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## On mechanism of antioxidant effect of fullerenols

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## ABSTRACT

Fullerenols are nanosized water-soluble polyhydroxylated derivatives of fullerenes, specific allotropic form of carbon, bioactive compounds and perspective pharmaceutical agents. Antioxidant activity of fullerenols was studied in model solutions of organic and inorganic toxicants of oxidative type – 1,4-benzoquinone and potassium ferricyanide. Two fullereneol preparations were tested:  $C_{60}O_{2-4}(OH)_{20-24}$  and mixture of two types of fullereneols  $C_{60}O_{2-4}(OH)_{20-24} + C_{70}O_{2-4}(OH)_{20-24}$ . Bacteria-based and enzyme-based bioluminescent assays were used to evaluate a decrease in cellular and biochemical toxicities, respectively. Additionally, the enzyme-based assay was used for the direct monitoring of efficiency of the oxidative enzymatic processes. The bacteria-based and enzyme-based assays showed similar peculiarities of the detoxification processes: (1) ultralow concentrations of fullereneols were active (ca  $10^{-17}$ – $10^{-4}$  and  $10^{-17}$ – $10^{-5}$  g/L, respectively), (2) no monotonic dependence of detoxification efficiency on fullereneol concentrations was observed, and (3) detoxification of organic oxidizer solutions was more effective than that of the inorganic oxidizer. The antioxidant effect of highly diluted fullereneol solutions on bacterial cells was attributed to hormesis phenomenon; the detoxification was concerned with stimulation of adaptive cellular response under low-dose exposures. Sequence analysis of 16S ribosomal RNA was carried out; it did not reveal mutations in bacterial DNA. The suggestion was made that hydrophobic membrane-dependent processes are involved to the detoxifying mechanism. Catalytic activity of fullereneol ( $10^{-8}$  g/L) in NADH-dependent enzymatic reactions was demonstrated and supposed to contribute to adaptive bacterial response.

## 1. Introduction

Fullerenols are bioactive compounds, polyhydroxylated water-soluble derivatives of fullerenes, specific allotropic form of carbon [1]. They are promising for application in different fields of physics, chemistry, nanobiotechnology, pharmacology, and medicine [2–5]. Chemical structure of a representative of fullereneol group,  $C_{60}(OH)_x$ , is presented in Fig. 1.

Similar to fullerene  $C_{60}$ , fullereneols behave like electron deficient agents and readily react with electron rich species. This feature makes fullereneols effective catalysts in chemical and biochemical processes. Fullereneols are amphiphilic structures: hydroxyl groups provide them with aqueous solubility while the fragments of fullerene skeleton – with affinity to lipid structures of cellular membranes [6,7]. The aque-

ous solubility of fullereneols depends on the amount of hydroxyl groups [8].

Due to hydrophilic properties and ability to scavenge free radicals, fullereneols could provide a serious alternative to the conventional pharmacological agents in chemotherapy, treatment of neurodegenerative diseases, and radiobiology [4,9]. A range of fullereneol biological effects is wide: from cell protection [4,6] to drug transport [10] and neutralization [4]. Biological activity of a series of fullereneols  $C_{60}(OH)_{12-14}$ ,  $C_{60}(OH)_{18-24}$ ,  $C_{60}(OH)_{30-38}$ , was studied by Eroshkin and co-workers [8]. Fullereneols  $C_{60}(OH)_{18-24}$  revealed maximal biological activity [3,4,11]. The fullereneols demonstrated antioxidant activity, neutralizing reactive oxygen and nitrogen species [5,11–13]. The antioxidant property endows fullereneols with ability to treat neurological

Abbreviations: NADH, nicotinamide adenine dinucleotide disodium salt reduced; FMN, flavinmononucleotide; GT, general toxicity; OxT, oxidative toxicity; F-60, fullereneol  $C_{60}O_{2-4}(OH)_{20-24}$ ; F-60, 70, mixture of fullereneols  $C_{60}O_{2-4}(OH)_{20-24}$  and  $C_{70}O_{2-4}(OH)_{20-24}$ .

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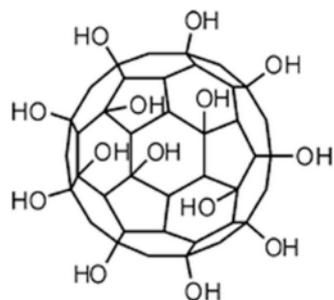


Fig. 1. Hypothetical structure of fullereneol  $C_{60}(OH)_x$  [1].

diseases [4,5,12,14], to function as radioprotectors [12] or antitumor agents [15].

Luminous marine bacteria are proper candidates for study the antioxidant activity of fullereneols. The bacteria have been used as a toxicity bioassay for several decades [16–20]. The tested parameter here is luminescence intensity; it can be easily measured instrumentally with simple physical devices. High rates of bioluminescence registration and simplicity of the test organism pave the way for simultaneous analyses of a lot of test-samples under comparable external conditions resulting in a proper statistical treatment.

