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ANTIBACTERIAL ACTIVITY OF CATIONIC CYCLEN-FUNCTIONALIZED FULLERENE DERIVATIVES: MEMBRANE STRESS

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We report a comparative investigation of the antibacterial activity of the novel water-soluble fullerene derivatives possessing cyclen attached to the cages of C_{60} via organic linkers. The posively charged cyclen-functionalized fullerene derivative (CF) showed significant antibacterial activity toword to both Gram negative (*E. coli*) and positive bacteria (*S. aureus*). Their antibacterial activities are time and concentration dependent. For cells, the IC_{50} for *E. coli* and *S. aureus* cells are 4.8 μg/mL and 7.4 μg/mL, respectively. The bacterial cytotoxicity may be attributed to membrane stress mediated by direct physical contacts. These results suggest that CF might be considered as a novel and promising type of antibacterial drugs.

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Keywords: Fullerene Derivative, Cyclen Moiety, Zeta potential, Bacterial cytotoxicity, Membrane stress

1. Introduction

Fullerene-C_{60} displays unique chemical and physical properties, and has proven to be important in the field of medical and biological sciences[1-5]. However, the potential application of fullerenes in biological systems has been restricted by their extremelypoor solubility in water. Thus, fullerene derivatives bearing a sufficient number of hydrophilic (or, even better, ionic) functional groups[6,7] have been intensively investigated for specific biological activities positioned in various fields, including neuroprotective agents[8], anti-HIV agents[9,10], photodynamic therapy agents[11-14], drug delivery systems, inhibitors of DNA enzymes[15,16], anticancer agents[17,18], and so on.

Interesting results were obtained while studying antimicrobial activity of fullerenes and their detivatives[19-24]. Fullerenes and their derivatives show antimicrobial activity against various bacteria, such as *E. coli*, *Salmonella* and *Streptococcus spp*[20]. It was proposed that inhibition of energy metabolism[25], respiratory chain inhibition[26], directing physical contact[22,24] and photosensitizing effects[28] of fullerene derivatives are responsible for the observed antibacterial action. The discovery of fullerenes ability to interact with biological membranes has encouraged many researchers to evaluate their antimicrobials applications. For example, Deryabin et al revealed that important correlations between these physicochemical characteristics and contacts of fullerene derivatives with the bacterial cell surface involved in bioenergetics violation and toxic effect[22]. In addition, cationic derivatves, such as some aminofullerenes, were active against Gram-positive (*Enterococcus faecalis*) and Gram-negative

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(*Escherichia coli*) bacteria[21]. However, 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 synthesis of a novel water-soluble fullerene derivatives possessing cyclen attached to the cages of C_{60} via organic linkers[29]. In the present work, we investigated the antibacterial activity of the derivatives in *Escherichia coli* (*E. coli*), a Gram-negative bacterium, and *Staphylococcus aureus* (*S. aureus*), a Gram-positive bacterium. We first characterized the cyclen-functionalized fullerene derivative (CF) in aqueous dispersions by dynamic light scattering analysis (DLS), and quantified its average sizes by SEM. The time and concentration dependent antibacterial activities were found, and then the material characteristics related to its antibacterial activities were identified. We suggest that the membrane stress mediated by direct physical contacts as their toxicity mechanism toward bacterials

2. Experimental section

2.1 Preparation and characterization of CF dispersions

The fullerene derivative CF, bearing the cyclen group, was synthesized according to the previously published procedure[29]. Aqueous suspensions of CF (4 mmol) were prepared in 0.5% DMSO solution in glass vials, vigorously vortexed and sonicated for 30 min in a water bath.

The size and zeta potential of CF dispersed in salt-free aqueous suspensions were assessed with a laser autocorrelation analyzer, Zetasizer Nano (Malvern Instruments Ltd, United Kingdom). SEM analysis was performed on an a JEOL field emission SEM (JSM-6700F), working at 5 kV.

2.2 Bactericidal Activity of CF

E. coli and *S.aureus* were grown in LB (Sigma-Aldrich) medium at 37 °C for 18-24 hours, after which the cells were harvested by centrifuged at 4000 rpm for 15 min, washed three times with sterile 0.9% NaCl to remove residual macromolecules and other growth medium constituents. Then the bacterial cell suspensions were diluted by 0.9% NaCl to achieve the optical density of 1.0 absorption units at 600 nm, which corresponds to the concentration of 10^7−10^8 colony-forming units (CFU) per 1 mL.

