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Research Article

The Activity of [60]Fullerene Derivatives Bearing Amine and Carboxylic Solubilizing Groups against *Escherichia coli*: A Comparative Study

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We report a comparative investigation of the antibacterial activity of two water-soluble fullerene derivatives bearing protonated amine (AF) and deprotonated carboxylic (CF) groups appended to the fullerene cage via organic linkers. The negatively charged fullerene derivative CF showed no tendency to bind to the bacterial cells and, consequently, no significant antibacterial activity. In contrast, the compound AF loaded with cationic groups showed strong and partially irreversible binding to the negatively charged *Escherichia coli* K12 TG1 cells and to human erythrocytes, also possessing negative zeta potential. Adsorption of AF on the bacterial surface was visualized by atomic force microscopy revealing the formation of specific clusters (AF aggregates) surrounding the bacterial cell. Incubation of *E. coli* K12 TG1 with AF led to a dose-dependent bactericidal effect with LD_{50} = 79.1 μM. The presence of human erythrocytes in the test medium decreased the AF antibacterial activity. Thus we reveal that the water-soluble cationic fullerene derivative AF possesses promising antibacterial activity, which might be utilized in the development of novel types of chemical disinfectants.

1. Introduction

Investigation of carbon-based nanomaterials (CBN) has made a great impact on the research in the fields of physics, material chemistry and technology, and also life sciences. Water-soluble forms of carbon have been studied intensively worldwide during recent decades. We would like to refer to [1], dedicated to the studies of biological activity of CBN [1]. Toxicity and biocompatibility of CBN were two major points addressed in this journal. Several review papers discussing the biological activities of CBN appeared later [2–4].

Fullerenes, the spherical carbon cages, and their functional derivatives attracted particular attention due to their unusual molecular structures and properties. Recent publications proved lower toxicity of fullerenes compared to other types of CBN, especially carbon nanotubes [5, 6]. It is known that pristine fullerenes such as C_{60} and C_{70} are very hydrophobic and possess virtually zero solubility in water. Classical fullerene derivatives bearing one organic addend appended to the fullerene cage typically have a strong tendency to aggregate in aqueous solutions [7]. Such aggregation lowers their activity significantly and hinders their practical applications. The problem can be solved by using chemically functionalized fullerene derivatives bearing a sufficient number of hydrophilic (or, even better, ionic) functional groups that significantly improve the solubility of these compounds in water [8, 9].

A number of studies have reported effective gene delivery [10] and antioxidative [11], neuroprotective [12], antitumour

[13], and antiviral [14] activities of fullerenes and their derivatives, which makes further exploration of this exciting family of CBN promising. Interesting results were obtained while studying antimicrobial activity of fullerenes [15]. It was proposed that membrane targeting [16], respiratory chain inhibition [17], and photosensitizing effects [18] of fullerene derivatives are responsible for the observed antibacterial action. Functionalizing the carbon cage with hydrophilic functional groups brings up new biological properties. For example, alkylated C_{60}-bis(N,N-dimethylpyrrolidinium iodide) adducts inhibited bacterial growth effectively [19]. Similarly, a carboxylic derivative of [60]fullerene bearing malonic acid fragments on the carbon cage protected mice from bacteria-induced meningitis without direct bacterial inhibition [20]. It should be emphasized that mechanisms of the observed antibacterial activity and selectivity of different types of fullerene derivatives are not currently well understood.

Recently we have succeeded in the selective synthesis of different types of water-soluble fullerene derivatives possessing 4–8 organic addends attached to the cages of C_{60} and C_{70} fullerenes [21–24]. These compounds became available on a large (multigram) scale sufficient for performing a thorough investigation of their biological activities [25, 26]. In the present work, we performed a comparative study of antibacterial activity of two different water-soluble derivatives of [60]fullerene bearing protonated amine (AF) and carboxylate (CF) groups appended to the fullerene cage via organic linkers (Figure 1).

2. Experimental

2.1. Fullerene Derivatives. The fullerene derivatives AF, bearing four protonated 4-(2-pyridyl)piperazine-1-yl groups, and CF, comprising five residues of phenylacetic acid, were synthesized according to the previously published procedures [25, 26]. Spectroscopic characteristics of the prepared samples were identical to the previously reported data [25, 26].

