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

Ekaterina S. Kovel1,*, Anna S. Sachkova2, Natalia G. Vnukova3,4, Grigoriy N. Churilov3,4, Elena M. Knyazeva2, Nadezhda S. Kudryasheva1,4

1 Institute of Biophysics SB RAS, FRC KSC SB RAS, Krasnoyarsk, 660036, Russia; n_qdr@yahoo.com (N.S.K.);
knazyeva@tpu.ru (E.M.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 basis for drug development. The aim of the study is to compare biological activity of fullerenols with different number of hydroxyl groups. Two clusters of fullerenols were under investigation: (I) C60(OH)x, C60,70(OH)x, where x+y=24–28 and (II) C60,70(OH)x, Fe0,5C60(OH)x, where x+y=40–42. Antioxidant activity and toxicity of the fullerenols were compared using simple cellular and enzymatic bioassays (luminous marine bacteria and their enzymatic reactions, respectively); a content of Reactive Oxygen Species (ROS) in fullerenol solutions was determined using chemiluminescence luminol method. Fullerenol’ antioxidant activity was investigated in model solutions of organic toxicant of oxidative type, 1,4-benzoquinone. Changes in toxicities of general and oxidative type were evaluated. Detoxification coefficients were calculated to analyze and compare the antioxidant activity of the fullerenols. 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 in fullerenol properties were attributed to their ability to affect the ROS balance in aqueous solutions. Toxic effect of the perspective endohedral metal-fullerenol with gadolinium atom involved, Gd@C82(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

Carbon nano-objects are of great interest for different fields of medicine, pharmacology, and biotechnology due to their specific biological activity [1-3]. It is known that high-dose exposures to bioactive compounds can inhibit physiological functions of multiple organisms and hence, produce toxic effects, while low-dose exposures can activate the physiological functions due to optimization of complex metabolic processes [4].

Fullerenols (F) are known to be rigid nanosize carbon particles, water-soluble polyhydroxylated derivatives of fullerenes. Scheme 1 presents hypothetical structure of F with 60 carbon atoms as an example.
Similar to fullerenes, fullerenols are electron deficient structures; and this property makes them efficient catalysts in biochemical reactions and perspective medical drugs. Fullerenols are amphiphilic structures: “hydroxyl groups provide them with aqueous solubility, while the fragments of fullerenol skeleton – with affinity to hydrophobic enzymatic fragments and lipid structures of cellular membranes” [1-2]. Amphiphilic properties and antiradical activity provide a wide range of fullerenol’ biological effects: from neutralization of free radicals [3] to cell protection and drug transportation [1,5-7]. Fullerenols can be used in radiobiology, chemotherapy, and neurology [5-6], ensuring an important alternative to the conventional pharmaceuticals. The antioxidant properties endow fullerenols with ability to neutralize reactive oxygen and nitrogen species [8-12], to function as radioprotectors [10], antitumor [13], or neurological [5,10-12] drugs.

Structural properties 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 affect their catalytic activity. This can result in different 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)_{12-24}, 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 properties. 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)_{34} is less effective despite the large number of hydroxyl groups. The authors concluded that involvement of ≥36 hydroxyl groups to the fullerene 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 were studied. Since the increase of number of hydroxyl substituents reduces the available π-electron system conjugation, it can reduce 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, such as cells, enzymes, low-molecular components, etc.

It is known that ROS group includes a number of free radicals or radical precursors, such as semiquinones, superoxide anion-radical (•O_2^{-}), hydroxyl (•OH) and peroxide (HOO•) radicals, hydrogen peroxide (H_2O_2), peroxide anion (HOO^{-}), singlet oxygen (O_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 a role of mediators of important intracellular signaling pathways [18], thereby regulating cellular processes (respiration, division, etc.), induce the immune system, mobilize ion transport systems, and trigger programmed cell death (apoptosis) [19]. As the ROS impact on living organismal functions is evident [20-28], a study of ROS content in fullerenol’ solutions can elucidate mechanism of fullerenol’ biological effects.
Radical scavenging can be a result of electron donation/acceptation which provide a catalyst activity of the fullerenols. For example, the process of $O_2^*$- neutralizing can be presented as following:

$$2O_2^* + 2H_2O \xrightarrow{F} H_2O_2 + 2OH^- + O_2.$$

Here F plays a role of a catalyst. Detailed decryption of possible mechanisms of neutralization of oxygen radicals is presented in [11]. Authors suggest “two different OH-scavenging mechanisms: … fullerenols with low degrees of hydroxylation prefer the OH addition mechanism, whereas those with high degrees of hydroxylation prefer the hydrogen abstraction”.

