 doi:10.20944/preprints201812.0348.v1

Article

Antioxidant Activity and Toxicity of Fullerenols with Different Number of Hydroxyl Substituents

Ekaterina S. Kovel[1,*], Anna S. Sachkova[2], Natalia G. Vnukova[3,4], Grigoriy N. Churilov[3,4], Elena M. Knyazeva[2], Nadezhda S. Kudryasheva[1,4]

[1] Institute of Biophysics SB RAS, FRC KSC SB RAS, Krasnoyarsk, 660036, Russia; n_qdr@yahoo.com (N.S.K.);
[2] National Research Tomsk Polytechnic University, Tomsk, 634050, Russia; as421@yandex.ru (A.S.S.); knyazeva@tpu.ru(E.M.K)
[3] Institute of Physics SB RAS, FRC KSC SB RAS, Krasnoyarsk, 660036, Russia; Nata_hd@rambler.ru(N.G.V); churilov@iph.krasn.ru (G.N.C.);
[4] Siberian Federal University, Krasnoyarsk, 660041, Russia
* Correspondence: kkovel@yandex.ru; Tel.: +7-3912- 494-242

Abstract: Fullerenols are nanosized water-soluble polyhydroxylated derivatives of fullerenes, specific allotropic form of carbon, bioactive compounds and perspective pharmaceutical agents. We studied biological effects of a series of fullerenols. Antioxidant activity and toxicity of the fullerenols were compared using bioluminescence assays (cellular and enzymatic); a content of Reactive Oxygen Species in fullerenol solutions was determined using chemiluminescence luminol method. Two groups of fullerenols with different number of hydroxyl substituents were under investigation: (I) $C_{60}O_y(OH)_x$, $C_{60,70}O_y(OH)_x$, where x+y=24–28 and (II) $C_{60,70}O_y(OH)_x$, $Fe_{0,5}C_{60}O_y(OH)_x$, where x+y=40–42. Toxicity of the fullerenols was evaluated using effective concentrations EC_{50}. Fullerenol' antioxidant activity was investigated in model solutions of organic toxicant of oxidative type, 1,4-benzoquinone. Detoxification coefficients were calculated to analyze and compare the antioxidant activity. Higher toxicity and lower antioxidant activity were demonstrated in the solutions of fullerenols with higher number of the oxygen substituents (x+y=40–42). The differences were concerned with fullerenol' ability to disturb Reactive Oxygen Species balance in aqueous solutions. Toxic effect of the prospective endohedral metal-fullerenol with gadolinium atom involved, $Gd@C_{82}O_y(OH)_x$, where x+y=40–42, was evaluated and explained by a high number of oxygen groups.

Keywords: bioactive compound; fullerenol; antioxidant activity; toxicity; reactive oxygen species; bioluminescence bioassay

1. Introduction

Biological activity of carbonic nano-objects is of great current interest for modern fields of medicine, biotechnology, pharmacology [1-3]. Biological activity presupposes that high-dose exposures inhibit physiological functions of organisms (toxic effect), but lower-dose exposures might activate physiological functions as a result of optimization of metabolic processes [4].

Fullerenols (F) are known to be rigid nanosize carbonic particles, water-soluble polyhydroxylated derivatives of fullerenes. Similar to fullerenes, fullerenols are electron deficient structures; and this property makes them as efficient catalyzers in biochemical reactions, and hence, perspective pharmaceutical agents. Fullerenols are amphiphilic structures: 'hydroxyl groups provide them with aqueous solubility, while the fragments of fullerene skeleton – with affinity to hydrophobic enzymatic fragments and lipid structures of cellular membranes' [1-2]. Due to the amphiphilic properties and ability to scavenge free radicals, fullerenols could provide a serious alternative to the conventional pharmacological agents in chemotherapy, treatment of neurodegenerative diseases, and radiobiology [5-6]. A range of fullerenol biological effects is wide:

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 December 2018 doi:10.20944/preprints201812.0348.v1

from cell protection [1,5] to drug transport [7] and free radicals neutralization [5]. The fullerenols demonstrated antioxidant activity, neutralizing reactive oxygen and nitrogen species [8-11]. The antioxidant property endows fullerenols with ability to treat neurological diseases [5,10-12], to function as radioprotectors [10] or antitumor agents [13].

Structural peculiarities influence the biological activity of fullerenols. Hydroxyl substituents distort the π-electron system conjugation of the fullerene carcass, change electron-acceptor ability of the nanoparticles, and hence, can decrease their catalytic activity. This can result in changes of toxicity and antioxidant activity of fullerenols with different number of hydroxyl substituents.

Biological activity of a series of fullerenols with different number of hydroxyl groups: $C_{60}(OH)_{12-14}$, $C_{60}(OH)_{18-24}$, $C_{60}(OH)_{30-38}$, was studied by Eropkin and co-workers [14]: $C_{60}(OH)_{12–14}$ was insoluble in water and did not show biological activity, while $C_{60}(OH)_{18-24}$ was soluble and showed maximum antiviral and protective activity. Maximal biological activity of $C_{60}(OH)_{18-24}$ was demonstrated in [5,8,15] as well. Hydration of fullerenol C_{60} with different number of hydroxyl groups (from 8 to 44) was theoretically studied in [16]. It was proved that hydration of $C_{60}(OH)_{44}$ is less effective despite the large number of hydroxyl groups. The authors concluded that involvement of >36 hydroxyl groups to the fullerenol structure results in effective intramolecular interactions of OH-groups, conflicting with the hydrogen bonds with the solvent.

The aim of the current study is to compare biological activity of fullerenols with different number of hydroxyl groups. Two clusters of fullerenols: with 24-28 and 40-42 hydroxyl groups are considered. Besides the water solubility, the higher number of hydroxyl substituents change available π-electron system conjugation, and hence, could decrease fullerenol' ability of reversible radical trapping [15]. This might be a reason of variation of content of Reactive Oxygen Species (ROS) in aeriated aqueous solutions with the following effects on biological structures – cells, enzymes, low-molecular components, so on.

It is known that ROS group includes a number of free radicals or radical precursors, such as semiquinones, superoxide anion-radical ($\bullet O_2^-$), hydroxyl ($\bullet OH$) and peroxide ($HOO\bullet$) radicals, hydrogen peroxide (H_2O_2), peroxide anion (HOO^-), singlet oxygen ($^1O_2$) [17], hypochloric acid (HOCl), peroxynitrite radical (ONOO-), and others. They are formed in cells as natural products of oxygen metabolism, their content is labile, and they can initiate formation of additional radicals. ROS play the role of mediators of important intracellular signaling pathways [18], thereby regulating cellular processes (cellular respiration, division, etc.), induce the immune system, mobilize ion transport systems, and trigger programmed cell death (apoptosis) [19]. Negative effects of ROS are studied in [20-25].

As the ROS impact on living organismal functions is evident [26-28], a study of ROS content in fullerenol' solutions can elucidate mechanism of fullerenol' biological effects.

Luminous marine bacterium is a proper bioassay for study biological activity of fullerenols. The bacteria have been used as a toxicity bioassay for several decades [29-34]. 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 and a proper statistical processing. This advantage is very important for biological types of analysis which are usually characterized by lower reproducibility than chemical assays.

Bacterial bioluminescence assays can be based on biological systems of different complexity – bacteria cells or their enzymes [30,35-37]. Along with water-soluble preparations, the solid preparations of immobilized bacteria and their enzymes have been developed [35,38-41]. 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 bioluminescence enzyme system, based on physicochemical, chemical, and biochemical processes, was suggested in [42] and developed in [43-46].