Bacterial bioluminescent assays can be based on biological systems of different complexity – bacteria or their enzymes [17,21,22]. Along with water-soluble bacterial preparations, the solid preparations of immobilized bacteria and their enzymes have been developed [23–27]. The bacteria-based and enzyme-based assays allow studying mechanisms of toxic effects at cellular and molecular levels, respectively. First classification of toxic effects in the bioluminescent enzyme system, based on physicochemical, chemical, and biochemical processes, was suggested in [28] and developed in [29–32].

Evaluation of antioxidant activity of bioactive compounds is a novel and prospective field in biosensor application [33,34]. The assay systems based on luminous marine bacteria and/or their enzyme reactions are proper candidates in this field. The both bioassays, cellular and enzymatic, can be used to evaluate general toxicity of test samples under conditions of oxidative stress. Additionally, the enzymatic assay is specific to oxidizers [32]; therefore it can be used for direct monitoring of oxidative toxicity of solutions. The oxidative toxicity is attributed to redox activity of toxic compounds, while the general toxicity considers, in a nonadditive way, all interactions of exogenous compounds with the components of the bioluminescent enzymatic assay system – redox reactions, hydrophobic and polar interactions. Previously [35,36] the general and oxidative toxicities of solutions of organic and inorganic oxidizers, quinones and polyvalent metals, were studied using the bioluminescent enzymatic assay. Decrease of both general and oxidative toxicities under addition of humic substances (products of natural decomposition of organic matter in soil and bottom sediments) was studied in [37–39]. Description of the enzyme bioluminescent technique to evaluate antioxidant activity and toxicity of bioactive compounds is presented in [40]. It should be noted that, similar to reactive oxygen species, active chlorine species can also produce toxic effects [41].

Current work uses fullereneols as bioactive compound models; the oxidative stress is simulated in solutions of model inorganic and organic oxidizers – complex salt potassium ferricyanide  $K_3[Fe(CN)_6]$  and 1,4-benzoquinone, respectively. Potassium ferricyanide was chosen because of its stability in water solutions (in contrast to unclustered iron salts) and mono-electron oxidative transition  $Fe^{3+}/Fe^{2+}$ . The 1,4-benzoquinone was chosen being a typical representative of the organic oxidizer group. In nature, quinones can appear as a result of oxidative transformation of various phenols [42,43], i.e. numerous group of hydroxylated aromatic compounds, third in the top list of widespread pol-

lutants (after metal salts and oil products), and frequent components of industrial wastewaters.

Current study evaluates changes of general and oxidative toxicities under addition of fullereneols as detoxifying agents. Cellular and enzymatic assays are used for the evaluation of the toxicities. A wide range of the fullereneol concentrations is tested, including ultralow concentrations. Detoxifying activities of two types of fullereneols ( $C_{60}O_{2-4}(OH)_{20-24}$  and a mixture of fullereneols  $C_{60}O_{2-4}(OH)_{20-24} + C_{70}O_{2-4}(OH)_{20-24}$ ) are under investigation. Additionally, our work focuses on chemical and biochemical processes taking place in the course of detoxification of the oxidizer solutions; the rates of chemical and biochemical processes in oxidizer + fullereneol solutions are analyzed.

The paper describes the results in the following sequence: Section 3.1 presents the evaluation of proper conditions for toxicity measurements focusing on fullereneol and oxidizer concentrations; mutagenicity of the oxidizer solutions is tested; Section 3.2 studies a response of bacterial cells to the fullereneol exposure in the oxidizer solutions; changes of general toxicity are determined; Section 3.3 studies the effects of the fullereneols on the bioluminescent enzymatic system; changes of general and oxidative toxicities are evaluated; rates of chemical and biochemical reactions are determined. Section Discussion considers peculiarities of fullereneol detoxifying effect (4.1) and presents speculations on mechanism of biological activity of highly diluted fullereneol solutions (4.2).

## 2. Materials and methods

### 2.1. Reagents and equipment

Toxicity of water solutions of model oxidizers  $K_3[Fe(CN)_6]$  (potassium ferricyanide) and 1,4-benzoquinone was assessed using two bioluminescent assay systems: (1) bacterial assay, i.e. Microbiosensor 677F, based on the lyophilized luminous bacteria *Photobacterium phosphoreum*, and (2) enzymatic assay, i.e. the preparation based on the coupled enzyme system NADH: FMN-oxidoreductase from *Vibrio fischeri* (0.15 a.u.) and luciferase from *Photobacterium leiognathi*, 0.5 mg/ml [44]. All the biological preparations were produced at the Institute of Biophysics SB RAS (Krasnoyarsk, Russia). The chemicals used were: NADH from ICN, USA; FMN, and tetradecanal from SERVA, Germany; potassium ferricyanide of analytical grade, Khimreaktiv, Russia; 1,4-benzoquinone, Aldrich, USA.

The enzymatic system includes two coupled reactions:



To construct the enzyme system, 0.1 mg/ml enzyme preparation,  $5 \cdot 10^{-4}$  M FMN,  $4 \cdot 10^{-4}$  M NADH, and 0.002% tetradecanal solutions were used. The assay was performed in a 0.05 M phosphate buffer (pH 6.8) at room temperature.