2.2. Preparation and Characterization of Aqueous Fullerene Suspensions. Aqueous suspensions of fullerene derivatives (4 mg/mL) were prepared in deionized water, filtered through syringe filters, and poured into vials that were prewashed several times with filtered water in order to remove dust particles. The suspensions were then incubated for about 2 hours at 20°C, thus allowing the colloidal systems to reach an equilibrium. The temperature control accuracy was 0.1°C.

The size of fullerene aggregates in aqueous suspension was measured using dynamic light scattering (DLS) with a Photocor Complex (Photocor Instruments Inc., USA) setup equipped with a He-Ne laser ($\lambda = 679.5$ nm). The mutual diffusion coefficients of fullerene aggregates were computed from the DLS data using the DynaLS program (Alango, Israel). Hydrodynamic diameters of the fullerene aggregates were calculated from the mutual diffusion coefficients using the Einstein-Stokes formula for diffusion coefficients of spherical particles.

Electrophoresis of fullerene derivatives was performed in a 1.5% agarose gel at a constant voltage of 150 V and current of 100 mA, so that the electric field strength was 5 V/cm. After 20 min of electrophoresis, migration of compounds was evaluated by visible and UV light on a transilluminator (Vilber Lourmat, France). The Smoluchowski equation was used to calculate the ζ-potential from the electrophoretic mobility.

Adsorption and fluorescence spectra of fullerene derivatives in aqueous suspensions were recorded with a Fluorat-02 Panorama spectrofluorometer (Lumex, Russia) in a spectral range of 220–400 nm.

2.3. Bacterial Strain and Cultures Preparation. The experiments involving bacterial cells were performed using the *Escherichia coli* K12 TG1 strain. The applicability of this strain for evaluation of bactericidal activity of a wide range of carbon-based nanomaterials has been demonstrated previously [26]. The bacteria were grown in LB-broth (Sigma-Aldrich, USA) for 18–24 hours at 37°C, after which the cells were harvested by centrifugation at 1000 g for 10 min, washed once with the distilled water, and diluted to achieve the optical density of 0.5 absorption units at 640 nm, which corresponds to the concentration of 3.5×10^9 colony-forming units (CFU) per 1 mL.

The human erythrocytes were collected from blood (0 Rh+) by centrifugation at 1000 g for 10 min and washing the precipitate with 0.85% NaCl solution twice. The final concentration of erythrocytes was 7×10^8 cells/mL, which is equal to the surface area of 0.02 m^2/mL.

2.4. Investigation of Adsorption/Desorption of Fullerene Derivatives on the Cell Surfaces. Fullerene derivatives AF and CF were introduced to the cell suspensions to prepare a series of samples with 2, 1, 0.5, 0.25, 0.12, 0.06, and 0.03 μM concentrations of the compounds. After incubation at 37°C for 60 minutes, the prokaryotic and eukaryotic cells modified with the fullerene derivatives were separated by centrifugation at 1000 g for 10 min. The concentration of unbound fullerene derivative in the supernatant was determined using fluorimetry in the case of AF and photometry in the case of CF. The value of the adsorption was calculated using

$$A = \frac{(C_0 - C_e)}{S}, \quad (1)$$

where C_0 is the starting concentration of the fullerene derivative, C_e is the equilibrium concentration of the fullerene derivative after partial adsorption to the cell membranes (determined in supernatant), and S is the surface area of the cells.

The evaluation of the fullerene derivative desorption from the bacterial cells surfaces was performed by dispersing the *E. coli* K12 TG1 cells in the solutions of AF ($C_0 = 1\,\mu$M) and CF ($C_0 = 10\,\mu$M); is incubation of these dispersions for 60 min is followed by centrifugation. The precipitated bacterial biomass was separated and dispersed again in an equal volume of distilled water, while the supernatant liquor