Evaluation of antioxidant activity of bioactive compounds is a novel and prospective field in biosensor application [47-48]. The assay systems based on luminous marine bacteria and/or their enzyme reaction are proper candidates in this field. The both bioassays, cellular and enzymatic, can be used to evaluate general toxicity of the test samples under the conditions of oxidative stress.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 December 2018 doi:10.20944/preprints201812.0348.v1

Additionally, the enzymatic assay is specific to oxidizers [46]; therefore it can be used for direct monitoring the 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 bioluminescence enzyme assay system – redox reactions, hydrophobic and polar interactions. Previously [49-50] the general and oxidative toxicities of solutions of organic and inorganic oxidizers, quinones and polyvalent metals, were studied using the bioluminescence enzymatic assay. Decrease of both general and oxidative toxicities under addition of humic substances (natural bioactive compounds, products of decomposition of organic matter in soils and bottom sediments) was studied in [51-53]. Description of the enzyme bioluminescence technique to evaluate antioxidant activity and toxicity of bioactive compounds is presented in [54-56].

The following fullerenols were chosen in this study: $C_{60}O_y(OH)_x$ and $C_{60,70}O_y(OH)_x$ where x+y =24–28; as well as $C_{60,70}O_y(OH)_x$, $Fe_{0,5}C_{60}O_y(OH)_x$, and $Gd@C_{82}O_x(OH)_y$ where x+y=40–42. They are attributed to two clusters involving different number of oxygen substituents: 24–28 and 40–42. Enzyme-based and cellular-based luminescent bioassays were applied to evaluate toxicity and antioxidant properties of fullerenols. Role ROS in effects of the fullerenols was studied, ROS content was evaluated using chemiluminescence lumonol method.

2. Materials and Methods

2.1. Preparations of fullerenols

Fullerenols $C_{60}O_y(OH)_x$ (**F1**) and $C_{60,70}O_y(OH)_x$ (**F2**) where x+y=24–28; $C_{60,70}O_y(OH)_x$ (**F3**), $Fe_{0,5}C_{60}O_y(OH)_x$ (**F4**) and $Gd@C_{82}O_y(OH)_x$ (**F5**) where x+y=40–42 were used as bioactive compounds.

F1 and F2 were produced by fullerene hydroxylation in nitric acid followed by the hydrolysis of the polynitrofullerenes [15,57-59]. Preparation of F2 involved 60% of $C_{60}O_y(OH)_x$ and 40% of $C_{70}O_y(OH)_x$. Fullerenes were preliminary synthesized by carbon helium high-frequency arc plasma at atmospheric pressure [58,60]. 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.

F3 was produced from a powder mixture of fullerene soot and acetylacetonate FeIII ($Fe(acac)_3$). The mixture was heated up to spontaneous ignition at 180°C. Then, the combustion process proceeded without additional heating. The product of the combustion reaction was exposed to boiling hydrochloric acid, the dissolved part of the product was removed. Retreatment with acid was provided to remove the metal salt. The solid residue of fullerenes was washed with water and used as a precursor in the synthesis of polyhydroxylated fullerenes (fullerenols). F3 was produced by precursor hydroxylation in nitric acid followed by the hydrolysis of the polynitrofullerenes [15,57-59].

F4 (two molecules of C_{60}-fullerenol are combined by an iron atom) was produced from the powder mixture of Fe-containing fullerene soot and acetylacetonate FeIII ($Fe(acac)_3$). The mixture was heated up to spontaneous ignition at 180°C. The process was continued in the smoldering regime with temperature increase up to 250°C. Then, the product was exposed to concentrated nitric acid at 90°C. The residuum was removed by filtration; red cinnamonic solution was evaporated and treated with distilled water to provide for the hydrolysis of polynitrofullerene to polyhydroxylated fullerene [15,57,59].

F5 (gadolinium atom is inside C_{82}-fullerenol) was produced by $Gd@C_{82}$-fullerene hydroxylation in nitric acid followed by the hydrolysis of the polynitrofullerenes [15,57,59,61]. Mixture of fullerenes, involving $Gd@C_{82}$-fullerene, was preliminary synthesized by carbon helium high-frequency arc plasma at 98 kPa [58,61]. The $Gd@C_{82}$-fullerene content in carbon soot was about 4.8%. The reaction of complexation with Lewis acids ($TiCl_4$) was used for enrichment of the extract of fullerene mixture by endohedral metallofullerenes ($Gd@C_{82}$) [62]. Then, $Gd@C_{82}$ was extracted with carbon disulfide from carbon soot.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 December 2018 doi:10.20944/preprints201812.0348.v1

The fullerenol preparations were characterized with IR and photoelectron spectroscopies [63-64].

2.2. Bioluminescence assay systems and experimental data processing

Antioxidant activity and toxicity of fullerenols were evaluated using bioluminescence assay systems, cellular and enzymatic: (1) bacterial assay, i.e. Microbiosensor 677F, based on the lyophilized luminous bacteria *Photobacterium phosphoreum*, and (2) enzyme 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 [65]. All the biological preparations were produced at the Institute of Biophysics SB RAS (Krasnoyarsk, Russia).

The chemicals were: NADH from ICN, USA; FMN, and tetradecanal from SERVA, Germany; 1,4-benzoquinone from Aldrich, USA; sodium chloride (NaCl) from Khimreactiv, Russia.

Antioxidant activity of fullerenols was assessed in water solutions of model oxidizer 1,4-benzoquinone.

To construct the enzyme system, 0.1 mg ml^{-1} 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 0.05 M phosphate buffer (pH 6.8) at room temperature.

Measurements of bioluminescence intensity were carried out with bioluminometers BLM-3606 (Nauka Special Design Bureau, Russia) and TriStar LB 941 (Berthold Technologies, Germany).

Toxic effects of fullerenols on bioluminescence of bacterial and enzymatic assay systems were evaluated by relative bioluminescence intensity, I_F^{rel} :

$$I_F^{rel} = I_F / I_{contr}, \quad (1)$$

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

To compare toxic effects of fullerenols, their effective concentrations decreasing bioluminescence intensity by 50% ($I_F^{rel} = 0.5$), EC_{50}, were determined.

General Toxicity (*GT*) of the model oxidizer solutions (1,4-benzoquinone) was evaluated with relative bioluminescence intensity, I_{Ox}^{rel} :

$$I_{Ox}^{rel} = I_{Ox} / I_{contr}, \quad (1a)$$

Here, I_{contr} and I_{Ox} are maximal bioluminescence intensities in the absence and presence of oxidizer, respectively, Figure 1. Effective concentration of model organic oxidizer (1,4-benzoquinone) decreasing bioluminescence intensity by 50% ($I_{Ox}^{rel} = 0.5$), EC_{50}, were determined with bacterial and enzymatic bioluminescence assays. The EC_{50} values of 1,4-benzoquinone were $2.5 \cdot 10^{-7}$ M and 10^{-4} M for bacterial and enzymatic assays, respectively. The values are close to those determined earlier [50,53].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 December 2018 doi:10.20944/preprints201812.0348.v1

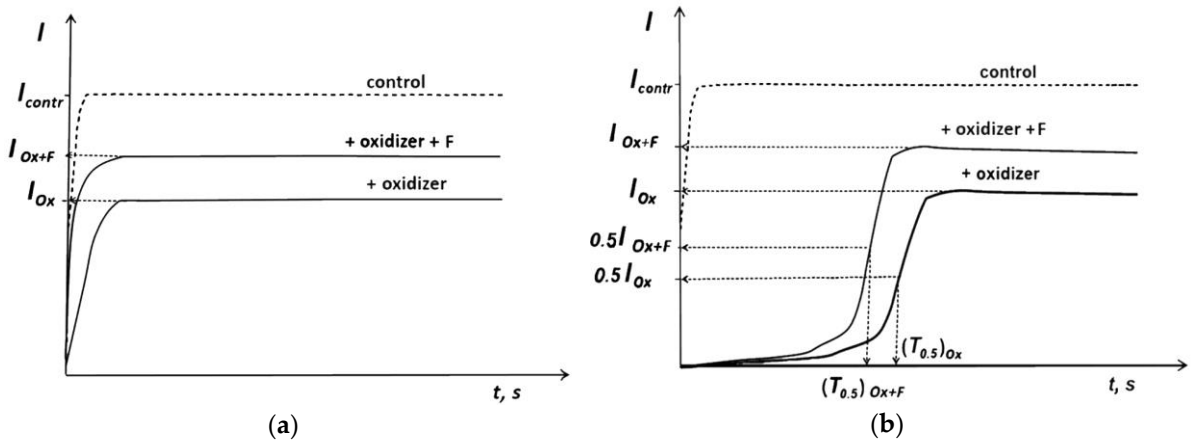

Figure 1. Bioluminescence kinetics in solution of model oxidizer 1,4-benzoquinone (Ox) and fullerenol (F): (**a**) cellular assay; (**b**) enzymatic assay.