Fullerenes were synthesized by carbon helium high-frequency arc plasma at atmospheric pressure [45,46]. The fullerene content in carbon soot was about 12.6%. Fullerene mixture was extracted with toluene, and the individual  $C_{60}$  fullerene was separated by liquid chromatography with turbostratic graphite (with interplanar distance 3.42 Å) as a stationary phase and toluene/hexane (4:6) mixture as a mobile phase. Fullereneol  $C_{60}O_{2-4}(OH)_{20-24}$  (F-60) and mixture of fullereneols  $C_{60}O_{2-4}(OH)_{20-24}$  and  $C_{70}O_{2-4}(OH)_{20-24}$  (with 60% of F-60) (F-60,70) were produced by fullerene hydroxylation in nitric acid followed by the hydrolysis of the polynitrofullerenes [3,46,47]. The fullereneol preparations were characterized with IR and photoelectron spectroscopies [48].

Measurements of bioluminescent intensity were carried out with bioluminometers BLM-3606 (Nauka Special Design Bureau, Russia) and TriStar LB 941 (Berthold Technologies, Germany). Optical density of solutions was measured by double-beam spectrophotometer UVIKON-943 (KONTRON Instruments, Italy).

## 2.2. Experimental data processing

Effects of fullerenols on bioluminescence of bacterial and enzyme assay systems were evaluated by relative bioluminescent intensity,  $I_F^{rel}$ :

$$I_F^{rel} = I_F / I_{contr} \quad (3)$$

Here,  $I_{contr}$  and  $I_F$  are maximal bioluminescent intensities in the absence and presence of fullerenols, respectively.

General Toxicity (GT) of the oxidizer solutions was evaluated by a similar way (Eq. (3a)), i.e. with relative bioluminescent intensity,  $I_{Ox}^{rel}$ :

$$I_{Ox}^{rel} = I_{Ox} / I_{contr} \quad (3a)$$

Here,  $I_{contr}$  and  $I_{Ox}$  are maximal bioluminescent intensities in the absence and presence of oxidizers, respectively.

Effective concentrations of oxidizers decreasing bioluminescent intensity by 50% ( $I_{Ox}^{rel} = 0.5$ ),  $EC_{50}$ , were determined.

To characterize the changes of General Toxicity (GT) under the exposure to fullerenols, both bioluminescent assays, bacterial and enzymatic, were applied. The detoxification coefficients,  $D_{GT}$ , were calculated as:

$$D_{GT} = I_{Ox+F}^{rel} / I_{Ox}^{rel}, \quad (4)$$

where  $I_{Ox+F}^{rel}$  and  $I_{Ox}^{rel}$  are relative bioluminescent intensities in ox-

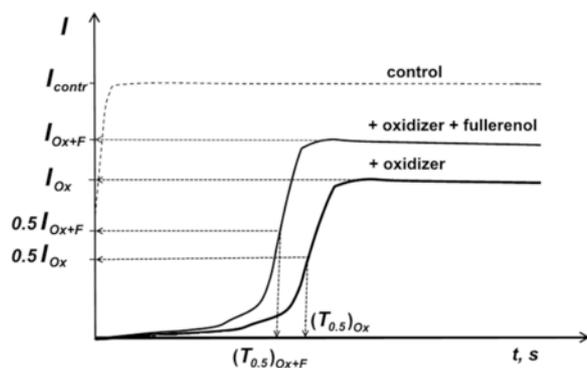


Fig. 2. Bioluminescent kinetics in solution of a model oxidizer (Ox) and fullereneol (F).

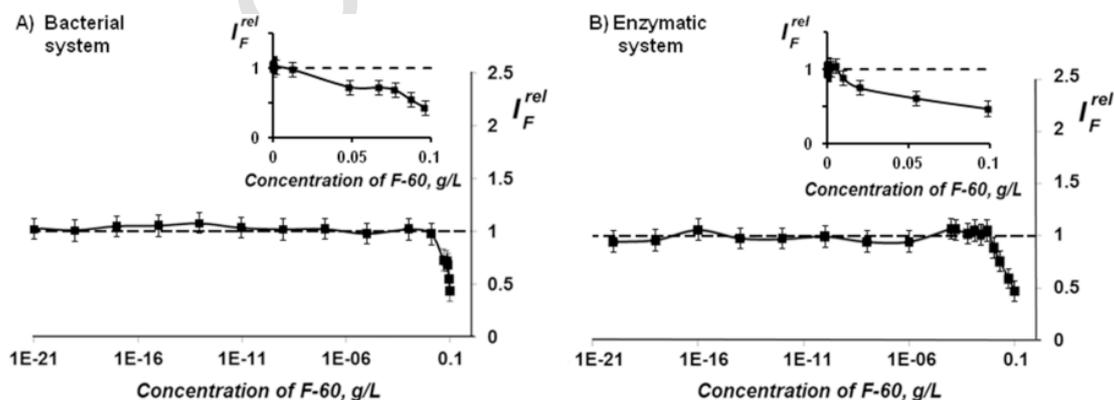


Fig. 3. Bioluminescent intensity  $I_F^{rel}$  at different concentrations of F-60 in bacterial (A) and enzymatic (B) systems.

dizer solutions at  $EC_{50}$ , in the presence and absence of fullerenols, respectively. Values of  $D_{GT}$  were determined at different fullereneol concentrations.

