophobic (in a particular case, hydrophobic) behavior of the carbon compounds under study, which formed polydisperse sedimentationally instable suspensions in aquatic environment.

The steps used in order to obtain higher dispersion of the CBN suspensions were sonication, formation of suspensions in organic solvents with the dielectric constant lower than that of water ($\varepsilon = 81$), and a combination of these approaches.

Sedimentation analysis of the nanocarbon suspensions obtained by ultrasound treatment (35 kHz) for various periods of time confirmed that 30-min treatment was sufficient, since longer sonication did not result in reliable increase of the parameters of dispersion. Formation of the CBN suspensions based on the solvents with the dielectric constant (ε) lower than that of water also resulted in an increased share of medium- and fine-dispersed fractions. In spite of its higher dielectric constant, the aprotonic solvent DMSO ($\varepsilon = 45$) was more efficient than the protonic solvent ethanol ($\varepsilon = 27$).

Dispersion analysis was used to determine the contribution of each of these approaches. Analysis revealed that the physicochemical properties of CBN were the major factor responsible for dispersion of the suspensions (43.71% of the degree of dispersion; $P < 0.001$). Thus, dispersion had a pronounced tendency to decrease with increased structuring of the nanocarbon particles and decreasing number of polar groups on their surface.

Ultrasound treatment was the second most important factor increasing the dispersion of suspensions (17.76%; $P < 0.01$), which assured mechanical disruption of the CBN aggregates.

The solvents with the dielectric constant lower than that of water had a lower impact (8.71%; $P < 0.05$). The effect revealed was probably caused by the adsorption–solvation mechanism, resulting in decreased surface energy during adsorption of the molecules of the disperse medium on the surface of carbon nanoparticles.

Finally, the combination of ultrasound treatment with primary dispersion of the CBN in organic solvents had no pronounced additive effect (2.92%; $P >$

0.05), although it increased the dispersion of suspensions to a higher degree than each approach alone.

Application of DMSO for primary dispersion of the CBN required determination of the solvent concentration which had no toxic effect on the bioluminescence of the bacterial biosensor, *E. coli* K12 TG1 with the cloned *P. leiognathi luxCDABE* genes. Comparison of the bioluminescence intensity of the test organism in the presence of different DMSO concentrations revealed the absence of its inhibitory effect in the concentrations of 2.5% and less from the total volume of the sample.

Thus, the following three-stage procedure may be recommended for production of highly dispersed suspensions of carbon nanocompounds under study: addition of the CBNs into the aprotonic solvent DMSO, sonication of the mixture (35 kHz, 30 min), and transfer of the formed suspension into the water phase with subsequent dilution in 2.5% water solutions of DMSO. These procedures resulted in a significant increase in the CBN dispersion, accompanied by enhanced biological activity as characterized by the toxicological parameters EC_{20} and EC_{50} (see below).

Enhanced dispersion of the CBN suspensions also resulted in changes in their optical properties, including a significant decrease in light transmittance and inadequate signal transfer from the biosensor to the light receiver.

The previously proposed solution for this problem [16] included measurement of the absorption parameters of the suspensions and a series of calculations, making the biotesting of significant numbers of the CBN suspension samples a labor-intensive task. In the present work, therefore, the calculation similar to that for assessment of bioluminescence in turbid and colored suspension [8] is proposed, using the equation $\frac{Ik_{0\min} \times Io_{n\min}}{Ik_{n\min} \times Io_{0\min}}$, where $Ik_{0\min}$ and $Ik_{n\min}$ are the luminescence intensities of the control sample at the *0*th and *n*th minutes, respectively, which reflects the kinetics of spontaneous quenching of the bacterial biosensor due to increasing consumption of the energy substrates for bioluminescence. $Io_{0\min}$ and $Io_{n\min}$ are the respective luminescence intensities at the 0th minute, prior to addition of the sensor microorganism to the CBN suspensions (which accounts for the contribution of their optical properties to a decrease in the registered parameter) and at the *n*th minute (which integrates the optical and toxic characteristics of the investigated nanocarbon compound).

The character of dynamics of the development of the toxic effect of CBN, which differs significantly from that of the "classical" water-soluble toxins, is an additional reason for the application of this algorithm. For instance, the registration of the luminescence of the sensor strain in nanocarbon suspensions revealed a decrease in the bioluminescence intensity during the first minutes of contact (figure). However, the quantitative parameters of the inhibition of bioluminescence exhibited a linear relationship with the intensity of light absorbance in the CBN suspensions, did not change significantly during the subsequent 15 min, and did not correlate with the development of the bactericidal effect. This suggests a critical attitude to the data of [9] concerning the possibility of "rapid" determination of the biological activity of multi-walled carbon nanotubes, since the authors probably misinterpreted the optical effects of CBN as a manifestation of their toxic properties.