E. coli and *S.aureus* cells were incubated with fresh fullerene derivatives suspensions at 37 °C for 2 h. The viability of *E. coli* and *S.aureus* cells was evaluated by the counting method. cell dilutions (100 μL each) were spread onto LB plates, and left to grow overnight (12 h) with 180 rpm shaking speed at 37 °C. Colonies were counted, and compared to those on control plates to calculate changes in the cell growth inhibition. All treatments were prepared in duplicate and repeated at least on three separate occasions. Loss of viability was calculated by the following formula：

Loss of viability % = (counts of control − counts of samples after incubation with suspensions)/counts of control.

2.3 Detection of Reactive Oxygen Species ($O_2^{\bullet-}$)

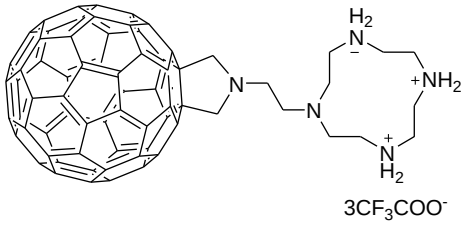
The possibility of superoxide radical anion ($O_2^{\bullet-}$) production was evaluated by monitoring the absorption of XTT (2,3-bis (2-methoxy-4-nitro-5-

sulfophenyl)-2H-tetrazolium-5-carboxanilide, Fluka). 80 μg/mL CF dispersion was mixed with 0.4 mM XTT in a phosphate buffered saline (PBS) buffer. The mixture was incubated in dark for 7 h. Afterwards, the mixture was filtered through a 0.22 μm polyethersulfone filter to remove CF. Filtered solution (2 mL) was then placed in a cuvette. The changes in absorbance at 470 nm were monitored on a PerkinElmer UV/VIS spectrophotometer.

3. Results and discussion

3.1 Characterization of cyclen-functionalized [60] fullerene derivatives

The synthesized cyclen-functionalized [60] fullerene derivatives (CF) used in this study is presented in Scheme 1. The compound consists of a hydrophobic "buckyball" cage and a ionic functional group attached to the [60]fullerene cage, thus including amphilic properties and significantly increasing solubility of the fullerene derivatives in water. The resulting 0.5% dimethylsulfoxide (DMSO) aqueous solution of CF was transparent and had a red-brown colour.



Scheme 1 Molecular structures of the cyclen-functionalized fullerene derivatives, CF

The SEM image of CF in Figure 1 shows the CF agglomerates slightly with a wide size range, from 150 to 320 nm in diameter. The CF aggregates were similar in size and shape to those published previously[22,23].

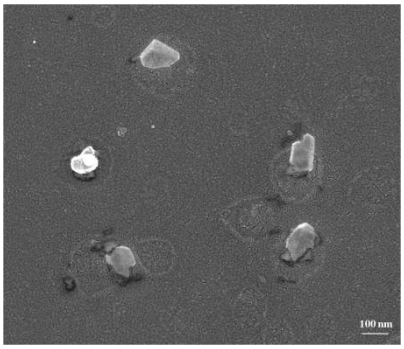


Fig. 1 SEM micrographs of CF

The dynamic light scattering (DLS) experiments allowed the determination of the hydrodynamic sizes of the fullerene derivative CF in the aqueous suspension. The DLS data indicated that particles with an average diameter of around 301.71 ± 139.26 nm predominated in the CF aqueous suspension (Figure 2a), suggesting the presence of supramolecular aggregates formed by billions of fullerene monomers. The result confirmed the particles size distribution determined by SEM. 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.

Zeta potential measurements, evaluated by electrophoretic mobility of colloidal particles of the fullerene derivative, indicated that CF acquired negative surface charge of +39.8 ± 0.4 mV. 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. Moreover, the functionalized part of the fullerene cage becomes hydrophilic, while the opposite side of the carbon sphere remains hydrophobic. Therefore, the peculiarity of the molecular structure of CF enable electrostatic dipole-dipole and hydrophobic-hydrophilic interactions. Thus, van der Waals attraction forces bring the molecules of the fullerene derivative CF together forming suspensions of solvated nanoparticles rather than truemolecular solutions[30].