To characterize the Oxidative Toxicity (OxT) of oxidizer solutions, the bioluminescent enzymatic assay was used; an example of the bioluminescent kinetics is schematically presented in Fig. 2. Bioluminescence delay period in oxidizer solutions,  $(T_{0.5})_{Ox}$ , is indicated in this Figure. Changes of Oxidative Toxicity (OxT) under fullereneol exposure were characterized with detoxification coefficients,  $D_{OxT}$ :

$$D_{OxT} = (T_{0.5})_{Ox} / (T_{0.5})_{Ox+F} \quad (5)$$

here,  $(T_{0.5})_{Ox+F}$  and  $(T_{0.5})_{Ox}$  are bioluminescence delay periods in oxidizer solutions in the presence and absence of fullerenols, respectively. Values of  $D_{OxT}$  were determined at different fullereneol concentrations.

Values of  $D_{GT} > 1$  and  $D_{OxT} > 1$  showed a decrease of GT and OxT in oxidizer solutions under the exposure to fullerenols, i.e. detoxification of the oxidizer solutions. Values of  $D_{GT} \approx 1$  and  $D_{OxT} \approx 1$  showed the absence of the fullereneol effects.

Values of SD for  $D_{GT}$  and  $D_{OxT}$  did not exceed 0.1. The data for the calculations of  $D_{GT}$  or  $D_{OxT}$  were obtained in three parallel experiments with five samplings for all fullereneol and control solutions. Values of SD for NADH oxidation rates were  $10^{-8}$  M/min; the data for the rate calculations were obtained in three experiments with five samplings in each of them.

## 2.3. Sequence analysis

Mutagenic effect of the oxidizer solutions was examined using sequence analysis of the 16S ribosomal RNA gene of *P.Phosphoreum*. This gene was chosen for genetic analysis as a model for evaluating nonspecific DNA damage. The analysis was performed on the samples of bacterial suspensions exposed to 1,4-benzoquinone ( $2 \cdot 10^{-7}$  M and  $10^{-6}$  M); the results were compared to those obtained on the control bacterial suspensions.

## 3. Results

### 3.1. Evaluation of fullereneol and oxidizer concentrations for toxicity measurements

#### 3.1.1. Influence of fullerenols on bioluminescent intensity

Fig. 3 presents a dependence of bioluminescent intensity ( $I_F^{rel}$ , Eq. (3)) of luminous bacterial and enzyme assay system on concentrations of fullereneol F-60. As is evident from this Figure, the fullereneol suppresses bioluminescence of the bacterial and enzymatic systems at con-

centrations  $>10^{-2}$  g/L and  $>5\cdot10^{-3}$  g/L, respectively. Similar results were obtained for the other fullerene preparation, F-60,70.

The suppression of bioluminescent intensity is an evidence of fullerene toxic effect; it is concerned with inhibition of membrane and intracellular processes (for bacterial cells) or chemical and biochemical reactions (for enzyme system). Additional reasons for the bioluminescence suppression can be: the effect of “optic filter” as a result of bioluminescence absorption/reabsorption [17] and “concentration quenching” resulted from collisional intermolecular interactions. The latter processes are concerned with peculiarities of the luminescence registration in solutions and do not contribute to toxic effects.

Basing on the experiments described, we chose the range of fullerene concentrations for further experiments ( $<10^{-4}$  g/L) providing the absence of the fullerene inhibiting effect. The fullerene concentrations varied in this range to decrease General Toxicity (GT) and Oxidative Toxicity (OxT) in solutions of oxidizers (organic and inorganic) as described below in Sections 3.2 and 3.3.

### 3.1.2. Oxidizers: effective concentrations ( $EC_{50}$ ) and mutagenicity

Effective concentrations of oxidizers decreasing bioluminescent intensity by 50% ( $I_{Ox}^{rel}=0.5$ ,  $EC_{50}$ ), were determined with bacterial and enzymatic bioluminescent assays. The  $EC_{50}$  values of 1,4-benzoquinone were  $2.5\cdot10^{-7}$  M and  $10^{-4}$  M for bacterial and enzymatic assays, respectively, while the  $EC_{50}$  values of potassium ferricyanide were  $4\cdot10^{-2}$  M and  $2\cdot10^{-4}$  M. Lower  $EC_{50}$  values of 1,4-benzoquinone reveal higher toxicity of this oxidizer. This result is supported by the differences in standard redox potentials (0.7 V and 0.36 V at neutral pH for 1,4-benzoquinone and potassium ferricyanide, respectively) [49] and hydrophobic characteristics of the oxidizers.

The  $EC_{50}$  values were applied in further experiments to monitor changes of General Toxicity (GT) in oxidizer solutions under addition of fullerenes, as described in Sections 3.2 and 3.3.1 for bacterial and enzymatic assays, respectively.

Mutagenic effect of organic oxidizer solutions (1,4-benzoquinone,  $2.5\cdot10^{-7}$  M and  $10^{-6}$  M) was examined using sequence analysis of the 16S ribosomal RNA gene; the results were compared to those obtained on the control bacterial suspension. No changes in the analyzed gene sequence were found under the conditions of the experiment. Hence, the analysis did not reveal any mutations in DNA of the bacteria exposed to the oxidizer.