Current paper uses physico-chemical approach to the toxic and antioxidant properties of fullerenols. We use a “structure- function” scientific attitude, that allows to find relations between structural peculiarities of fullerenols and their biological activity, and hence, to predict their toxic and antioxidant properties. The relations can help in further applied studies focused on proper structure fullerenols with targeted properties. Additionally, the relations can help to minimize further routine experiments with organs and whole organisms, which are usually time-consuming, expensive and have a low reproducibility.

The physico-chemical approach assumes an application of simple biological assays as models. We applied two types of bioassays of different level of organization - cellular and enzymatic. Both bioassays use luminescence intensity as a test parameter. The simplicity and luminescence registration provide high rates, low costs, and convenience of the bioassay procedure; this paves a way for simultaneous multiple analyses and, hence, statistical reliability.

Cellular bioassay is based on luminous marine bacterium. This bioassay is classic, it has been widely used for more than five decades [29-34]. Enzymatic bioluminescence assay progresses from early 90-s [30,35-37]. Solid immobilized bacterial and enzymatic preparations are developed now as a basis for bioluminescent biosensors [35,38-41]. The bacterial and enzymatic bioassays are tools for investigation the toxic mechanisms at cellular and molecular levels, respectively. Classification of toxic effects was suggested first in [42] and developed later in [43-46]; it describes (1) physicochemical, (2) chemical, and (3) biochemical basis for the toxic effects in the bioluminescence assay systems.

Antioxidant property of bioactive compounds provides a new perspective for their application as biosensors [47-48]. The bioassay systems based on the luminous bacterium or its enzymatic reactions are proper candidates for this application. The both assays can evaluate a general toxicity in the test samples under conditions of the oxidative stress. Additionally, the enzymatic bioassay is specific to oxidizers [46]; it can be applied to monitor the oxidative toxicity in the solutions. This type of toxicity is attributed to redox properties of the toxic compounds only, while the other toxicity type, general toxicity, considers all interactions of toxic compounds with the bioluminescent assay system: redox reactions, polar and non-polar interactions, etc. Previously [49-50] we evaluated the general and oxidative toxicities in solutions of inorganic and organic oxidizers (polyvalent metals and quinones) using the bioluminescent enzymatic assay system. Changes in general toxicity and oxidative toxicity under exposure to humic substances (bioactive compounds of natural origin, products of organics decomposition in soils) were studied in [51-53]. The bioluminescence technique for evaluation an antioxidant activity of bioactive compounds was described in [54-56].

The following fullerenols were chosen in this study: $C_{60}O_x(OH)_y$ and $C_{60}O_{60}(OH)_y$, where $x+y=24-28$; as well as $C_{60}O_x(OH)_y$, Fe$^{3+}C_{60}O_x(OH)_y$, and Gd@C$_{60}O_x(OH)_y$, where $x+y=40-42$. The fullerenols 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 the 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 $C_{60}O_3(OH)_x$ (F1) and $C_{60,70}O_3(OH)_x$ (F2) where $x+y=24-28$; $C_{60}O_3(OH)_y$ (F3), $Fe_{60}C_{60}O_3(OH)_x$ (F4) and $Gd@C_{60}O_3(OH)_y$ (F5) where $x+y=40-42$ were studied.

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 $C_{60}O_3(OH)$ and 40% of $C_{60}O_3(OH)_y$. Fullerenes were preliminary synthesized by carbon helium high-frequency arc plasma at atmospheric pressure [58,60]. The carbon soot included 12.6% of fullerene. The fullerene mixture was extracted by toluene. Then, the individual fullerene $C_{60}$ was isolated by liquid chromatography based on turbostratic graphite with 3.42 Å interplanar distance (as a stationary phase) and toluene/hexane mixture (as a mobile phase).