Antioxidant activity of fullerenols was evaluated in model solutions of oxidizer (1,4-benzoquinone). EC_{50} of the oxidizer was used in these experiments. Concentration range of the fullerenols decreasing bioluminescence intensity less than 10% (I_F^{rel} >0.9) was preliminary determined and used in the experiments, to exclude the peculiar toxic effects of the fullerenols.

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

$$D_{GT} = I_{Ox+F}^{rel} / I_{Ox}^{rel}, \quad (2)$$

where I_{Ox}^{rel}, I_{Ox+F}^{rel} are relative bioluminescence intensities in oxidizer solutions at EC_{50}, in the absence and presence of fullerenols, respectively, calculated according to Eq.1a. Values of D_{GT} were determined at different fullerenol concentrations.

To characterize the Oxidative Toxicity (*OxT*) in the oxidizer solutions, the bioluminescence enzyme assay was used. Changes of *OxT* under fullerenols exposure were characterized with detoxification coefficients, D_{OxT}:

$$D_{OxT} = (T_{0.5})_{Ox} / (T_{0.5})_{Ox+F}, \quad (3)$$

Here, $(T_{0.5})_{Ox}$, $(T_{0.5})_{Ox+F}$ are bioluminescence delay periods in the oxidizer solutions in the absence and presence of fullerenols, respectively, Figure 1B. Values of D_{OxT} were determined at different fullerenol 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} ≈1 and D_{OxT} ≈1 showed an absence of the fullerenol 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 fullerenol and control solutions.

It should be paid attention that all experiments with 'colored' solutions of fullerenols excluded effect of 'optic filter' [30], and this effect did not skew the results the toxicological measurements.

2.3. Luminol chemiluminescence assay

Luminol from Sigma-Aldrich, Russia, potassium hydroxide (KOH) from Khimreactiv, Russia, and 3% hydrogen peroxide solution (H_2O_2) from Tula Pharmaceutical Factory, Russia, were used. Concentration of aqueous alkaline luminol solution was 10^{-4} M.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 December 2018 doi:10.20944/preprints201812.0348.v1

The chemiluminescence reaction was initiated with $K_3[Fe(CN)_6]$ solution through TriStar LB 941 bioluminometer injector system. Maximal chemiluminescence intensity was determined. Measurements of chemiluminescence intensity were performed in 25-40 replicates for all solutions. Average and SD values were calculated, they did not exceed 0.05.

Dependence of chemiluminescence intensity on H_2O_2 concentration was initially determined; it was used as a calibration dependence in the following experiments to evaluate concentrations of peroxide compounds in the solutions of fullerenols. Peroxides were considered as components of ROS. The ROS content was plotted vs. concentrations of fullerenols.

To compare effects of fullerenols on ROS content, their effective concentrations decreasing chemiluminescence intensity by 50%, EC_{50}, were determined.

3. Results and discussion

3.1. Toxicity and antioxidant activity of fullerenols via bioluminescence assays

3.1.1. Fullerenol toxicity

To compare the toxicity of fullerenol solutions, cellular and enzymatic bioluminescence assays were used. Suppression of bioluminescence intensity was considered as an evidence of fullerenol toxic effect. This suppression is concerned with inhibition of membrane and intracellular processes (for bacterial cells) or chemical and biochemical reactions (for enzyme system).

Dependencies of relative bioluminescence intensities I_F^{rel} (Eq. 1) on concentration of the fullerenols were obtained. Examples of these dependencies are presented in Figures 2 and 3; fullerenols F2 and F3 were chosen here as nanostructures with similar carbon carcass, but different number of hydroxyl substituents. It is seen that F2 and F3 suppress bioluminescence of the cellular system at concentrations >0.002 g L^{-1} and >0.001 g L^{-1} (Figure 2), and suppress bioluminescence of the enzymatic system at concentrations >0.010 g L^{-1} and >0.003 g L^{-1}, respectively (Figure 3). The results demonstrate higher toxicity of F3, i.e. fullerenol with higher number of hydroxyl substituents.

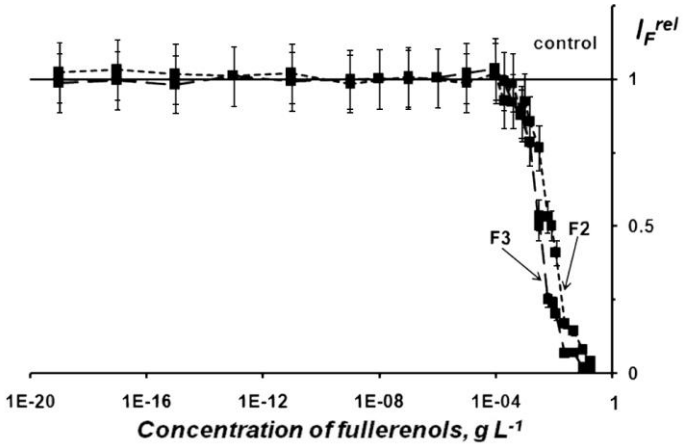

Figure 2. Bioluminescence intensity, I^{rel}, at different concentrations of fullerenols F2 and F3. Cellular assay.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 December 2018 doi:10.20944/preprints201812.0348.v1

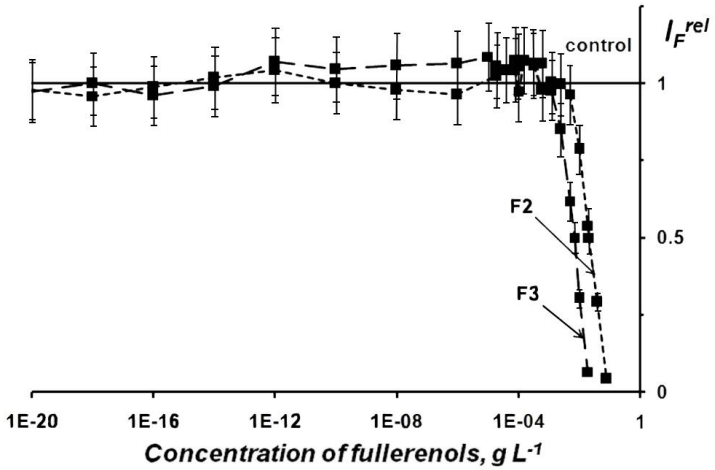

Figure 3. Bioluminescence intensity, I^{rel}, at different concentrations of fullerenols F2 and F3. Enzymatic assay.

Fullerenol' effective concentrations EC_{50} were determined and presented in Table 1. It is seen that F1 and F2 (i.e. fullerenols with lower hydroxyl number) are characterized by higher EC_{50} values and, hence, produce lower toxic effects, as compared to fullerenols F3, F4, and F5. The value of F4 in bacterial assay (0.021 g L^{-1}, Table 1) is an exclusion. Probably, iron atoms involved into the fullerenol preparation affect specifically on metabolism of the bacterial cells, according to their microelement properties.

Table 1. Values of EC_{50} of fullerenols F1-F5 and concentration range (CR) of F1-F5 decreasing bioluminescent intensity less than 10%. Enzymatic and cellular assays.

Fullerenols	Cellular assay		Enzymatic assay	
	Fullerenol concentration (g L^{-1})			
	EC_{50}	CR	EC_{50}	CR
F1	0.031	<0.010	0.092	<0.010
F2	0.008	<0.002	0.021	<0.010
F3	0.003	<0.001	0.007	<0.003
F4	0.021	<0.012	0.007	<0.001
F5	-	-	0.018	<0.005

Basing on the experiments described, we chose the range of fullerenol concentrations providing the absence of the fullerenol inhibiting effect to compare an antioxidant activity of fullerenols in further experiments (Section 3.1.2. below). These concentration ranges of fullerenols are presented in Table 1 (CR) for both cellular and enzymatic systems.