### 3.2. Bacterial response to the fullerene exposure in solutions of oxidizers

Detoxifying ability of fullerenes was studied using the bacteria-based assay. Bioluminescent intensity of the bacteria was measured in solutions of model oxidizers (1,4-benzoquinone and potassium ferricyanide) at  $EC_{50}$  in the absence and presence of fullerenes F-60 and F-60,70. Concentrations of the fullerenes varied in a wide range as shown in Fig. 4. Detoxification coefficients  $D_{GT}$  were calculated according to Eq. (4).

Fig. 4 shows that 1,4-benzoquinone solutions were detoxified ( $D_{GT}>1$ ) in the concentration ranges of  $10^{-17}$ – $10^{-8}$  and  $10^{-17}$ – $10^{-4}$  g/L for F-60 and F-60,70, respectively. Maximal values of  $D_{GT}$  were about 1.5 and 1.8, respectively. In solutions of potassium ferricyanide, the values of  $D_{GT}$  were lower: they are not more than 1.3 for F-60,70 and close to “1” at all F-60 concentrations. No monotonic dependencies of  $D_{GT}$  on fullerene concentrations were observed in all cases.

### 3.3. Effects of fullerenes on enzymatic processes

This section presents fullerene effects on the bioluminescent system of coupled enzymatic reactions (reactions (1), (2)). It studies: (1) enzymatic activity characterized by bioluminescent intensity of the coupled enzymatic system ( $I$ , Figs. 2), (2) redox properties of the enzyme sys-

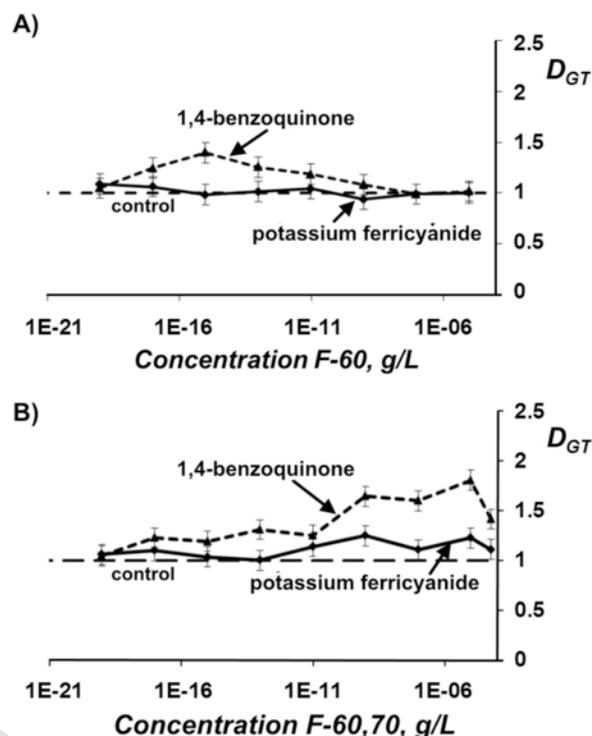


Fig. 4. Detoxification coefficients  $D_{GT}$  vs. concentration of fullerenes F-60 (A) and F-60,70 (B) in solutions of 1,4-benzoquinone ( $2.5\cdot10^{-7}$  M) and potassium ferricyanide ( $4\cdot10^{-2}$  M). Bacteria-based assay.

tem evaluated by the bioluminescence induction period ( $T_{0.5}$ , Figs. 2), and (3) rates of oxidation of NADH, endogenous reducer, a low-molecular component of the bioluminescent enzyme system. Changes of these characteristics are presented in Sections 3.3.1, 3.3.2, and 3.3.3, respectively.

#### 3.3.1. Changes of enzymatic activity under the action of fullerenes in oxidizer solutions

Bioluminescent intensity was measured in solutions of oxidizers in the absence and presence of fullerenes:  $I_{ox}$  and  $I_{ox+F}$ , respectively, Fig. 2. Detoxification coefficients  $D_{GT}$  calculated with Eq. (4), are presented in Fig. 5. Fig. 5A demonstrates detoxifying effect of F-60 ( $D_{GT} > 1$ ) in oxidizer solutions in at different fullerene concentrations. Lower detoxification coefficients  $D_{GT}$  were found under exposure to F-60,70, Fig. 5B. It is evident that  $D_{GT}$  values in solutions of organic oxidizer, 1,4-benzoquinone, are higher than those of inorganic oxidizer, potassium ferricyanide. Probably, hydrophobic properties of the solution components (fullerene + oxidizer) are critical for the enzymatic process, similar to the cellular process.

#### 3.3.2. Changes of redox properties of the bioluminescent enzyme system in oxidizer + fullerene solutions

Bioluminescent kinetics of the enzymatic system was studied in solutions of model organic and inorganic oxidizers. Induction periods were measured in the absence and presence of fullerenes:  $(T_{0.5})_{ox}$  and  $(T_{0.5})_{ox+F}$ , respectively, Fig. 2. Detoxification coefficients  $D_{OxT}$  were calculated with Eq. (5).