F3 was produced from a powder mixture of fullerene soot and acetylacetone $Fe$III ($Fe(acac)_3$). 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 $C_{60}$-fullerol are combined by an iron atom) was produced from the powder which involved Fe-containing $C_{60}$ fullerene soot and acetylacetone of $Fe$III ($Fe(acac)_3$). This mixture was heated up to spontaneous ignition (180°C); then the temperature increased up to 250°C in the smoldering regime. The product was treated with concentrated nitric acid (90°C). The red cinnamonic solution was evaporated and treated by distilled water. The procedure provided the hydrolysis of poly-nitro-fullerene to poly-hydroxylated fullerene [15,57,59].

F5 (gadolinium atom is inside $C_{60}$-fullerol) was produced by $Gd@C_{60}$-fullerene hydroxylation in nitric acid followed by the hydrolysis of the polynitrofullerenes [15,57,59,61]. Mixture of fullerenes, involving $Gd@C_{60}$-fullerene, was preliminary synthesized by carbon helium high-frequency arc plasma at 98 kPa [58,61]. The $Gd@C_{60}$-fullerene content in carbon soot was about 4.8%. The reaction of complexation with Lewis acids (TiCl) was used for enrichment of the extract of fullerene mixture by endohedral metallofullerenes ($Gd@C_{60}$) [62]. Then, $Gd@C_{60}$ was extracted with carbon disulfide from carbon soot.

The fullerol 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 enzymatic assay system, we used 0.1 mg ml⁻¹ enzyme preparation, 4·10⁻⁴ M NADH, 5·10⁻⁴ M FMN, and 0.002 % tetradecanal solutions. The enzymatic assay was performed in 0.05 M phosphate buffer, pH 6.8, at 20°C.

The enzymatic assay system is based on the following coupled enzymatic reactions:

$$NADH + FMN \xrightarrow{NADH-FMN-oxidoreductase} FMN \cdot H^- + NAD^+ \quad \text{(reaction 1)}$$

$$FMN \cdot H^- + RCHO + O_2 \xrightarrow{luciferase} \text{FMN} + \text{RCOO}^- + H_2O + h\nu \quad \text{(reaction 2)}$$
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 characterized by relative bioluminescence intensity, \(I_{\text{rel}}^{I_F}\):

\[
I_{\text{rel}}^{I_F} = \frac{I_F}{I_{\text{contr}}^{I_F}}.
\]

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

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

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

\[
I_{\text{rel}}^{I_{\text{Ox}}} = \frac{I_{\text{Ox}}}{I_{\text{contr}}^{I_{\text{Ox}}}}.
\]

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

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 inhibiting the bioluminescence intensity less than 10% (\(I_{\text{rel}}^{I_F} > 0.9\)) was preliminary determined and used in the experiments, to exclude the peculiar toxic effects of the fullerenols.

Both bioluminescent assays (bacterial and enzymatic) were applied to study changes in General Toxicity (GT) under addition of fullerenols. Detoxification coefficients \(D_{GT}\) were determined as follows:

\[
D_{GT} = \frac{I_{\text{rel}}^{I_{\text{Ox}}+F}}{I_{\text{rel}}^{I_{\text{Ox}}}}.
\]
where \( I_{\text{rel}}^{\text{CE}} \) and \( I_{\text{rel}}^{\text{CE+F}} \) 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 \( D_{\text{GT}} \) were determined at different fullerenol concentrations.

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

\[
D_{\text{GT}} = \frac{(T^*)_{\text{CE}}}{(T^*)_{\text{CE+F}}} ,
\]

where \((T^*)_{\text{CE}}\) and \((T^*)_{\text{CE+F}}\) are bioluminescence induction periods in oxidizer solutions in the absence and presence of fullerenols, respectively (Figure 1b). The \( D_{\text{GT}} \) values were determined and plotted vs. fullerenol concentrations.