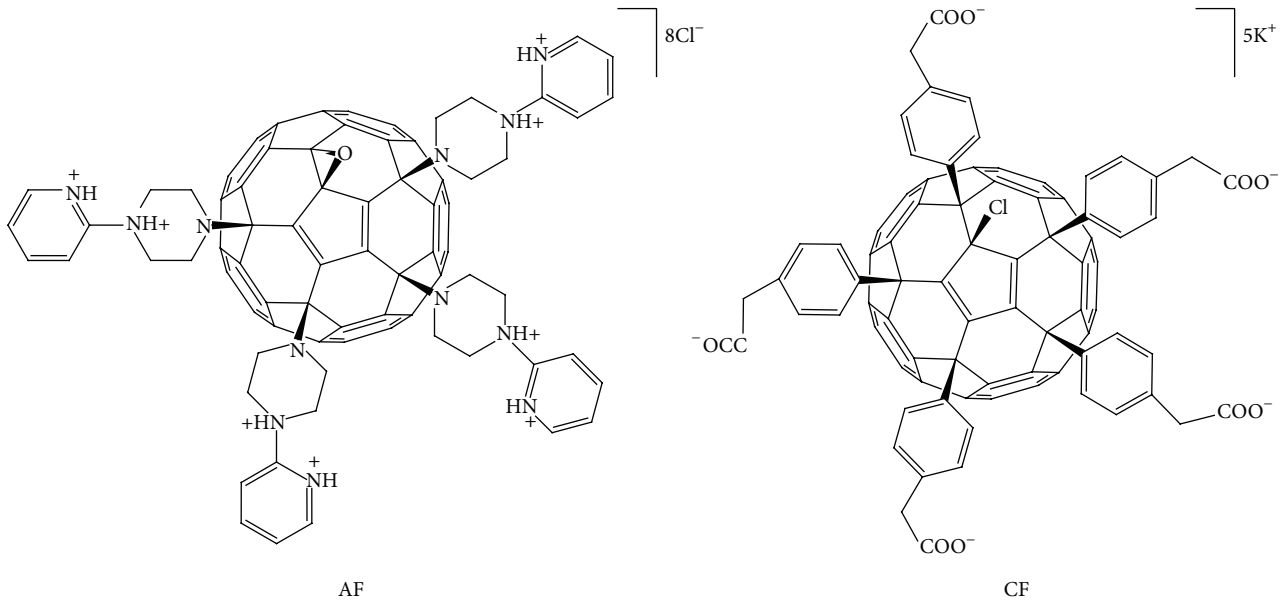


Figure 1: Molecular structures of the fullerene derivatives AF and CF.

was analysed using fluorimetry or photometry to reveal the concentrations of the AF and CF, respectively. The bacterial cells that were redispersed in distilled water were subjected again to centrifugation and the concentration of the fullerene derivative in the supernatant liquid was determined. Washing the bacterial biomass with distilled water was repeated ten times and each time the fullerene derivative concentration in the supernatant was determined in order to reconstruct the desorption profiles.

2.5. Atomic Force Microscopy. Visualization of the bacterial cells in the intact form or after incubation with the fullerene derivatives was performed using an atomic force microscope (SMM-2000; Proton-MIET, Russia). An aliquot (20 μL) of the suspension comprising either intact cells of *E. coli* K12 TG1 or their mixtures with AF and CF was applied to the freshly prepared mica at 95% relative humidity and 20–22°C. The samples were scanned in a contact mode using V-shaped silicon nitride cantilevers MSCT-AUNM (Veeco Instruments Inc., USA) with a spring constant of 0.01 N/m and a tip curvature of 150–250 Å. Quantitative morphometrical analysis of the images was performed using standard software provided with the microscope.

2.6. Bactericidal Activity of Fullerene Derivatives. The test samples comprised a suspension of *E. coli* K12 TG1 at a concentration of 3.5×10^9 CFU/mL and the fullerene derivatives were added in concentrations ranging from 25 to 200 μM. In a separate series of experiments, *E. coli* was incubated with fullerene derivatives in the presence of human erythrocytes at a concentration 7×10^8 cells/mL. This concentration of erythrocytes provided an equal surface area compared to the total area of the bacterial cell membranes (0.02 m^2/mL). Lysis of erythrocytes was induced by addition of 0.05% saponin prior to plating the prepared cultures in the BCP-agar medium.

The 100 μL aliquots taken from the experimental samples 60 min after the fullerene derivative addition and the control samples comprising no fullerene derivatives were diluted with 900 μL of distilled water. Small portions of the obtained test samples (10 μL) were inoculated on the plates with BCP-agar (Bio-Merieux, France). The percentage of the bacterial cells retaining their viability during incubation with fullerenes (calculated as the total number of CFU in an experimental sample divided by the number of CFU in the control series) was determined after an additional 18–24 h incubation of the samples at 37°C. The dose of the fullerene derivative causing the death of 50% of the microbial cells (LD_{50}) was obtained from these experiments.