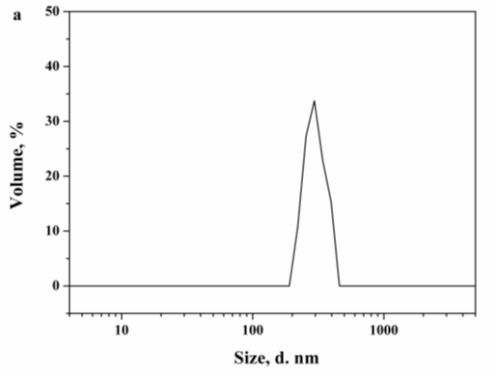


Fig. 2 Example of size distribution in the aqueous suspension of CF.
The diameter of CF aggregates is 301.71 ± 139.26 nm.

3.2 Antibacterial Activity of CF against *E. Coli.* and *S. aureus* cells

We examine the antibacterial activity of CF against two well-studied laboratory bacteria, specifically *Escherichia coli* (*E. coli*), a Gram-negative bacterium, and *Staphylococcus aureus* (*S. aureus*), a Gram-positive bacterium. The bactericidal effect of fullerene derivatives was evaluated by investigating the loss of the viability of *E. coli* or *S. aureus* cells after 2 h incubation with C_{60}, cyclen and CF, respectively. The viability of cells was determined by the colony forming units (CFU) counting method. The isotonic saline solution without CF-based materials was used as a control. As shown in Figure 3, the cyclen solution exhibits a moderate cytotoxicity with the *E. coli* and *S. aureus* cells inactivation percentage at 19.5 ± 6.1% and 18.2 ± 4.3%, respectively. The C_{60} dispersion shows a slight weaker antibacterial activity compared with cyclen solution, having the *E. coli* and *S. aureus* cells inactivation percentage at 14.0 ± 4.5% and 7.3 ± 2.2%, respectively. CF have a much stronger bacterial activity compared with C_{60} and cyclen. The loss of *E. coli* cells

viability increases to 86.1 ± 1.7%, which is more than 6-fold compared with that of C_{60}. On the other hand, the loss of *S. aureus* cells viability increases to 40.7 ± 2.7%, which is more than 6-fold compared with that of C_{60}. It also should be noted that the shaking speed of 180 rpm was used in all antibacterial assays. Although some C_{60} and CF particles precipitate when the dispersions stand still for 2 h, under the shaking condition, the particles are well suspended in the saline solution interacting with cells in all assays. Besides, the *S. aureus* cells seem to show less susceptive toward the CF dispersions compared to the *E. coli* cells.

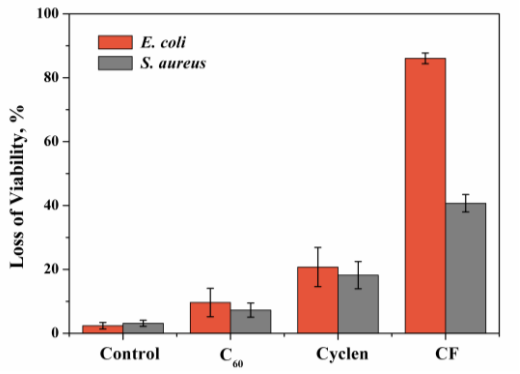


Fig. 3 Cell viability of E. coli or S. aureus cells (10^7 ~ 10^8 CFU/mL) after incubation with CF, C_{60} and cyclen suspensions (7.5 μg/mL) for 2 h with 180 rpm shaking speed at 37 °C, respectively. Loss of viability was calculated by the following formula: loss of viability % = (counts of control − counts of samples after incubation with suspensions)/counts of control.