Fig. 6 demonstrates the dependences of  $D_{OxT}$  on fullerene concentrations. Detoxification coefficients  $D_{OxT}$  in the solutions of organic oxidizer 1,4-benzoquinone (Fig. 6A,B) were found to be higher than these in ferricyanide solutions; they reached up to “2” in the cases of both F-60 and F-60,70. These data demonstrate an effective decrease of oxidative toxicity in the range of  $ca\ 10^{-12}$ – $10^{-6}$  g/L for F-60, and  $ca\ 10^{-16}$ – $10^{-4}$  g/L for F-60,70.

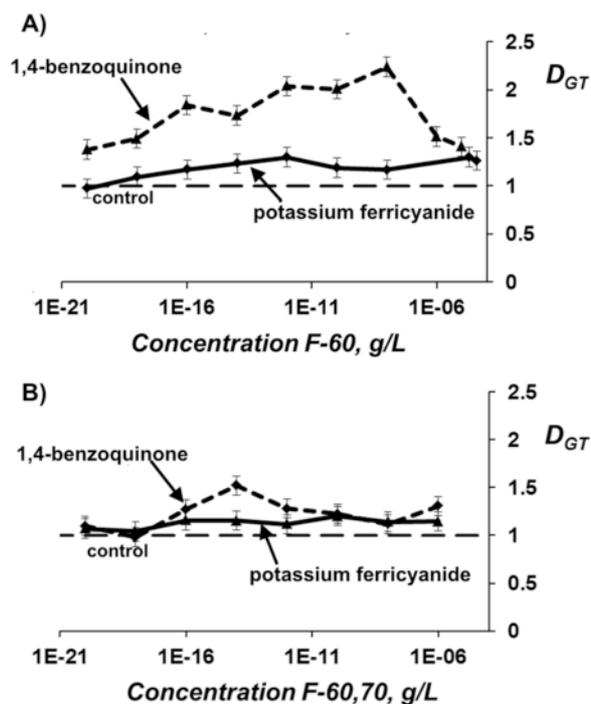


Fig. 5. Detoxification coefficients  $D_{GT}$  vs. concentration of fullereneols F-60 (A) and F-60,70 (B) in solutions of 1,4-benzoquinone ( $10^{-4}$  M) and potassium ferricyanide ( $2 \cdot 10^{-4}$  M). Enzyme-based assay.

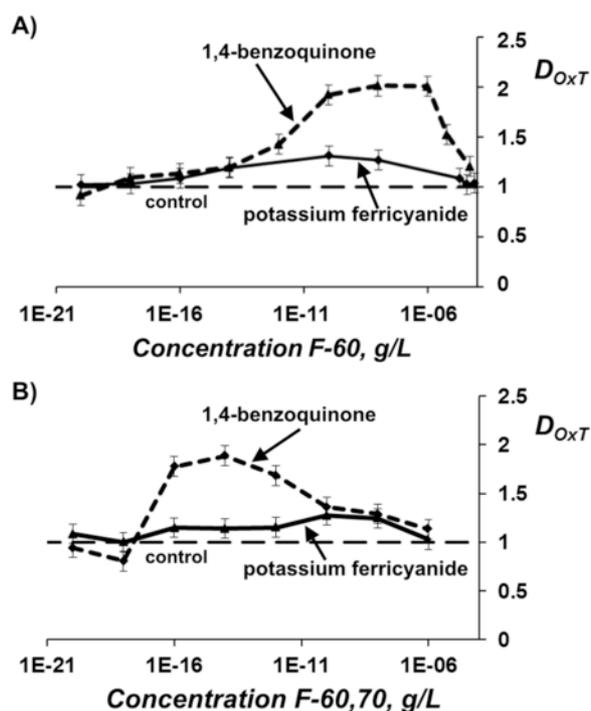


Fig. 6. Detoxification coefficients  $D_{OxT}$  vs. concentration of fullereneols F-60 (A) and F-60,70 (B) in solutions of 1,4-benzoquinone ( $10^{-4}$  M) and potassium ferricyanide ( $2 \cdot 10^{-4}$  M). Enzyme-based assay.

### 3.3.3. Reaction rates in enzymatic assay system in solutions of oxidizers and fullereneols

The rates of NADH oxidation were studied in the presence and absence of fullereneol F-60, in enzymatic and nonenzymatic processes, Table 1.

Table 1

Rates of NADH oxidation in the absence ( $V$ ) and presence ( $V_F$ ) of fullereneol – F-60,  $C_{NADH} = 1.6 \cdot 10^{-4}$  M,  $C_{F-60} = 10^{-8}$  g/L,  $C_{FMN} = 5.4 \cdot 10^{-5}$  M, E – enzyme preparation. Registration wavelength 340 nm. SD for  $V$  and  $V_F$  was  $10^{-8}$  M/min.

No	Components of solution	$V \cdot 10^8$ , M/min		
		$V$	$V_F$	$V_F - V$
<b>Nonenzymatic processes</b>				
1	NADH (auto-oxidation)	6	10	4
2	NADH + FMN	32	32	0
<b>Enzymatic processes</b>				
1E	NADH + E	4	9	5
2E	NADH + FMN + E	317	374	57

The rates of NADH auto-oxidation in the absence (No. 1) and presence (No. 1E) of the enzymes are presented in the Table 1. These values are close to those determined previously [36,39]. The data show that the addition of F-60 to NADH solutions increases the auto-oxidation rates by  $4 \cdot 10^{-8}$  and  $5 \cdot 10^{-8}$  M/min, respectively.