3. Results and Discussion

3.1. Characterization of Aqueous Fullerene Suspensions. The attachment of the cationic (protonated amine, AF) or anionic (COO–, CF) functional groups to the [60]fullerene cage significantly increased the solubility of the fullerene derivatives in water. The resulting aqueous solutions of AF and CF (concentration 4 mg/mL) were transparent and had a bright orange-brown colour. No fullerene precipitation was observed in the course of the experiments indicating that the prepared aqueous systems were rather stable with respect to aggregation and sedimentation of the dissolved/dispersed compounds.

The dynamic light scattering (DLS) experiments allowed the determination of the hydrodynamic sizes of the fullerene nanoparticles in aqueous suspensions. The DLS experiments showed that both [60]fullerene derivatives form aggregates in aqueous solutions with diameters of 2–200 nm (AF) and 70–100 nm (CF) (Figure 2). Larger particles of 10^4–10^6 nm (AF)

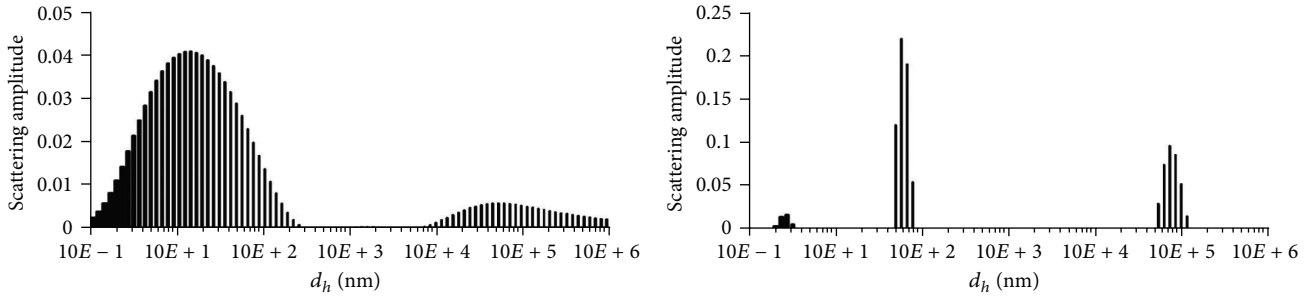


Figure 2: DLS profiles for aqueous suspensions of AF (a) and CF (b) fullerene derivatives.

and ~10^5 nm (CF) correspond, most likely, to some larger aggregates composed of the fullerene derivative aggregates.

The DLS measurements indicated that the fullerene aggregates formed in aqueous systems have rather high polydispersity, particularly in the case of AF. These data correspond well to the previous reports describing spherical and irregularly shaped fullerene-based nanoparticles as revealed by DLS, TEM, and high-resolution TEM [26–30]. Very similar nanoclusters were also observed in this work using atomic force microscopy (AFM) (see Section 3.4); therefore, DLS and AFM measurements showed that fullerene derivatives AF and CF undergo aggregation in aqueous solutions. Taking into account the typical size of these aggregates, the bicomponent systems comprising the fullerene derivative and water should be called a colloidal solution or even suspension rather than a true solution.

3.2. Detection of Electric Charge and Zeta Potentials of Fullerene Derivatives. The agarose gel electrophoresis performed for the water-soluble fullerene derivatives AF and CF showed that they represent highly charged cationic and anionic species (Table 1). Motion of the carboxylic fullerene derivative towards the anode was clearly detectable with the naked eye. Movement of the amino fullerene towards the cathode was visualized with a UV transilluminator owing to the reasonably strong fluorescence of the AF compound (see Section 3.3).

The calculated zeta potentials (ζ) of AF and CF were +41.5 mV and −52.2 mV, respectively. It is known that aqueous fullerene suspensions are stable if the zeta potentials of the dispersed particles are smaller than −15 mV or higher than +15 mV [31]. Very pronounced cationic and anionic properties of AF and CF, respectively, reflected in the values of their zeta potentials, explain the high solubility of these compounds in water. It should also be noted that compounds of AF and CF have rather large dipole moments since organic addends bearing ionic groups are located at one hemisphere of the fullerene cage.

Moreover, the functionalized part of the fullerene cage becomes hydrophilic, while the opposite side of the carbon sphere remains hydrophobic. Therefore, peculiarities of the molecular structures of AF and CF enable electrostatic dipole-dipole and hydrophobic-hydrophilic interactions. Thus, van der Waals attraction forces [32] bring the molecules of the fullerene derivatives together forming suspensions of solvated nanoparticles rather than true molecular solutions.