3.3 Time-dependent and concentration-dependent antibacterial activity

The incubation of *E. coli* or *S. aureus* with CF led to the time and concentration-dependent death of the bacterial cells. First, we examined the time-dependent antibacterial behavior of CF. CF dispersions (7.5 μg/mL) were incubated with *E. coli* or *S. aureus* for 4 h. The loss of *E. coli* or *S. aureus* viability were counted at hourly intervals. Figure 4a indicates the loss of *E. coli* or *S. aureus* viability steadily increases with extending incubation time. For *E. coli*, the loss viability increases from 50.2 ± 3.0% after 1 h incubation to 78.6 ± 2.4% after 2 h, and further increases to 84.3± 3.9% after 3 h and 90.5± 4.2% after 4 h. The antiactivity of *S. aureus* cells displays a similar trend. The loss of *S. aureus* viability is 23.6 ± 3.5% after 1 h, and increases to 36.8 ± 2.7%, 42.8%±4.5%, and 43.5 ± 3.2% after 2, 3, and 4 h, respectively. For CF, a large fraction of cell death occurs in the first two hours of incubation.

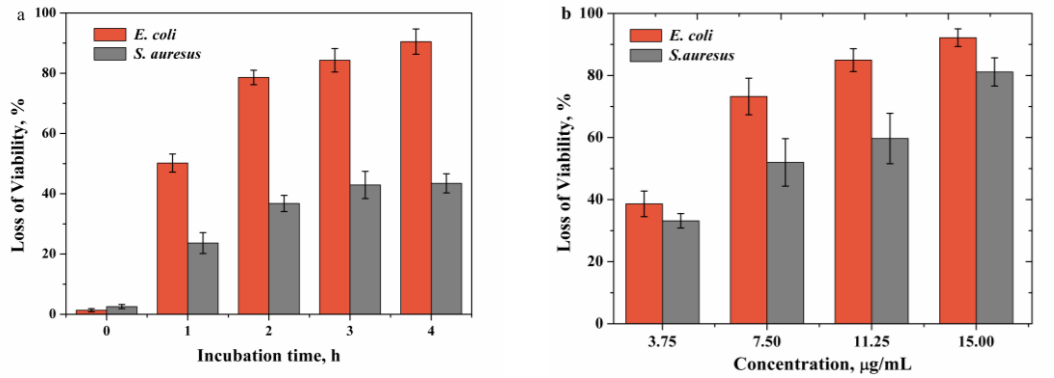


Fig. 4 (a) Time-dependent antibacterial activity of CF suspensions. CF were incubated with E. coli or S. aureus cells (10^7 ~ 10^8 CFU/mL, 100 μL) for 4 h (the concentration of CF in mixtures is 7.5 μg/mL), respectively. The loss of viability was measured at 0, 1, 2, 3, and 4 h, respectively. (b) Concentration-dependent antibacterial activities of CF. 5 mL of CF (at 3.75, 7.50, 11.25, and 15.00 μg/mL) was incubated with E. coli or S. aureus (10^7 to 10^8 CFU/mL, 100 μL) for 2 h, respectively, with 180 rpm shaking speed at 37 °C

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Furthermore, the concentration-dependent antibacterial activities on CF was studied. CF dispersions at different concentrations (3.75, 7.50, 11.25, and 15.00 μg/mL) were incubated with *E. coli* or *S. aureus* cells (10^7 to 10^8 CFU/mL) for 2 h at 37℃ under the 180 rpm shaking speed, respectively. As shown in Figure 4b, the loss of *E. coli* viability progressively goes up with the increases of CF concentration. The loss of *E. coli* viability jumps from 38.6 ± 3.2% at the CF concentration of 3.75 μg/mL to 92.2 ± 3.1% at 15 μg/mL. The majority of *E. coli* was killed after incubation with CF at the concentration of 15 μg/mL. The majority of *E. coli* was killed after incubation with CF at the concentration of 15 μg/mL. In a similar manner, CF dispersion at the concentration of 3.75 μg/mL kills 33.2 ± 2.8% of *S. aureus*, while 15 μg/mL CF dispersion kills only 81.8 ± 4.8% of *S. aureus*. These results suggest that antibacterial activities of CF are also concentration dependent and the E. coli cells seem to show more susceptive toward the CF dispersions compared to the S. aureus cells.