Toxicity of newly synthesized preparation, F5, endohedral metal-fullerenol with gadolinium atom involved, was studied using enzyme-based bioassay, Table 1. Perspectivity of this compound is concerned with unique paramagnetic properties of gadolinium, its application as an agent for magnetic resonance imaging and antitumor agent [66]. However, high toxicity of gadolinium-based chemotherapeutic drugs limits their clinical application. They are known to lead to severe skin and systemic diseases, renal dysfunction [67], intracranial deposition of gadolinium [68], and so on. Endoendeal involvement of gadolinium into fullerenol carcass might be a basis for actual gadolinium-based preparations of lower toxicity. Basing at the results of this study, selection and synthesis of the fullerenol-based gadolinium preparation with less number of hydroxyl substituents and, hence, lower toxicity can be recommended as a next step of the investigation.

3.1.2. Fullerenol antioxidant activity

3.1.2.1. Change of General Toxicity (GT) of oxidizer solutions under exposure to fullerenols

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 December 2018 doi:10.20944/preprints201812.0348.v1

Antioxidant ability of fullerenols was studied using the cellular and enzyme-based assays. Bioluminescence intensity of the cellular and enzymatic system was measured in solutions of model oxidizer 1,4-benzoquinone at EC_{50} in the absence and presence of fullerenols. Concentrations of the fullerenols varied in a wide range as shown in Figures 4 and 5 for bacterial and enzymatic systems, respectively. Detoxification coefficients D_{GT} were calculated according to Eq. 2. The results are presented in Figures 4 and 5, with F2 and F3 taken as examples. As discussed before [51], difference in responses of cells and enzyme reactions can be attributed to active role of the bioassay systems in the detoxification processes. Antioxidant activity of low-concentration fullerenol solutions was discussed in [55] in terms of hormesis phenomenon.

Cellular assay Figure 4 shows that the oxidizer' solutions were detoxified (D_{GT}>1) in the concentration ranges 10^{-19}–10^{-3} and 10^{-15}–$4{\cdot}10^{-4}$ g L^{-1} for F2 and F3, respectively. Maximal values of D_{GT} were about 1.8 and 1.3, respectively.

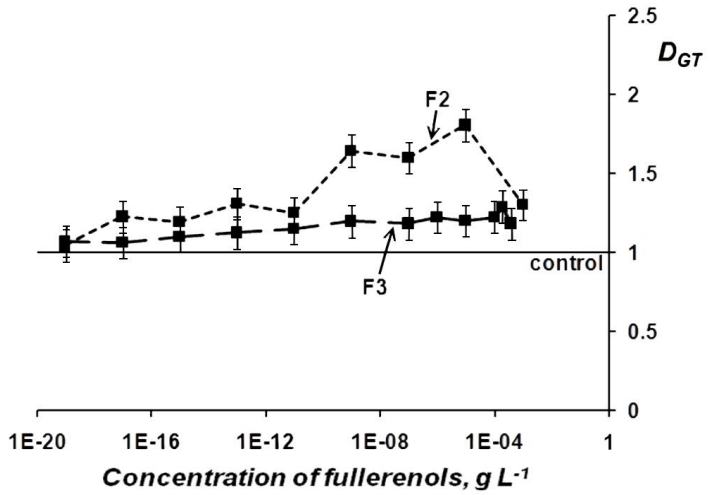

Figure 4. Detoxification coefficients D_{GT} vs. concentration of fullerenols F2 and F3 in solutions of 1,4-benzoquinone ($2.5{\cdot}10^{-7}$ M). Cellular assay.

The lower values of D_{GT} were obtained using enzyme-based assay. Figure 5 demonstrates detoxifying effect of F2 and F3 in 1,4-benzoquinone solutions (D_{GT}>1) in the concentration ranges of 10^{-19}–10^{-3} and 10^{-20}–10^{-10} g L^{-1}, respectively. Maximal values of D_{GT} were about 1.5 and 1.3, respectively.

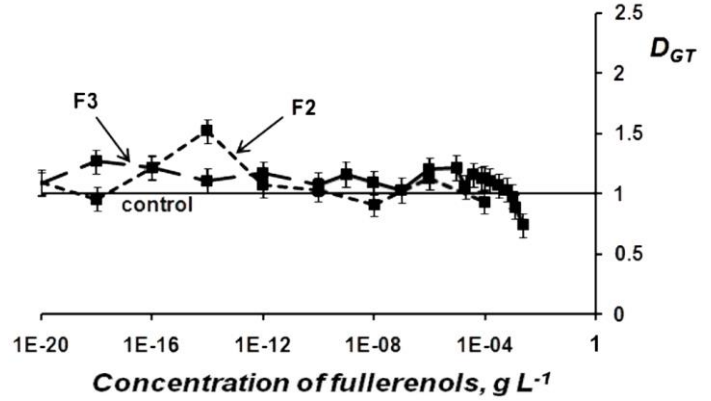

Figure 5. Detoxification coefficients D_{GT} vs. concentration of fullerenols F2 and F3 in solutions of 1,4-benzoquinone (10^{-4} M). Enzyme-based assay.

Maximal values of D_{GT} of fullerenols F1, F2, F3, and F4 in model solutions of organic oxidizer (1,4-benzoquinone) obtained with enzyme and bacterial assays are presented in Table 2. In is evident

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 December 2018 doi:10.20944/preprints201812.0348.v1

that higher D_{GT} values and, hence, detoxification efficiency was observed for F1 and F2, i.e. fullerenols with lower number of oxygen groups are more active.

Table 2. Maximal values of D_{GT} of fullerenols (F1-F4) in model solutions of organic oxidizer (1,4-benzoquinone). Enzymatic and cellular assays.

Fullerenols	Maximal value of D_{GT}	
	Enzymatic assay	Cellular assay
F1	2.2	1.4
F2	1.5	1.8
F3	1.3	1.3
F4	1.1	1.3

3.1.2.2. Change of Oxidative Toxicity (OxT) of oxidizer solutions under exposure to fullerenols

Bioluminescence kinetics of the enzymatic system was studied in solutions of model organic oxidizer 1,4-benzoquinone. Induction periods were measured in the absence and presence of fullerenols: $(T_{0.5})_{Ox}$ and $(T_{0.5})_{Ox+F}$, respectively, Figure 1. Detoxification coefficients D_{OxT} were calculated according to Eq. 3.

Figure 6 demonstrates the dependences of D_{OxT} on fullerenol concentrations. Detoxification coefficients D_{OxT} in the solutions of organic oxidizer 1,4-benzoquinone are not more than 1.9 for F2 and close to '1' at all F3 concentrations.

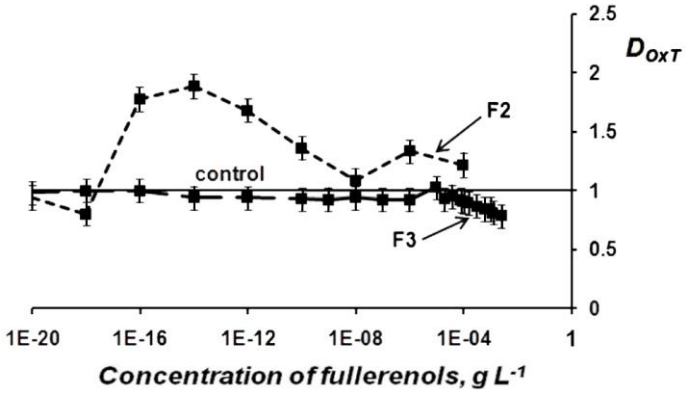

Figure 6. Detoxification coefficients D_{OxT} vs. concentration of fullerenols F2 and F3 in solutions of 1,4-benzoquinone (10^{-4} M). Enzyme-based assay.