3.3. Spectroscopic Study of Fullerene Derivatives. An aqueous suspension of CF exhibits one distinct adsorption band with the maximum at 230 nm, while the AF suspension shows two bands with maxima at 240 and 300 nm (Figure 3(a)).

It is surprising that the amino fullerene showed unusually strong fluorescence with maxima at 360 nm under excitation with ultraviolet light at 240 or 300 nm. An excitation of AF solution at 240 nm produced 1.8 times higher fluorescence intensity compared to the excitation at 300 nm. The fluorescence intensity linearly depended on the concentration of AF in the range of 0–2 μM (Figure 3(b)).

The fluorescence was observed previously for pristine [60]fullerene and its derivatives. Compared to the week fluorescence of pristine C60 (λ_{max} = 689 nm) [33], the functionalized fullerenes show stronger emission bands shifted to the short-wave spectral range. For instance, blue-shifted fluorescence was reported for the fullerene derivatives bearing eight or ten pyridyl groups attached to the carbon cage [34].

In this study we used the fluorescence spectroscopy to perform an accurate investigation of AF adsorption and desorption on the surface of prokaryotic or eukaryotic cells. In the case of the nonfluorescent CF derivative, a photometric method was used for determination of its concentration in the experimental solutions.

3.4. Adsorption of the Fullerene Derivatives on the Surface of Prokaryotic or Eukaryotic Cells. The prokaryotic (*E. coli*) or eukaryotic (human erythrocyte) cells were incubated with the suspensions of the fullerene derivative AF at concentrations ranging from 0.03 to 1 μM. Then the cells loaded with the fullerene derivative were separated by centrifugation and the supernatant liquid was subjected to fluorescence analysis.

The fluorescence measurements revealed the residual amount of amino fullerene that was not adsorbed by the cells. These experiments show that AF has similarly strong but not identical affinity to both types of cell surfaces. An increase in the AF concentration resulted in a stronger binding of this compound to the cell membranes reflected by higher binding indexes (Table 2).

Table 1: Fullerene derivatives electrophoretic mobility and zeta-potentials.

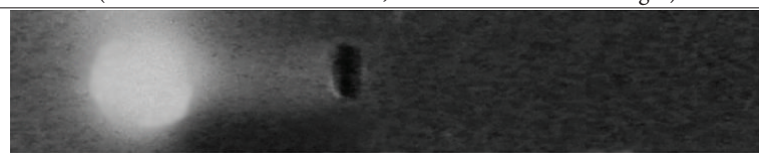
Compound	Mobility in the electric field (the cathode side is on the left; the anode side is on the right)	Zeta potential, ζ, mV
AF		+41.5
CF		−52.2

Table 2: AF adsorption values on *E. coli* K12 TG1 cells and human erythrocytes.

Concentration of AF, μM			Adsorption values, μM/m^2	
Initial	Bound to cells			
	E. coli	Erythrocytes	*E. coli*	Erythrocytes
0.03	0.03 ± 0.01	0.02 ± 0.01	1.50	1.00
0.06	0.06 ± 0.01	0.06 ± 0.01	3.00	3.00
0.12	0.11 ± 0.02	0.10 ± 0.02	5.50	5.00
0.25	0.18 ± 0.01	0.16 ± 0.01	9.00	8.00
0.50	0.40 ± 0.02	0.23 ± 0.03	20.00	11.50
1.00	0.54 ± 0.17	0.39 ± 0.11	27.00	19.50

It is seen from Table 2 that AF when used in high concentrations has somewhat stronger affinity to the *E. coli* cells compared to the human erythrocytes. For example, the maximal adsorption values of 27.0 μM/m^2 and 19.5 μM/m^2 were obtained for *E. coli* and erythrocytes, respectively, when AF was added at an initial concentration of 1 μM.

Similar experiments were also performed for CF incubated with prokaryotic and eukaryotic cells. Due to the lower affinity of CF to the cell membranes, its initial concentration was increased to 10 μM. However, the fullerene derivative CF showed very weak adsorption on bacterial and erythrocyte cell surfaces, as characterized by maximal adsorption values of 1.91 μM/m^2 and 2.64 μM/m^2, respectively.

The binding of AF to the bacterial cells was confirmed by atomic force microscopy. Contact mode AFM allowed us to visualize the cells of *E. coli* before (Figure 4(a)) and after (Figure 4(b)) incubation with AF.