Reactions No. (2) and 2E (Table 1) present endogenous processes in the bioluminescent assay system, i.e. nonenzymatic and enzymatic redox reactions of the endogenous reducer and oxidizer (NADH and FMN, respectively, reaction (1), Section 2.1). Addition of F-60 increased the rate of the enzymatic reaction by  $57 \cdot 10^{-8}$  M/min, Table 1. Similar results were obtained with fullereneol F-60,70.

Light-absorption spectra of oxidizer solutions ( $EC_{50}$ , Section 3.1.2) were studied under exposure to fullereneols of different concentrations:  $10^{-17} - 10^{-4}$  g/L. No changes in oxidizer concentrations were observed during the 4-h exposure.

## 4. Discussion

### 4.1. Peculiarities of fullereneol detoxifying effect

Figs. 4–6 demonstrate that  $D_{GT}$  and  $D_{OxT}$  exceed “1” in a wide range of fullereneol concentrations, revealing detoxification ability of fullereneols in oxidizer solutions. Both cellular and enzymatic assays demonstrate the detoxifying activity of the fullereneols. Three peculiarities of the fullereneol detoxifying effect are evident from these experiments: (1) highly diluted fullereneol solutions were active, (2) no monotonic dependencies of  $D_{GT}$  or  $D_{OxT}$  on fullereneol concentrations were observed, and (3) detoxification of organic oxidizer solutions was more effective.

The NADH, organic reducer, is a component of a lot of biochemical reactions, including reaction (1) in the bioluminescent system of coupled enzymatic reactions (Section 2.1). The change of rate of NADH oxidation serves as an indicator of intensification (or slowdown) of redox processes in the assay system. Section 3.3.3 studied rates of NADH oxidation in the absence and presence of fullereneol ( $10^{-8}$  g/L), in enzymatic and nonenzymatic processes, Table 1. Acceleration of auto-oxidation processes (No. 1 and 1E, Table 1) by the fullereneol was found. This result shows that the fullereneol stimulates an oxidation of the endogenous reducer, NADH, withdrawing it from the biochemical process, decreasing the bioluminescent intensity in accordance with reactions (1) and (2) (Section 2.1), and contributing to toxicity increase ( $D_{GT} < 1$ ). On the other hand, fullereneol increased the rate of the enzymatic redox reaction of endogenous reducer and endogenous oxidizer (NADH and FMN, respectively, reaction (1), Section 2.1) by  $57 \cdot 10^{-8}$  M/min (No. 2E, Table 1). So, the experiments demonstrate that fullereneols are able to increase toxicity of aqueous solutions by acceleration of NADH auto-oxidation, or decrease the toxicity by acceleration of endogenous NADH-dependent biochemical processes. In both cases, fullereneol in highly diluted solutions ( $10^{-8}$  g/L, corresponding to  $ca 10^{-11}$  M) can be considered as a catalyzer of the redox reactions.

Light-absorption measurements in oxidizer solutions showed independence of 1,4-benzoquinone or ferricyanide concentrations on the exposure to fullerene and, hence, did not confirm the neutralization of the oxidizers in the solutions, outside the bioassay systems.

All the peculiar experimental results suggest a specific mechanism of the fullerene biological influence.

#### 4.2. On mechanism of antioxidant effects in highly diluted solutions of fullerenes

Ultralow bioactive concentrations of fullerenes are a subject of a special interest. Previous sections showed that antioxidant effects of the fullerenes take place within  $ca\ 10^{-17}$ – $10^{-4}$  and  $10^{-17}$ – $10^{-5}$  g/L for bacteria-based and enzyme-based assays, respectively. The active concentrations may correspond to several tens of fullerene molecules per liter.

Activation of vital functions of various organisms by low-intensive exposures is a well-known effect, common to all living organisms. It is attributed to triggering of cell defense response under the influence of low concentrations of toxins, low dose radiation, and other stressors. It is known that low doses of bioactive substances can serve as effective drugs [50–52].

Generally, low-intensive exposures are described in terms of hormesis phenomenon. “Hormesis” is a term for “a generally favorable biological response to low exposures to toxins and other stressors.” “It is characterized by low-dose stimulation and high-dose inhibition, resulting in either a J-shaped or an inverted U-shaped dose response” [51,53–56]. The rapid and extensive exponential growth of hormesis citations in the biomedical community takes place for the last two decades [55]. Evidence emerged that hormesis is highly generalizable, independent on biological model or endpoint measured, inducing agent and level of biological organization (e.g. cell, organ, organism). The hormesis mechanism is not understood yet and still lacks strong experimental support, which leads to uncertainty as to the exact underlying causes of hormesis [57]. There exist two models explaining mechanism of hormesis; they consider the adaptive response as related with DNA damage or cell membrane processes [50,58–61].