Maximal values of D_{OxT} of fullerenols F1, F2, F3, and F4 are presented in Table 3. Similar to D_{GT} (Table 2), the D_{OxT} values of F1 and F2 are higher than these of F3 and F4, revealing higher ability to decrease oxidative toxicity.

Table 3. Maximal values of D_{OxT} of fullerenols (F1-F4) in model solutions of organic oxidizer (1,4-benzoquinone). Enzymatic assay.

Fullerenols	Maximal value of D_{OxT}
F1	2.0
F2	1.9
F3	1.0
F4	1.3

3.2. ROS content in fullerenol solutions. Luminol chemiluminescence assay

We suppose that ROS content is directly concerned with oxidative toxicity (*OxT*) of the solutions. Additionally, ROS contribute to general toxicity (*GT*) in a more complex way. Similar to excess of ROS, the lack of ROS can suppress bioluminescence of enzymatic systems since the peroxide compounds are

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 December 2018 doi:10.20944/preprints201812.0348.v1

involved to the bioluminescence reaction as intermediates [45]. The same effects can take place in other (non-bioluminescence) redox enzymatic reactions in cells.

We used luminol chemiluminescence method to compare the content of peroxides (as representative of ROS) in the solutions of fullerenols F1, F2, F3, and F4. Dependencies of ROS content on fullerenol concentrations were studied; suppression of chemiluminescence signal was demonstrated at higher peroxide concentrations. The effects of F2 and F3 on chemiluminescence signal are presented in Figure 7 as an example.

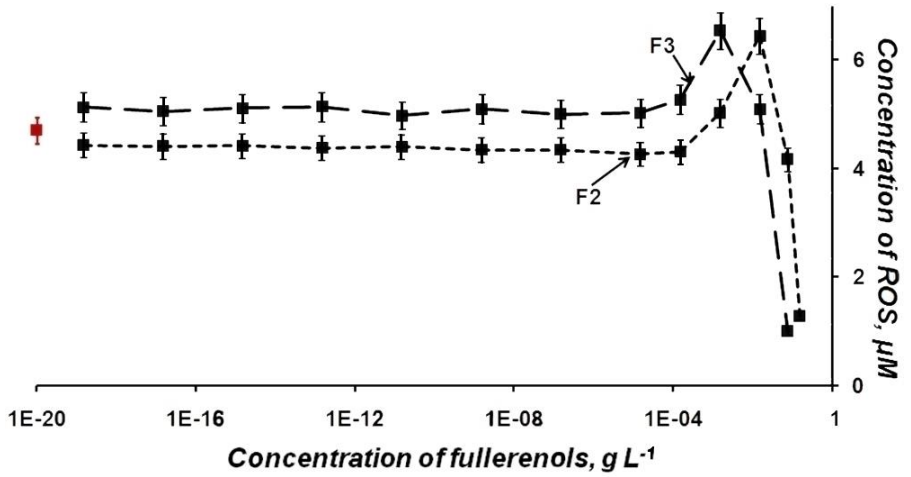

Figure 7. Concentration of ROS vs. concentrations of fullerenols F2 and F3. Chemiluminescence assay. The content of ROS in distilled water is indicated by a maroon point in the left part of figure.

Concentrations of fullerenols decreasing ROS content by 50%, EC_{50}, were determined and presented in Table 4. The table shows that F1 and F2 suppress ROS less effectively than F3 and F4 (values of EC_{50} are 0.179 and 0.124 as compared to 0.056 and 0.105 g L^{-1}, respectively).

Inhibition and activation of bacterial bioluminescence signal by ROS was reported previously for bacterial and enzymatic assays [69-70]; hydrogen peroxide was applied by the authors as a representative of ROS.

Table 4. Values of EC_{50} of fullerenols in chemiluminescence assay.

Fullerenols	EC_{50}, g L^{-1}
F1	0.179
F2	0.124
F3	0.056
F4	0.105

Correlation coefficients between EC_{50} of fullerenols F1-F4 in chemiluminescence assay (Table 4) and EC_{50} of fullerenols in bioluminescence systems – cellular and enzymatic assays (Table 1) were calculated as 0.88 and 0.83, respectively. These results might support the suggestion on the involvement of ROS to the bioluminescence inhibition by the fullerenols and hence, to the toxic effect of the fullerenols.

Correlation coefficients between EC_{50} of fullerenols F1-F4 in chemiluminescence assay (Table 4) and detoxification coefficients D_{GT} and D_{OxT} in enzyme-based assay system (Tables 2,3) were 0.83 and 0.91, respectively. These results support the suggestion on the involvement of ROS to antioxidant effect of fullerenols. Correlation coefficient between EC_{50} of fullerenols F1-F4 in chemiluminescence assay (Table 4) and values of D_{GT} obtained using cellular assay (Table 2) was 0.13. This result does not demonstrate the dependence on ROS content; probably due to complicated structure and processes responsible for the antioxidant response in the cells (as compared to the enzymatic processes).

Hence, the data demonstrate that toxicity of fullerenols might be concerned with their extra ability to neutralize oxygen radicals under the conditions of high-concentration solutions. Fullerenols with higher number of hydroxyl substituents (F3 and F4) suppress ROS more effectively, producing more toxic effect on cellular and enzymatic systems. Antioxidant activity of fullerenols in low-concentration solutions is

probably concerned with their ability to regulate ROS content reversibly; fullerenols with lower number of hydroxyl substituents (F1 and F2) are characterized by higher activity. The results might predict a higher antioxidant activity of non-substituted fullerenes, i.e. carbonic nanostructures with holistic π-system apportioned evenly over the spherical macromolecule. Additional experiments under similar conditions should be provided to confirm this suggestion. Previously, the biological activity of hydrated C-60 fullerene was studied [71-73]; it was attributed to specific structure of hydrated shell of the C-60 fullerene.

4. Conclusions

Current study demonstrates difference in properties of water-soluble fullerenols with different number of hydroxyl groups. Two clusters of fullerenols differing in the number of oxygen substituents were under study; their toxicity and antioxidant activity were compared using bioluminescence assays, cellular and enzymatic. Lower toxicity and higher antioxidant activity were demonstrated for the fullerenols with lower number of the substituents: $C_{60}O_y(OH)_x$ and $C_{60,70}O_y(OH)_x$, where x+y=24–28. The differences were supposedly attributed to fullerenol' ability to disturb ROS balance in aqueous solutions. Further investigations, including theoretical ones, should be carried out to understand physics and chemistry of these differences. Probably, the investigations are to be aimed at such structural fullerenol peculiarities as interrelation between a number of oxygen-containing groups and hydrophobic π-conjugated surface fragments, with the latter to be responsible for the reversible electron acceptation and, hence, nonspecific catalytic activity in chemical and biochemical processes.

Basing on the results of this study, a recommendation can be done for selection and synthesis of fullerene' water-soluble derivatives, including pharmaceutically prospective endohedral metal-fullerenes: high number of oxygen substituents (up to 40 and higher) provides high toxicity and low catalytic activity.

Author Contributions: E.S.K. and A.S.S. were involved in experimental studied using bioluminescence and chemiluminescence methods, data processing, interpretation, writing and editing manuscript. E.M.K.produced bacterial and enzyme preparations for the experiments. N.G.V. provided qualitative and quantitative analysis of fullerenes. G.N.C. provided fullerene and fullerenol synthesis. N.S.K. was involved in general leadership of the work, data analysis, manuscript preparation. All authors read and approved the final manuscript.