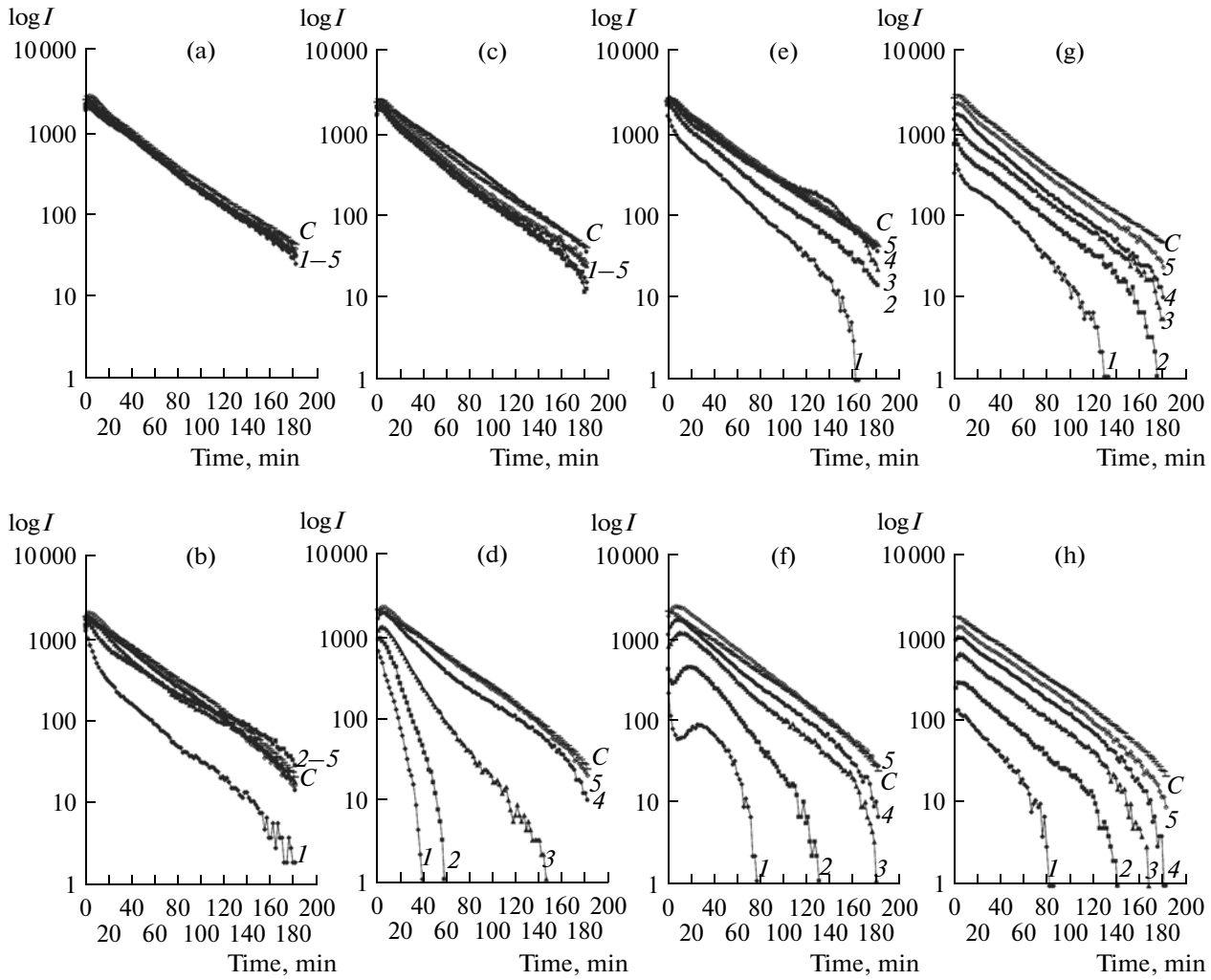
In some cases, increased time of contact between CBN and the test organism to 30–45 min resulted in a relative stimulation of bioluminescence, rather than its suppression. This effect was especially pronounced in the case of some SWNT preparations (figure, f), in agreement with the results of [7], where the possibility of increased bioluminescence intensity was shown for a recombinant strain *E. coli* K12 TG1 during short-term contact with single-walled carbon nanotubes. In this case, however, the stimulation of bioluminescence indicated only the biological activity of the investigated nanomaterials, rather than their toxic effect.