Values of \( D_{\text{GT}}>1 \) or \( D_{\text{GT}}>1 \) revealed a decrease of \( GT \) or \( OxT \) under the exposure to fullerenols, i.e. detoxification of solutions of oxidizers. Values of \( D_{\text{GT}} \approx 1 \) or \( D_{\text{GT}} \approx 1 \) revealed the absence of the fullerenol effect.

The SD-values for \( D_{\text{GT}} \) or \( D_{\text{GT}} \) did not exceed 0.1. The data for the \( D_{\text{GT}} \) or \( D_{\text{GT}} \) processing were obtained in three experiments with five samplings from all control and fullerenol 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 was obtained from Sigma-Aldrich, potassium hydroxide – from Khimreactiv (Russia), and 3% solution of hydrogen peroxide – from Tula Pharmaceutical Factory (Russia). The \( 10^4 \) M aqueous alkaline luminol solution was used in the experiments.

The chemiluminescence luminol reaction was initiated by solution of \( K_2[Fe(CN)_{6}] \); maximal value of chemiluminescence intensity was determined. All measurements were carried out in 25-40 replicates using TriStar LB 941 bioluminometer with injector system. Average and SD values 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. Fullerene toxicity

We examined toxicity factor of fullerenols with different number of hydroxyl groups using cellular and enzymatic bioluminescence assays. Suppression of bioluminescence intensity was considered as an evidence of fullerene 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 \( I_{\text{rel}}^{\text{CE}} \) (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 assay at concentrations >0.002 g L\(^{-1}\) and >0.001 g L\(^{-1}\) (Figure 2), and suppress bioluminescence of the
enzymatic assay 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 more hydroxyl substituents.

![Figure 2](image2.png)

**Figure 2.** Bioluminescence intensity, \(I_{\text{rel}}\), at different concentrations of fullerenols F2 and F3. Cellular assay.

![Figure 3](image3.png)

**Figure 3.** Bioluminescence intensity, \(I_{\text{rel}}\), 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.

<table>
<thead>
<tr>
<th>Fullerenols</th>
<th>Cellular assay</th>
<th>Enzymatic assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(EC_{50})</td>
<td>(EC_{50})</td>
</tr>
<tr>
<td>F1</td>
<td>0.031</td>
<td>0.092</td>
</tr>
<tr>
<td>F2</td>
<td>0.008</td>
<td>0.021</td>
</tr>
<tr>
<td>F3</td>
<td>0.003</td>
<td>0.007</td>
</tr>
<tr>
<td>F4</td>
<td>0.021</td>
<td>0.007</td>
</tr>
<tr>
<td>F5</td>
<td>-</td>
<td>0.018</td>
</tr>
</tbody>
</table>
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 fresh synthesized F5 (gadolinium atom is inside fullerenol) was studied using enzyme-based bioassay, Table 1. The result demonstrate moderate toxicity of this compound.

It is known that gadolinium is perspective in magnetic resonance imaging and cancer research due to its unique paramagnetic properties [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. Endoendal involvement of gadolinium into fullerenol carcass might be a basis for actual gadolinium-based preparations of low toxicity. The results of this study predict lower toxicity of the preparations with lower number of hydroxyl substituents. Therefore, selection and synthesis of endohedral gadolinium-fullerenol preparations with fewer hydroxyl groups is recommended as a next step of investigations.

3.1.2. Fullerenol antioxidant activity

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

Antioxidant ability of fullerenols was studied using the cell-based and enzyme-based assays. Bioluminescence intensity of the cellular and enzymatic systems was measured in solutions of model oxidizer 1,4-benzoquinone (at EC50) 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 $10^{-15}$–$4\times10^{-4}$ g L$^{-1}$ for F2 and F3, respectively. Maximal values of $D_{GT}$ were about 1.8 and 1.3, respectively.

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 4](https://example.com/figure4.png)
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 brought together in Table 2. It is evident that higher $D_{GT}$ values were observed for F1 and F2. This result demonstrates that fullerenols with fewer oxygen groups are characterized by higher detoxification ability.