Funding: This work was supported by the state budget allocated to the fundamental research at the Russian Academy of Sciences, project 0356-2017-0017; PRAN-32, Program: 'Nanostructures: physics, chemistry, biology, technological basis'; RFBR 18-29-19003.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Foley, S.; Crowley, C.; Smaihi, M.; Bonfils, C.; Erlanger, B.F.; Seta, P.; Larroque, C. Cellular localization of a water-soluble fullerene derivative. *Biochem. Biophys. Res. Commun.* **2002**, *294*, 116–119, DOI: 10.1016/S0006-291X(02)00445-X.
2. Grebowski, J.; Krokosz, A.; Puchala, M. Fullerenol $C_{60}(OH)_{36}$ could associate to band 3 protein of human erythrocyte membranes. *Biochim. Biophys. Acta (BBA) – Biomembr.* **2013**, *1828*, 2007–2014, DOI: 10.1016/j.bbamem.2013.05.009.
3. Zheng, Y.; Hou, L.; Liu, M.; Newell, S.E.; Yin, G.; Yu, C.; Zhang, H.; Li, X.; Gao, D.; Gao, J.; Wang, R.; Liu, C. Effects of silver nanoparticles on nitrification and associated nitrous oxide production in aquatic environments. *Sci. Adv.* **2017**, *3(8):e1603229*, 1-12, DOI: 10.1126/sciadv.1603229.
4. Iavicoli, I.; Leso, V.; Fontana, L.; Calabrese, E.J. Nanoparticle exposure and hormetic dose–responses: an update. *Int. J. Mol. Sci.* **2018**, *19(3)*, 805, DOI: 10.3390/ijms19030805.
5. Grebowski, J.; Kazmierska, P.; Krokosz, A. Fullerenols as a new therapeutic approach in nanomedicine. *Biomed. Res. Int.* **2013**, *2013*, 1–9. DOI: 10.1155/2013/751913.
6. Cai, X.; Hao, J.; Zhang, X.; Yu, B.; Ren, J.; Luo, C.; Li, Q.; Huang, Q.; Shi, X.; Li, W.; Liu, J. The polyhydroxylated fullerene derivative $C_{60}(OH)_{24}$ protects mice from ionizing-radiation-induced immune

and mitochondrial dysfunction. *Toxicol. Appl. Pharmacol*. **2010**, *243(1)*, 27–34, DOI: 10.1016/j.taap.2009.11.009.

7. Slavic, M.; Djordjevic, A.; Radojicic, R.; Milovanovic, S.; Orescanin-Dusic, Z.; Rakocevic, Z.; Spasic, M.B.; Blagojevic, D. Fullerenol$C_{60}(OH)_{24}$ nanoparticles decrease relaxing effects of dimethyl sulfoxide on rat uterus spontaneous contraction. *J. Nanopart. Res.* **2013**, *15(5)*, 1–10, DOI: 10.1007/s11051-013-1650-1.
8. Mirkov, S.M.; Djordjevic, A.N.; Andric, N.L.; Andric, S.A.; Kostic, T.S.; Bogdanovic, G.M.; Vojinovic-Miloradov, M.B; Kovacevic, R.Z. Nitric oxide-scavenging activity of polyhydroxylated fullerenol, $C_{60}(OH)_{24}$. *Nitric Oxide* **2004**, *11(2)*, 201–207, DOI: 10.1016/j.niox.2004.08.003.
9. Injac, R.; Prijatelj, M.; Strukelj, B. Fullerenol nanoparticles: toxicity and antioxidant activity. *Methods Mol. Biol.* **2013**, *1028*, 75–100, DOI: 10.1007/978-1-62703-475-3_5.
10. Djordjevic, A.; Srdjenovic, B.; Seke, M.; Petrovic, D.; Injac, R.; Mrdjanovic, J. Review of synthesis and antioxidant potential of fullerenol nanoparticles. *J. Nanomater.* **2015**, *2015*, 1–15, DOI: 10.1155/2015/567073.
11. Wang, Z.; Wang, S.; Lu, Z.; Gao, X. Syntheses, structures and antioxidant activities of fullerenols: knowledge learned at the atomistic level. *J. Clust. Sci.* **2015,** *26(2)*, 375–388, DOI: 10.1007/s10876-015-0855-0.
12. Djordjevic, A.; Canadanovic-Brunet, J.M.; Vojinovic-Miloradov, M.; Bogdanovic, G. Antioxidant properties and hypothetic radical mechanism of fullerenol $C_{60}(OH)_{24}$. *Oxid. Commun.* **2005**, *27(4)*, 806–812.
13. Jiao, F.; Liu, Y.; Qu, Y.; Li, W.; Zhou, G.; Ge, C.; Li, Yu.; Sun, B.; Chen C. Studies on anti-tumor and antimetastatic activities of fullerenol in a mouse breast cancer model. *Carbon* **2010**, *48(8)*, 2231–2243, DOI: 10.1016/j.carbon.2010.02.032.
14. Eropkin, M.Yu.; Melenevskaya, E.Yu.; Nasonova, K.V.; Bryazzhikova, T.S.; Eropkina, E.M.; Danilenko, D.M.; Kiselev, O.I. Synthesis and biological activity of fullerenols with various contents of hydroxyl groups. *Pharm. Chem. J.* **2013**, *47(2)*, 87–91, DOI: 10.1007/s11094-013-0901-x.
15. Goncharova, E.A.; Isakova, V.G.; Tomashevich, E.V.; Churilov, G.N. Obtaining of water-soluble polyhydroxylated fullerenols with iron nanoparticles as catalyzers. *Vestn. SibGAU* **2009**, *22*, 90–93 (In Russian).
16. Chaban, V.V.; Fileti, E.E. Which fullerenols are water soluble? Systematic atomistic investigation. *New J. Chem.* **2017**, *41*, 184-189, DOI: 10.1039/C6NJ02813F.
17. Hayyan, M.; Hashim, M.A.; AlNashef, I.M. Superoxide ion: generation and chemical implications. *Chem. Rev.* **2016**, *116(5)*, 3029–3085, DOI: 10.1021/acs.chemrev.5b00407.
18. Hancock, J.T.; Desikan, R.; Neill, S.J. Role of reactive oxygen species in cell signalling pathways. *Biochem. Soc. Trans.* **2001**, *29 (Pt 2)*, 345–350, DOI: 10.1042/bst0290345.
19. Devasagayam, T.P.; Tilak, J.C.; Boloor, K.K.; Sane, K.S.; Ghaskadbi, S.S; Lele, R.D. Free radicals and antioxidants in human health: current status and future prospects. *J. Assoc. Physicians India* **2004**, *52*, 794-804.
20. Klaunig, J.E.; Kamendulis ,L.M. The role of oxidative stress in carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 239–267, DOI: 10.1146/annurev.pharmtox.44.101802.121851.
21. Sinha, K.; Das, J.; Pal, P.B.; Sil, P.C. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. *Arch. Toxicol.* **2013**, *87(7)*, 1157–1180, DOI: 10.1007/s00204-013-1034-4.
22. Anderson, G.; Maes, M. Neurodegeneration in Parkinson's disease: interactions of oxidative stress, tryptophan catabolites and depression with mitochondria and sirtuins. *Mol. Neurobiol*. **2014**, *49(2)*, 771–783, DOI: 10.1007/s12035-013-8554-z.
23. Giacco, F.; Brownlee M. Oxidative stress and diabetic complications. *Circ. Res*. **2010**, *107(9)*, 1058–1070, DOI: 10.1161/CIRCRESAHA.110.223545.
24. Zenker, M.; Aigner, T.; Wendler, O.; Tralau, T.; Müntefering, H.; Fenski, R.; Pitz, S.; Schumacher, V.; Royer-Pokora, B.; Wühl, E.; Cochat, P.; Bouvier, R.; Kraus, C.; Mark, K.; Madlon, H.; Dötsch, J.; Rascher, W.; Maruniak-Chudek, I.; Lennert, T.; Neumann, L.M.; Reis, A. Human laminin β2 deficiency causes congenital nephrosis with mesangial sclerosis and distinct eye abnormalities. *Hum. Mol. Genet.* **2004**, *13(21)*, 2625–2632, DOI: 10.1093/hmg/ddh284.
25. Caldeira, G.L.; Ferreira, I.L.; Rego, A.C. Impaired transcription in Alzheimer's disease: key role in mitochondrial dysfunction and oxidative stress. *J. Alzheimer's Dis*. **2013**, 34(1), 115–131, DOI: 10.3233/JAD-121444.
26. Dharmaraja, A.T. Role of reactive oxygen species (ROS) in therapeutics and drug resistance in cancer and bacteria. *J. Med. Chem.* **2017**, 60(8), 3221−3240. DOI: 10.1021/acs.jmedchem.6b01243.