Further increase in the duration of existence of the reaction systems resulted in real suppression of the luminescence of the sensor microorganism. This effect increased with the concentration of the tested CBN and resulted in the death of bacterial cells. The registered parameters depended significantly on the nature of the tested nanocarbon compounds (figure, a, c, e, g) and on the degree of their dispersion determined by the combination of sonication and initial suspending in the aprotonic solvent DMSO (figure, b, d, f, h).

These results demonstrate that the duration of biotesting should be increased from the usual 5–30 min to 180 min. This change will result in more complete detection of the biological activity of nanocarbon, which is characterized by slower rates of development compared to molecular toxicants. On the other hand, increasing the time of contact over 180 min is technically impossible due to exhaustion of the luminescence potential of the bacterial sensor, which may be observed as a decrease in the luminescence (to the values undistinguished from zero) of intact cells in the control samples.

In this context, the results of bioluminescence testing of SWNT during 24–72 h, which is comparable to the duration of assessment of chronic toxicity on growth media, but without addition of growth substrates for the sensor strain *E. coli* K12 TG1, presented in [7], look questionable. However, the introduction of the components of nutrient media into the analyzed samples may result in aggregation of nanocarbon particles [17], creating addition obstacles for detection of their toxicity by luminescent and other bacterial models.

Thus, the above data made it possible to determine the ways of modification of the bioluminescence anal-



Parameters of the bioluminescent dynamics of *E. coli* K12 TG1 with the cloned *P. leiognathi luxCDABE* genes contacting with the CBN suspensions based on distilled water (a, c, e, g) and 2.5% DMSO (b, d, f, h). Designations: C60-fullerene (a, b); MWNT (c, d); SWNT-4 (e, f); and SWNT-3 (g, h) at 100 (*1*), 50 (*2*), 25 (*3*), 12.5 (*4*), and 6.25 (*5*) μM for C60-fullerene or μg/mL for MWNT and SWNT; control (*C*). The X value shows time of contact, min. The Y value shows the registered luminescence intensities (*I*).

ysis for assessment of integral toxicity of carbon-based nanomaterials, considering the physicochemical characteristics and biological activity of the objects under study. The proposed approach was based on: (i) special procedure for the preparation of the nanocarbon samples by combined sonication and initial suspending in DMSO in order to obtain finely dispersed suspensions; (ii) increased duration (to 180 min) of the contact between the sensory luminescent microorganism and CBN; and (iii) the special algorithm for the calculation of the index of bioluminescence suppression, which makes it possible to exclude the effect of the optical properties of the CBN on the results of analysis.

The results of testing the biological toxicity of investigated CBN samples using the proposed procedure are presented in the table. The toxicological parameters EC_{20} and EC_{50} calculated after 60, 120, and 180 min of contact were used as integral characteristics of the registered biological activity. The results are presented in comparison to the biotesting variant without DMSO.

Thus, only weak biological toxicity or none was detected in some CBN suspensions formed in distilled water with sonication but without DMSO. This applies to the C60-fullerene and SWNT-2, which had no reliable effect on the luminescence of the bacterial biosensor within the range of experimental concentrations at any duration of interaction. For water suspensions of C70-fullerene, MWNT, and NF, the final EC_{20} values were within the range of 70–100 μg/mL, while the probable EC_{50} values were still above the maximal tested concentration of 100 μg/mL. SWNT-1, -3, and -4 behaved as much more active

Values of the toxicological parameters EC_{20} and EC_{50} (μg/mL or μM for fullerenes) determined for CBN suspensions obtained by different techniques assessed by their effect on bioluminescence of the sensor strain *E. coli* K12 TG1 with the cloned *P. leiognathi luxCDABE* genes