The analysis of the morphological characteristics of the bacterial cell incubated with AF showed a significant increase in the surface roughness. This change was caused by the granules (35–160 nm in diameter) that accumulated on the cell surface. We believe that these granules are represented by nanoclusters composed of AF molecules, which were also revealed by the DLS measurements (see above). In addition, the *E. coli* cells changed their length, width, and height, thus supporting additionally the action of the AF derivative. It is important to note that no signs of leakage of the cytosolic content of the bacterial cells to the environment were observed. This observation implies that the membrane disruption is not the leading mechanism of antibacterial activity of fullerenes [35, 36] in contrast to the action of other types of CBN [37].

Analysis of the morphological characteristics of *E. coli* cells incubated with CF did not reveal any significant changes in the cell surface roughness or size (Figure 4(c)). At the same time, scanning the substrate (mica) around the bacterial cells revealed freely located round-shaped features with a mean diameter of 217.90 ± 89.71 nm. These features might be attributed to the aggregates of the CF molecules also evident from the DLS data.

The sharply different behaviour of AF and CF with respect to the prokaryotic (bacterial) and eukaryotic (erythrocyte) cells can be explained mainly by the electrostatic forces. Indeed, the cationic AF (ζ = +41.5 mV) should have strong Coulomb attraction to the negatively charged *E. coli* cells (ζ = −50.0 mV) [38] and somewhat weaker attraction to the less negatively charged human erythrocytes (ζ = −13.5 mV) [39]. At the same time, the anionic fullerene derivative CF bearing a negative charge itself (ζ = −52.2 mV) cannot interact effectively with the surface of the prokaryotic and eukaryotic cells due to the Coulomb repulsion. The proposed mechanism relying on electrostatic interactions between the fullerene derivatives (or their aggregates) and cell membranes can explain both antibacterial [40] and cytotoxic [41, 42] effects of the fullerene derivatives. However, one should keep in mind that electrostatic interactions could be just an initial step of a complicated cascade of processes that occur when prokaryotic or eukaryotic cells are incubated with the fullerene derivatives.

3.5. Desorption of Fullerene Derivatives from the E. coli Cell Surface. In order to check the strength and reversibility of the AF and CF binding to the bacterial cell surface, we analysed the desorption of these compounds using a very simple experiment (see Section 2). The obtained AF and CF desorption values are shown in Table 3.

It was shown that the binding of the amino fullerene AF to the bacterial cells is strong and partially irreversible. It is seen from Table 3 that only 64.7 ± 1.6% of initially adsorbed AF was desorbed from the bacterial cells after 10 washing cycles. Moreover, excretion of fullerene occurs gradually and the last cycles showed very little and decreasing desorption of AF,