27. Forrester, S.J.; Kikuchi, D.S.; Hernandes, M.S.; Xu, Q.; Griendling, K.K. Reactive oxygen species in metabolic and inflammatory signaling. *Circ. Res.* **2018**, *122(6)*, 877-902, DOI: 10.1161/CIRCRESAHA.117.311401.
28. Nugud, A.; Sandeep, D.; El-Serafi, A.T. Two faces of the coin: Minireview for dissecting the role of reactive oxygen species in stem cell potency and lineage commitment. *J. Adv. Res.* **2018**, *14*, 73–79, DOI: 10.1016/j.jare.2018.05.012.
29. Bulich, A.A.; Isenberg, D.L. Use of the luminescent bacterial system for rapid assessment of aquatic toxicity. *ISA Trans.***1981**, 20(1), 29–33.
30. Fedorova, E.; Kudryasheva, N.; Kuznetsov, A.; Mogil'naya, O.; Stom, D. Bioluminescent monitoring of detoxification processes: activity of humic substances in quinone solutions. *J. Photochem. Photobiol. B* **2007**, *88*, 131–136, DOI:10.1016/j.jphotobiol.2007.05.007.
31. Girotti, S.; Ferri, E.N.; Fumo, M.G.; Maiolini, E. Monitoring of environmental pollutants by bioluminescent bacteria. *Anal. Chim. Acta* **2008**, 608(1), 2–29, DOI: 10.1016/j.aca.2007.12.008.
32. Kudryasheva, N.; Kratasyuk, V.; Esimbekova, E.; Vetrova, E.; Nemtseva, E.; Kudinova, I. Development of bioluminescent bioindicators for analyses of environmental pollution. *Field Anal. Chem. Tech.* **1998**, *2(5)*, 277–280, DOI: 10.1002/(SICI)1520-6521(1998)2:5<277::AID-FACT4>3.0.CO;2-P.
33. Roda, A.; Pasini, P.; Mirasoni, M.; Michchelini, E.; Guardigli, M. Biotechnological application of bioluminescence and chemiluminescence. *Trends Biotech.* **2004**, *22*(6), 295–303, DOI: 10.1016/j.tibtech.2004.03.011.
34. Abbas, M.; Adil, M.; Ehtisham-Ul-Haque, S.; Munir, B.; Yameen, M.; Ghaffar, A.; Shar, G.A.; Asif Tahir, M.; Iqbal, M. Vibrio fischeri bioluminescence inhibition assay for ecotoxicity assessment: A review. *Sci. Total Environ.***2018,***626*, 1295–1309, DOI:10.1016/j.scitotenv.2018.01.066.
35. Kratasyuk, V.A.; Esimbekova, E.N. Applications of luminous bacteria enzymes in toxicology. *Comb. Chem. High Throughput Screen.* **2015**, *18*(10), 952–959, DOI: 10.2174/1386207318666150917100257.
36. Rozhko, T.V.; Kudryasheva, N.S.; Kuznetsov, A.M.; Vydryakova, G.A.; Bondareva, L.G.; Bolsunovsky, A.Ya. Effect of low-level α-radiation on bioluminescent assay systems of various complexity. *Photochem. Photobiol. Sci.* **2007**, *6*, 67–70, DOI: 10.1039/B614162P.
37. Selivanova, M.A.; Mogilnaya, O.A.; Badun, G.A.; Vydryakova, G.A.; Kuznetsov, A.M.; Kudryasheva, N.S. Effect of tritium on luminous marine bacteria and enzyme reactions. *J. Environ. Radioact*. **2013,** *120*, 19–25, DOI: 10.1016/j.jenvrad.2013.01.003.
38. Esimbekova, E.N.; Kondik, A.M.; Kratasyuk, V.A. Bioluminescent enzymatic rapid assay of water integral toxicity. *Environ. Monit. Assess*. **2013,** *185(7)*, 5909–5916, DOI: 10.1007/s10661-012-2994-1.
39. Efremenko, E.N.; Maslova, O.V.; Kholstov, A.V.; Senko, O.V.; Ismailov, A.D. Biosensitive element in the form of immobilized luminescent photobacteria for detecting ecotoxicants in aqueous flow-through systems.*Luminescence***2016**, *31(6)*, 1283-1289, DOI:10.1002/bio.3104.
40. Ismailov, A.D.;Aleskerova, L.E. Photobiosensors containing luminescent bacteria. *Biochemistry* **2015**, *80(6)*, 733–744, DOI: 10.1134/S0006297915060085.
41. Ranjan, R.; Rastogi, N.K.; Thakur, M.S. Development of immobilized biophotonic beads consisting of Photobacterium leiognathi for the detection of heavy metals and pesticide. *J. Hazard. Mater.* **2012**, *225–226,* 114–123, DOI: 10.1016/j.hazmat.2012.04.076.
42. Kudryasheva, N.S. Bioluminescence and exogenous compounds: physicochemical basis for bioluminescence assay. *J. Photochem. Photobiol. B* **2006**, *83(1),* 77–86,DOI: 10.1016/j.jphotobiol.2005.10.003.
43. Kirillova, T.N.; Kudryasheva, N.S. Effect of heavy atom in bioluminescent reactions. *Anal. Bioanal. Chem.* **2007**, *387(6)*, 2009–2016. DOI: 10.1007/s00216-006-1085-y.
44. Kirillova, T.N.; Gerasimova, M.A.; Nemtseva, E.V.; Kudryasheva, N.S. Effect of halogenated fluorescent compounds on bioluminescent reactions. *Anal. Bioanal. Chem*. **2011**, *400(2),* 343–351, DOI: 10.1007/s00216-011-4716-x.
45. Nemtseva, E.V.; Kudryasheva, N.S. The mechanism of electronic excitation in bacterial bioluminescent reaction. *Russ. Chem. Rev.* **2007**, *76(1)*, 91–100, DOI: 10.1070/RC2007v076n01ABEH003648.
46. Vetrova, E.V.; Kudryasheva, N.S.; Kratasyuk, V.A. Redox compounds influence on the NAD(P)H:FMN-oxidoreductase-luciferase bioluminescent system. *Photochem. Photobiol. Sci*. **2007**, *6(1),* 35–40, DOI: 10.1039/b608152e.
47. Fedorova, G.F.; Kancheva, V.D.; Menshov, V.A.; Naumov, V.V.; Vasil'ev, R.F.; Veprintsev, T.L.; Trofimov, A.V.; Tsaplev, Yu.B.; Yablonskaya, O.I. Exogenous and endogenous mediators of oxygen

metabolism: alternatives for chemical and biological activity. *Stud. Nat. Prod. Chem.* **2016**, *47*, 357–385, DOI: 10.1016/B978-0-444-63603-4.00011-5.