Carbon-based nanomaterials	Dispersion method	Contact duration					
		60 min		120 min		180 min	
		EC_{20}	EC_{50}	EC_{20}	EC_{50}	EC_{20}	EC_{50}
Single-walled carbon nanotubes with 2–5% of terminal COOH-groups (SWNT-1)	US	>100	>100	>100	>100	30 ± 1.56	62 ± 3.62
	US + DMSO	>100	>100	63 ± 2.78	77 ± 4.05	12 ± 0.64	18 ± 0.72
Single-walled carbon nanotubes with 2–5% of terminal NH_2-groups (SWNT-2)	US	>100	>100	>100	>100	>100	>100
	US + DMSO	>100	>100	>100	>100	35 ± 1.54	48 ± 2.22
Shortened single-walled carbon nanotubes with 5–10% of terminal COOH-groups (SWNT-3)	US	>100	>100	52 ± 2.39	85 ± 5.01	22 ± 1.07	54 ± 2.81
	US + DMSO	70 ± 3.55	>100	45 ± 2.54	66 ± 3.82	10 ± 4.59	17 ± 0.98
Single-walled carbon nanotubes with annealed COOH-groups (SWNT-4)	US	>100	>100	66 ± 3.22	90 ± 4.98	50 ± 5.03	78 ± 3.89
	US + DMSO	67 ± 3.33	92 ± 4.58	39 ± 2.04	57 ± 2.55	30 ± 1.27	46 ± 2.98
Carbon nanofibres (CNF)	US	>100	>100	>100	>100	90 ± 0.45	>100
	US + DMSO	>100	>100	52 ± 2.63	60 ± 3.04	14 ± 0.65	20 ± 0.97
Multi-walled carbon nanotubes (MWNT)	US	>100	>100	>100	>100	99 ± 0.46	>100
	US + DMSO	52 ± 2.53	65 ± 3.37	38 ± 1.57	48 ± 2.26	23 ± 0.13	28 ± 0.11
C60-fullerene	US	>100	>100	>100	>100	>100	>100
	US + DMSO	>100	>100	87 ± 4.85	>100	72 ± 3.59	84 ± 4.34
C70-fullerene	US	>100	>100	>100	>100	70 ± 3.48	>100
	US + DMSO	65 ± 3.71	71 ± 3.66	64 ± 3.84	69 ± 4.06	44 ± 2.14	50 ± 2.53
Amorphous carbon (AC)	US	>100	>100	48 ± 2.28	98 ± 0.63	8 ± 0.05	40 ± 0.23
	US + DMSO	65 ± 3.36	>100	32 ± 1.56	44 ± 1.99	4 ± 0.01	8 ± 0.05

compounds, with shortened single-walled carbon nanotubes with 5–10% of the terminal COOH-groups being the most toxic (the final EC_{20} and EC_{50} values were 22 and 54 μg/mL, respectively). The highest level of toxicity was detected in the sample of amorphous carbon, for which the EC_{20} and EC_{50} values after 180 min were 8 and 40 μg/mL, respectively.

Increased dispersion of the CBN by sonication, initial suspension in DMSO, and subsequent transfer of the resulting suspension to aquatic environment resulted in higher rate and expression of the toxic effects. Under such conditions, amorphous carbon was the most toxic compound, with the EC_{20} and EC_{50} values of 4 and 8 μg/mL, respectively. The SWNT samples exhibited similar levels of toxicity, with C_{20} values within the 10–35 μg/mL range and C_{50} from 17 to 48 μg/mL. Increased dispersion of the CBN resulted in toxicity of NF, MWNT, and C70-fullerenes, for which EC_{50} values increased 1.5–5-fold, to 23, 20, and 50 μg/mL, respectively. Under these conditions, C60-fullerene remained the most biologically inert nanocarbon compound, with the inhibition of bioluminescence only at its high concentrations and the EC_{20} and EC_{50} values of 72 and 84 μM, respectively.

The results obtained made it possible to scale the investigated nanocarbon compounds according to their toxic effect on the sensory luminescent strain *E. coli* K12 TG1 with cloned *luxCDABE* genes of the marine luminescent bacterium *P. leiognathi*. While the absolute values of the CBN biological toxicity clearly depended on the degree of dispersion of nanocarbon compounds achieved by different techniques, the relative differences between them depended on the original characteristics of the compounds. This conclusion is confirmed by the results of statistical analysis showing a pronounced correlation between the EC_{20} ($r = 0.650$; $P < 0.05$) and EC_{50} ($r = 0.615$; $P < 0.05$) values determined in two experimental series: in CBN suspensions in aquatic medium obtained by sonication and in those involving additional application of the aprotonic solvent DMSO. It was also shown that high levels of carbon structuring in the nanomaterials under study did not result in their increased toxicity for the

test microorganism compared to unstructured (amorphous) carbon, which was the most biologically active compound in this system of biological indication.

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