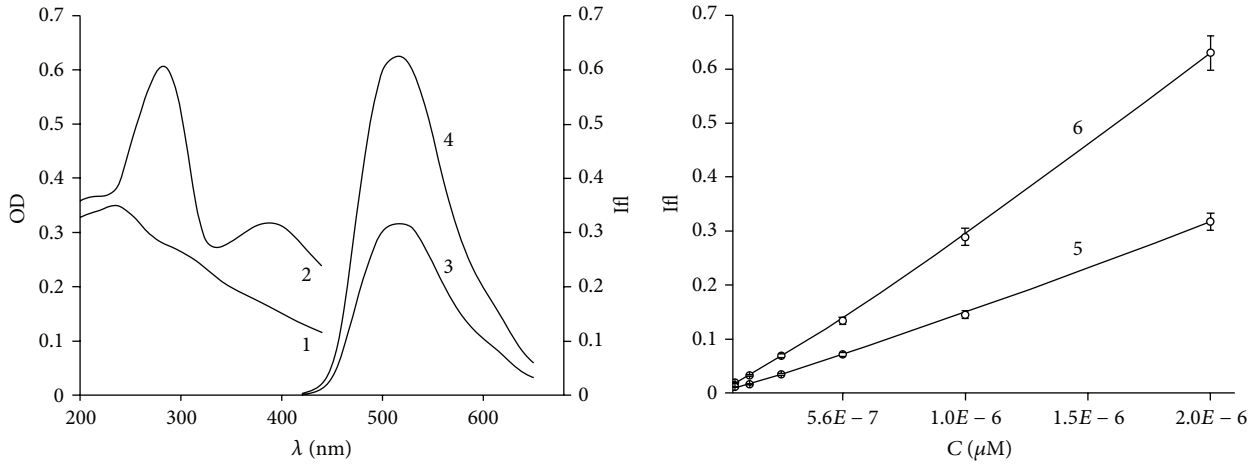


Figure 3: Optical spectra of the fullerene derivatives. (a) Adsorption spectra of CF, $C = 2 * 10^{-6}$ M (1), and AF, $C = 2 * 10^{-5}$ M (2), and the fluorescence spectra of AF obtained under excitation at 300 nm (3) and 240 nm (4). (b) Dependence of AF fluorescence intensity on the concentration of AF in the solution for $\lambda_{ex.} = 300$ nm (5) and $\lambda_{ex.} = 240$ nm (6).

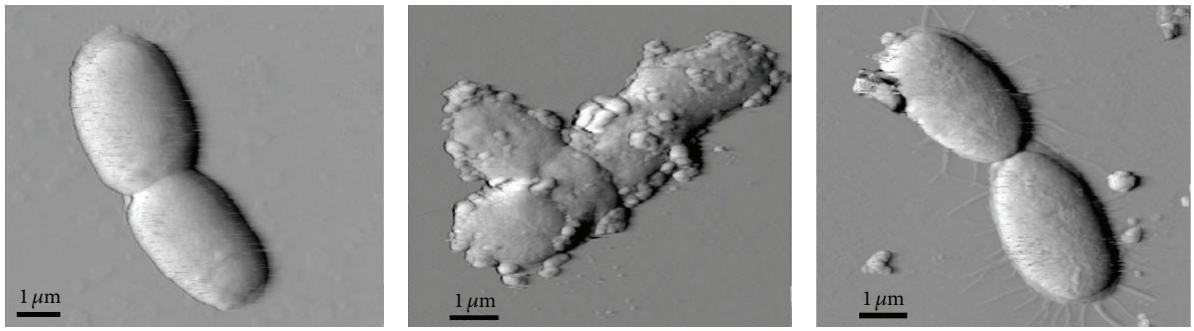


Figure 4: AFM images of *E. coli* K12 TG1 before (a) and after incubation with AF (b) and CF (c).

thus indicating that the remaining amount of this fullerene derivative is absorbed irreversibly. It can be estimated from these data that each *E. coli* cell absorbed irreversibly about 10^5 molecules of AF.

The observed partially irreversible absorption of AF suggests the possibility of its penetration into the microbial cell, which was reported previously for some other fullerene derivatives [43]. The transmembrane AF diffusion could develop due to the concentration gradient and subsequently lead to the electrostatic binding of AF to the intracellular structures and molecules bearing negative charges (including DNA) [44].

This is our assumption at the present stage; further research will be performed to check this idea experimentally.

Similar desorption experiments were performed with CF. The obtained desorption values shown in Table 3 illustrate that four washing cycles removed 94.7 ± 5.5% of CF applied in a very high initial concentration of 10 μM. Most of the fullerenes (80.9 ± 3.3%) were already washed out at the first cycle. This observation suggests that interactions of CF with the bacterial cells are very weak, which is in line with the experimental results described above.

3.6. Antibacterial Activity of Fullerene Derivatives against E. coli. The bactericidal effect of fullerene derivatives was evaluated by investigating the loss of the viability of *E. coli* K12 TG1 cells after a 60-minute incubation with different concentrations of AF and CF. Carboxylic fullerene derivative CF did not cause any bactericidal effect at concentrations of up to 400 μM. In contrast, the amino fullerene derivative showed notable bactericidal activity. The incubation of *E. coli* with AF led to the time- and concentration-dependent death of the bacterial cells (Table 4). The characteristic LD_{50} value of 79.1 μM was calculated from the obtained data. Use of higher concentrations of this compound (400 μM and higher) resulted in the death of at least 99% of *E. coli* cells. These results suggest that AF might be considered as a novel and promising type of chemical bactericide.

Table 3: Fullerene derivatives desorption from *E. coli* K12 TG1 cells.