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

<table>
<thead>
<tr>
<th>Fullerenols</th>
<th>Maximal value of $D_{GT}$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Enzymatic assay</td>
</tr>
<tr>
<td>F1</td>
<td>2.2</td>
</tr>
<tr>
<td>F2</td>
<td>1.5</td>
</tr>
<tr>
<td>F3</td>
<td>1.3</td>
</tr>
<tr>
<td>F4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

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: $(T_{st})_A$ and $(T_{st})_{Ox+F}$, 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 were 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 antioxidant ability of fullerenols with lower hydroxyl number.

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

<table>
<thead>
<tr>
<th>Fullerenols</th>
<th>Maximal value of $D_{OxT}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>2.0</td>
</tr>
<tr>
<td>F2</td>
<td>1.9</td>
</tr>
<tr>
<td>F3</td>
<td>1.0</td>
</tr>
<tr>
<td>F4</td>
<td>1.3</td>
</tr>
</tbody>
</table>

3.2. ROS content in fulleren solutions. Luminol chemiluminescence assay

We suppose that ROS content is directly related 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 the enzymatic system since the peroxide compounds are involved to the bioluminescence reaction as intermediates [45]. Similar effects can take place in other (non-bioluminescence) redox enzymatic reactions in cells.

We used luminol chemiluminescence method to compare a 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. The effects of F2 and F3 on ROS content are presented in Figure 7 as an example.

![Figure 7](image)

**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.

Table 4 presents fullerenol concentrations which reduce ROS content by 50%, $EC_{50}$. 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).

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

<table>
<thead>
<tr>
<th>Fullerenols</th>
<th>$EC_{50}$, g L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.179</td>
</tr>
<tr>
<td>F2</td>
<td>0.124</td>
</tr>
<tr>
<td>F3</td>
<td>0.056</td>
</tr>
<tr>
<td>F4</td>
<td>0.105</td>
</tr>
</tbody>
</table>
Inhibition and activation of bacterial bioluminescence intensity by ROS was reported previously for bacterial and enzymatic assays [69-70]; hydrogen peroxide was applied by the authors as a representative of ROS.

Correlations between characteristics of chemiluminescence and bioluminescence assays (Table 4 and Tables 1-3, respectively) were studied to confirm involvement of ROS to toxic and antioxidant properties of fullerenols F1-F4. Values of EC50, DC50, and D0.5 were used to calculate correlation coefficients.

The correlation coefficient between EC50-values in chemiluminescence assay (Table 4) and bioluminescence cellular assay (Table 1) was determined as 0.88, while this between chemiluminescence assay (Table 4) and bioluminescence enzymatic assay (Table 1) was determined as 0.83. These results support the hypothesis on ROS involvement to the bioluminescence inhibition by the fullerenols, resulted in toxic effect of these compounds.

The correlation coefficients between EC50 -values in chemiluminescence assay (Table 4) and detoxification coefficients DC50 and D0.5 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 EC50-values in chemiluminescence assay (Table 4) and values of DC50 obtained using bioluminescence cellular assay (Table 2) was 0.13. This result does not demonstrate the dependence on ROS content, probably due to complicated structure and antioxidant response of the cells (as compared to the enzymatic processes).

Our results show that toxicity of fullerenols in high-concentration solutions might be related to their extra ability to neutralize oxygen radicals. 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 antioxidant activity. The results might predict a higher antioxidant activity of non-substituted fullerenes, i.e. carbon nanostructures with holistic π-system apportioned evenly over the spherical macromolecule. Additional experiments under similar conditions should be provided to confirm this suggestion. Previously [71-74], the biological activity of hydrated C60 fullerene was studied; it was attributed to specific structure of hydrated shell of the 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 cellular and enzymatic bioluminescence assays. Lower toxicity and higher antioxidant activity were demonstrated for the fullerenols with fewer substituents: C60Ox(HO)y and C60:70Ox(HO)y, where x+y=24–28. The differences were attributed to fullerol’ ability to disturb ROS balance in aqueous solutions. Further investigations, including theoretical studies, should be carried out to understand physical and chemical basis of these differences. The investigations should be aimed at such structural fullerol 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 acceptance and, hence, nonspecific catalytic activity in chemical and biochemical processes.

As an outlook, a recommendation can be done for selection and synthesis of fullerene’ water-soluble derivatives: high number of oxygen substituents (up to 40 and more) provides high toxicity and low antioxidant 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.
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