48. Slavova-Kazakova, A.K.; Angelova, S.E.; Veprintsev, T.L.; Denev, P.; Fabbri, D.; Dettori, M.A; Kratchanova, M.; Naumov, V.V.; Trofimov, A.V.; Vasil'ev, R.F.; Delogu, G.; Kancheva, V.D. Antioxidant potential of curcumin-related compounds studied by chemiluminescence kinetics, chain-breaking efficiencies, scavenging activity (ORAC) and DFT calculations. *Beilstein J. Org. Chem*. **2015**, *11*, 1398–1411, DOI: 10.3762/bjoc.11.151.
49. Kudryasheva, N.; Vetrova, E.; Kuznetsov, A.; Kratasyuk, V.; Stom, D. Bioluminescent assays: effects of quinones and phenols. *Ecotoxicol. Environ. Saf.* **2002**, 53(2), 221–225, DOI: 10.1006/eesa.2002.2214.
50. Tarasova, A.S.; Stom, D.I.; Kudryasheva, N.S. Effect of humic substances on toxicity of inorganic oxidizer bioluminescent monitoring. *Environ. Toxicol. Chem*. **2011**, *30(5)*, 1013–1017, DOI: 10.1002/etc.472.
51. Kudryasheva, N.S.; Tarasova, A.S. Pollutant toxicity and detoxification by humic substances: mechanisms and quantitative assessment via luminescent Biomonitoring. *Environ. Sci. Pollut. Res. Int*. **2015**, *22(1)*, 155–167, DOI: 10.1007/s11356-014-3459-6.
52. Tarasova, A.S.; Stom, D.I.; Kudryasheva, N.S. Antioxidant activity of humic substances via bioluminescent monitoring in vitro. *Environ. Monit. Assess*. **2015**, *187(3)*, 89, DOI: 10.1007/s10661-015-4304-1.
53. Tarasova, A.S.; Kislan, S.L.; Fedorova, E.S.; Kuznetsov, A.M.; Mogilnaya, O.A.; Stom, D.I.; Kudryasheva, N.S. Bioluminescence as a tool for studying detoxification processes in metal salt solutions involving humic substances. *J. Photochem. Photobiol. B* **2012**, *117*, 164–170, DOI: 10.1016/j.jphotobiol.2012.09.020.
54. Kudryasheva, N.S.; Kovel, E.S.; Sachkova, A.S.; Vorobeva, A.A.; Isakova, V.G.; Churilov, G.N. Bioluminescent enzymatic assay as a tool for studying antioxidant activity and toxicity of bioactive compounds. *J. Photochem. Photobiol.* **2017**, *93(2)*, 536-540, DOI: 10.1111/php.12639.
55. Sachkova, A.S.; Kovel, E.S.; Churilov, G.N.; Guseynov, O.A.; Bondar, A.A.; Dubinina, I.A.; Kudryasheva, N.S. On mechanism of antioxidant effect of fullerenols. *Biochem. Biophys. Rep*. **2017**, *9*, 1–8, DOI: 10.1016/j.bbrep.2016.10.011.
56. Sachkova, A.S.; Kovel, E.S.; Churilov, G.N.; Stom, D.I.; Kudryasheva, N.S. Biological activity of carbonic nano-structures—comparison via enzymatic bioassay. *J. Soils Sediments* **2018**, DOI: 10.1007/s11368-018-2134-9.
57. Sun, D.; Huang, H.; Yang, S. Synthesis and characterization of a water-soluble endohedral metallofullerol. *Chem. Mater.* **1999**, *11(4),* 1003-1006, DOI: 10.1021/cm980669t.
58. Churilov, G.N.; Kratschmer, W.; Osipova, I.V.; Glushenko, G.A.; Vnukova, N.G.; Kolonenko, A.L.; Dudnik A.I. Synthesis of fullerenes in a high-frequency arc plasma under elevated helium pressure. *Carbon* **2013**, *62*, 389–392, DOI: 10.1016/j.carbon.2013.06.022.
59. Isakova, V.G.; Goncharova, E.A.; Bayukov, O.A.; Churilov, G.N. Hydroxylation of fullerenes modified with iron nanoparticles. *Russ. J. Appl. Chem.* **2011**, *84(7)*, 1165–1169, DOI: 10.1134/S107042721107007X.
60. Churilov, G.N.; Popov, A.A.; Vnukova, N.G.; Dudnik, A.I.; Glushchenko, G.A.; Samoylova, N.A.; Dubinina, I.A.; Gulyaeva U.E. A method and apparatus for high-throughput controlled synthesis of fullerenes and endohedral metal fullerenes. *Tech. Phys. Lett.* **2016**, *42(5)*, 475-477, DOI: 10.1134/S1063785016050072.
61. Churilov, G.; Popov, A.; Vnukova, N.; Dudnik, A.; Samoylova, N.; Glushenko,G. Controlled synthesis of fullerenes and endohedral metallofullerenes in high frequency arc discharge. *Fuller. Nanotub. Car. N.* **2016**, *24(11)*, 675-678, DOI: 10.1080/1536383X.2016.1207062.
62. Akiyama, K.; Hamano, T.; Nakanishi, Y.; Takeuchi, E.; Noda, S.; Wang, Z.; Kubuki, S.; Shinohara, H. Non-HPLC rapid separation of metallofullerenes and empty cages with $TiCl_4$ Lewis acid. *J. Am. Chem. Soc.* **2012**, *134(23)*, 9762-9767, DOI: 10.1021/ja3030627.
63. Li, J.; Zhang, M.; Sun, B.; Xing, G.; Song, Yan; Guo, HaiLi; Chang, Ya.; Ge, Yo.; Zhao, Yu. Separation and purification of fullerenols for improved biocompatibility. *Carbon* **2012**, *50(2)*, 460–469, DOI: 10.1016/j.carbon.2011.08.073.
64. Li, J.; Wang, T.; Feng, Y.; Zhang, Y.; Zhen, M.; Shu, C.; Jiang, L.; Wang, Y.; Wang, C. A water-soluble gadolinium metallofullerenol: facile preparation, magnetic properties and magnetic resonance imaging application. *Dalton trans.* **2016**, *45(21)*, 8696-8699, DOI: 10.1039/c6dt00223d.
65. Kuznetsov, A.M.; Rodicheva, E.K.; Shilova, E.V. Bioassay based on lyophilized bacteria. *Biotekhnologiya* **1996**, *9*, 57–61 (in Russian).

66. Meng, J.; Liang, X.; Chen, X.; Zhao, Y. Biological characterizations of $[Gd@C_{82}(OH)_{22}]_n$ nanoparticles as fullerene derivatives for cancer therapy. *Integr. Biol.* **2013**, *5(1)*, 43-47, DOI: 10.1039/c2ib20145c.
67. Sosnovik, D.E.; Caravan, P. Molecular MRI of the Cardiovascular System in the Post-NSF Era. *Curr. Cardiovasc. Imaging Rep.* **2013**,*6(1)*, 61-68, DOI: DOI: 10.1007/s12410-012-9182-0.
68. Kanda, T.; Osawa, M.; Oba, H.; Toyoda, K.; Kotoku, J.; Haruyama, T.; Takeshita, K.; Furui, S. High signal intensity in dentate nucleus on unenhanced T1-weighted MR Images: association with linear versus macrocyclic gadolinium chelate administration. *Radiology* **2015**, *275(3)*, 803-809, DOI: 10.1148/radiol.14140364.
69. Remmel', N.N.; Titova, N.M.; Kratasyuk, V.A. Oxidative stress monitoring in biological samples by bioluminescent method. *Bull. Exp. Biol. Med.* **2003**, *136(2)*, 209-211, DOI: 10.1023/A:1026347830283.
70. Alexandrova, M.; Rozhko, T.; Vydryakova, G.; Kudryasheva, N. Effect of americium-241 on luminous bacteria. Role of peroxides. *J. Environ. Radioact.* **2011**, *102 (4)*, 407-411, DOI: 10.1016/j.jenvrad.2011.02.011.
71. Andrievsky, G.V.; Bruskov, V.I.; Tykhomyrov, A.A.; Gudkov, S.V. Peculiarities of the antioxidant and radioprotective effects of hydrated C_{60} fullerene nanostuctures in vitro and in vivo. *Free Radic. Biol. Med.* **2009**, *47(6)*, 786—793, DOI: 10.1016/j.freeradbiomed.2009.06.016.
72. Andrievsky, G.; Klochkov, V.; Derevyanchenko, L. Is the C_{60} fullerene molecule toxic?! *Fuller. Nanotub. Car. N.* **2005**, *13(4)*, 363-376, DOI: 10.1080/15363830500237267.
73. Voeikov, V.L.; Yablonskaya, O.I. Stabilizing effects of hydrated fullerenes C_{60} in a wide range of concentrations on luciferase, alkaline phosphatase, and peroxidase in vitro. *Electromagn. Biol. Med*. **2015**, 34(2), 160-166, DOI: 10.3109/15368378.2015.1036077.