Number of the consecutive washing (separation/redispersion) cycles	Fullerene derivative desorption, %	
	AF	CF
1	16.5 ± 1.0	80.9 ± 3.3
2	13.7 ± 0.7	6.7 ± 0.5
3	8.3 ± 0.5	5.4 ± 0.4
4	7.1 ± 0.5	1.7 ± 0.1
5	4.9 ± 0.4	0
6	4.2 ± 0.3	0
7	3.7 ± 0.2	0
8	2.7 ± 0.2	0
9	2.2 ± 0.1	0
10	1.4 ± 0.1	0
Total	64.7 ± 1.6	94.7 ± 5.5

Table 4: Percentage of the *E. coli* K12 TG1 cells that lost their vitality after 60 min of incubation with AF in the absence and in the presence of human erythrocytes.

Concentration of AF, μM	Bactericidal effect, %	
	E. coli	*E. coli* + erythrocytes
25	12.0 ± 3.7	1.8 ± 4.4
50	31.9 ± 3.1	5.6 ± 4.3
100	58.1 ± 2.4	6.0 ± 4.3
200	77.6 ± 1.4	66.8 ± 1.8

The selectivity of the antibacterial action of AF was investigated in a three-component test mixture comprising fullerene derivative, *E. coli* cells, and human erythrocytes. The concentrations of the cells in the test samples were adjusted to equalize the total areas (0.02 m^2/mL) of the *E. coli* and the erythrocyte cell surfaces. Indeed, the bactericidal activity of AF was essentially decreased in the presence of human erythrocytes in the test samples together with the *E. coli* cells (Table 4). The effect of the erythrocytes suppressing the antibacterial activity of AF was the most pronounced for concentrations ≤100 μM.

This observation is explained by nonspecific AF binding to the bacterial cells and erythrocytes. Such a concurrent pathway decreases the bactericidal activity of AF and increases LD_{50} value more than twofold for the system comprising both *E. coli* cells and human erythrocytes. The observed tendency of AF to bind to the erythrocytes hinders *in vivo* applications of this compound for fullerene-mediated therapy. Nevertheless, AF still can be considered as a promising chemical disinfectant for various types of surfaces.

4. Conclusion

Highly water-soluble derivatives of [60]fullerene bearing cationic (protonated amine, AF) or anionic (COO–, CF) functional groups appended to the fullerene cage have been investigated. It was shown that both fullerene derivatives form nanoclusters in aqueous solutions with hydrodynamic diameters of 2–200 nm (AF) and 70–100 nm (CF) as revealed by the DLS measurements.

The obtained experimental data implied that initial interactions of the fullerene derivatives and their nanoclusters with prokaryotic and eukaryotic cells are governed mainly by electrostatic Coulomb forces. Moreover, electrostatic interactions explain the appearance of the antibacterial activity of fullerene derivatives. Indeed, the cationic fullerene derivative AF (having zeta potential $\zeta = +41.5$ mV) undergoes strong binding to the negatively charged *E. coli* cells ($\zeta = -50$ mV) due to the attractive electrostatic interactions that result in the appreciable bactericidal activity of this compound. In contrast, the anionic fullerene derivative CF ($\zeta = -52.2$ mV) does not bind to the bacterial cell membranes because of the repulsive Coulomb interactions and subsequently shows no bactericidal effect. The binding of the amino fullerene (AF) clusters to the bacterial cells and also the absence of such binding in the case of CF were visualized by atomic force microscopy.

The fullerene derivative AF induced time- and concentration-dependent death of the bacterial *E. coli* cells characterized by an LD_{50} value of 79.1 μM. However, the antibacterial activity mechanism of [60]fullerene and fullerene derivatives is unclear and still debated [45]. Like Aquino et al. [46] we confirmed AF influence on the bacteria viability without cell membrane disruption. On the other hand, our results reaffirm AF intercalation into the cell wall that enables membrane stress [40], subsequent respiratory chain inhibition [47], and ROS-dependent toxicity [48].

The bactericidal activity of AF decreased significantly in the presence of human erythrocytes. Indeed, human erythrocytes also bear a negative charge ($\zeta = -13.5$ mV), which facilitates their electrostatic interactions with the positively charged AF molecules (and/or nanoclusters). The efficient binding of AF (and, presumably, other positively charged fullerene derivatives) to both prokaryotic (*E. coli*) and eukaryotic (erythrocyte) cells hinders *in vivo* applications of such compounds for fullerene-mediated therapy. Nevertheless, AF can still be considered as a promising chemical disinfectant for various types of surfaces. In contrast, CF (and probably other anionic fullerene-based compounds) shows no ability to bind to the bacterial cells or erythrocytes and might have big potential for *in vivo* studies and, possibly, for some biomedicinal applications.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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