The concentration dependence curve is a common tool used in toxicology to determine the effective concentration at which 50% of the bacteria exhibit a response, which in this case is loss of cell viability (IC_{50}). For *E. coli* cells, the IC_{50} is around 4.8 μg/mL which is lower than the IC_{50} for *S. aureus* cells at 7.4 μg/mL, indicating that *E. coli* cells is more susceptive toward the CF dispersions. These results suggest that CF might be considered as a novel and promising type of chemical bactericide.

3.4 Antibacterial Mechanism of CF

Oxidative stress is often suggested as a key antibacterial mechanism of carbon nanomaterials, such as fullerene, carbon nanotubes and graphehe sheets[31]. In general, oxidative stress mediated by fullerene based materials may come from several paths, reactive oxygen species (ROS) are believed to be responsible for eukaryotic cell menmbrane disruption and eukaryotic lipid perioxidation. This is the mechanism proposed in the previous fullerene toxicity study[32].

XTT(2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxan-ilide) is a widely used superoxide probe that offers the advantage of being specific, water-soluble, and resistant to auto-oxidation. XTT can be reduced by superoxdie anion ($O_2^{\bullet-}$) to form water-soluble

XTT-formazan with the maximum absorption at 470 nm that can be used to quantify the relative amount of superoxide present[33]. Therefore, XTT method was used to measured the possibility of $O_2^{\bullet-}$ production in this work. In this assay, 40 μg/mL TiO_2 dispersion was exposed to a UV light source as a positive control. As shown in Figure 5, no noticeable absorption is detected during the entire 7 h incubation period, which indicates that no $O_2^{\bullet-}$ is produced by CF. TiO_2 under UV radiation as a positive control validated our XTT tests. On the basis of the XTT results, we conclude that CF mediate little superoxide anion production. Therefore, oxidative stress plays a minor role in the antibacterial activity of CF. These results are in a good agreement with the data on the antibacterial action of the fullerene derivatives studied previously[24].

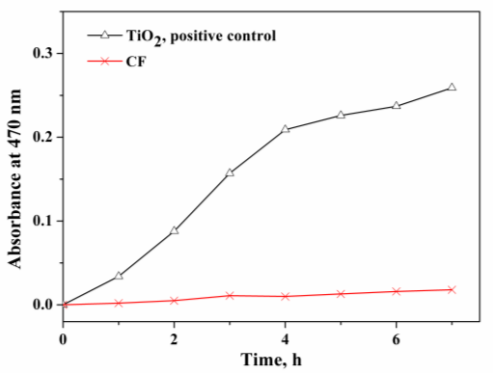

Fig. 5 Production of superoxide radical anion ($O_2^{\bullet-}$) by CF dispersions. The $O_2^{\bullet-}$ production was monitored during the incubation of XTT (0.4 mM) with CF (80 μg/mL) dispersions at pH 7.0 in dark. Incubation with TiO_2 (40 μg/mL) under UV radiation was carried out as a positive control.

Other than oxidative stress mediated by ROS, previous studies on fullerene derivatives cytotoxicity has cited membrane stress mediated by direct physical contacts as their toxicity mechanism toward bacterials[21,22]. In this work, the experimental zeta potential of CF measured in an aqueous suspension (Fig. 2) corresponds to the surface potential of +39.8 ± 0.4 mV. On the other hand, the surface potentials of *E. coli* and *S. aureus* cells measured in an aqueous suspension are −49 mV and −31.7 mV[34], respectively. Therefore, we assumed that electrostatic (Colomb) attraction plays a major role in the antibacterial action of CF. In general, Gram-negative bacteria have a more negative charge than Gram-positive bacteria. As a result, the *S. aureus* cells seem to show less susceptive toward the CF dispersions compared to the *E. coli* cells in this work. However, it should be emphasized that mechanisms of the observed antibacterial activity of CF are not currently well understood.

4. Conclusions

Fullerene derivative CF possesses antibacterial activity against both Gram negative (*E. coli*) and positive bacteria (*S. aureus*) in this work. Their antibacterial activities are time and concentration dependent. Most of bacterial inactivation happens in the first hour of incubation, and cell death rate increases continuously with the increase of material concentration. The bacterial

cytotoxicity may be attributed to membrane stress mediated by direct physical contacts. These results suggest that CF might be considered as a novel and promising type of antibacterial drugs.

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