In our previous studies, we demonstrated a positive response of luminous bacteria to low-dose radiation exposure. The results of a series of investigations were summarized in a review [62]. Independence of the bacterial luminescent response on active concentration of beta-emitting radionuclide tritium was demonstrated for intact and lyophilized bacteria in a wide radioactivity interval:  $10^{-4}$ –200 MBq/L [22]. Similar low-dose radiation effects, current study showed (a) positive response of the luminous bacteria to fullerene exposure and (b) absence of linear dose-effect dependencies: the bacterial and enzyme luminescent responses did not depend on fullerene concentrations in a wide concentration interval:  $ca\ 10^{-17}$ – $10^{-4}$  and  $10^{-17}$ – $10^{-5}$  g/L, respectively.

Biological efficiency of ultralow concentrations of hydrated fullerenes was determined and discussed earlier in [63,64]. This effect was attributed to fullerene ability to adjust dynamic structure of aqueous media and to “regulate redox processes (especially those involving oxygen) in aqueous systems”. Earlier [65], a role of aqueous medium in antiradical activity of fullerenes was discussed. According to [62,66], reactive oxygen species in aqueous media might contribute to bacterial bioluminescence activation under low-dose exposures.

Sequence analysis did not reveal any mutagenic effect of the oxidizer solutions under the conditions of the experiments. Therefore, the low-concentration fullerene effect might be due to “nongenetic” mechanism of the cellular response. The results of this study might be interpreted using the novel “exosome” concept, that complements the genome and encompasses the totality of environmental (i.e. nongenetic) exposures [67].

The difference in effects of fullerenes in solutions of organic and inorganic oxidizers might be concerned with membrane processes in the luminous bacteria. Probably, the combination of amphiphilic compounds (1,4-benzoquinone + fullerene) in water solutions promotes membrane processes resulting in the intensification of protecting metabolic response of the bacterial cells. Changes in structural organization and fluidity of lipid bilayers in hydrophobic parts of a membrane by F-60 were previously reported in [68]. In our recent experiments under conditions excluding penetration of radionuclide tritium to the bacterial cells [69], a preference of membrane process for the bacterial bioluminescence activation was proved.

Hence, the low-concentration antioxidant effects of fullerenes on bacterial cells can be attributed to hormesis phenomenon, while the intensification of biochemical reactions is explained in terms of fullerene catalytic activity. Cellular and enzymatic bioluminescent assays showed that solutions of organic oxidizer are detoxified more effectively than those of inorganic oxidizer, indicating the importance of hydrophobic interactions in the detoxification mechanism.

## 5. Conclusions

The study considers antioxidant properties of bioactive compounds, fullerenes – nanosized water-soluble derivatives of fullerenes, specific allotropic form of carbon. Two fullerene preparations were tested:  $C_{60}O_{2-4}(OH)_{20-24}$  and mixture of two types of fullerenes  $C_{60}O_{2-4}(OH)_{20-24} + C_{70}O_{2-4}(OH)_{20-24}$ . Fundamental and applied aspects of the fullerene antioxidant activity were under consideration.

The study promotes application of bioluminescent assays to evaluate detoxifying activity of bioactive compounds, with fullerenes taken as an example. Bacteria-based assay was used to demonstrate a decrease in cellular toxicity, while the enzyme-based assay – in biochemical toxicity. Additionally, the enzyme-based assay was used for the direct monitoring of the efficiency of oxidative enzymatic processes.

Bacteria-based and enzyme-based assays have demonstrated similar peculiarities of the detoxification processes: (1) ultralow concentrations of fullerenes were active ( $ca\ 10^{-17}$ – $10^{-4}$  and  $10^{-17}$ – $10^{-5}$  g/L, respectively), (2) no monotonic dependencies of detoxification efficiency on fullerene concentrations were observed, and (3) detoxification of organic oxidizer solutions was more effective than that of the inorganic oxidizer. First and second peculiarities attribute the antioxidant effects of fullerenes to hormesis phenomenon, which is generally considered as a basis for biological adaptive response. Since the sequence analysis of 16S ribosomal RNA gene did not reveal any mutagenic effects of oxidizer solutions, the results support the concept of a “nongenetic” mechanism of the cellular response to the low-concentration fullerene exposure.

Higher detoxification efficiency in solutions of the organic oxidizer supports a suggestion on involving hydrophobic (probably membrane-dependent) processes to the detoxification mechanism.

Catalytic activity of fullerene ( $10^{-8}$  g/L) in NADH-dependent enzymatic reactions was demonstrated and supposed to contribute to the adaptive bacterial response.

Current work elaborates physicochemical, biochemical and cellular basis for bioluminescence-based sensors aimed at evaluation of antioxidant activity of bioactive compounds. The study develops application of luminous marine bacteria and their enzyme reactions for the antioxidant activity monitoring. High potential of the bioluminescence systems for studying the biological activity in ultra-diluted solutions is demonstrated. The bioluminescent bacteria- and enzyme-based assays are suitable for low-dose effect evaluation due to simplicity and high rates of bioluminescence measurements. These properties ensure higher reliability of the biological measurements.

Apart from the quantitative evaluation, molecular mechanisms of adaptive response in the bacterial cells should be studied in further ex-

periments using a number of methods applicable for intracellular processes: from membrane penetrability to gene regulation, enzyme activity, ATP consumption and crystallinity of intracellular macromolecules.

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## Appendix A. Transparency document

Transparency data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2016.10.011.

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