Induced temperature-dependent DNA degradation by C₆₀ without photoactivation

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This study shows that C₆₀ can degrade pBR322 plasmid DNA at room temperature without photoactivation. The degradation was enhanced by increasing incubation temperature, reaction time or C₆₀ concentration. We also found that superoxide radical anions (O₂^{•−}) were formed in the C₆₀ solution. Superoxide dismutase significantly inhibited DNA cleavage and O₂^{•−} generation induced by C₆₀. These results suggest that DNA cleavage was caused by the formation of reactive oxygen species induced by C₆₀ at room temperature. Furthermore, we demonstrate that the DNA degradation was significantly inhibited by acid amide chemicals such as formamide, and by increased ionic strength of the reaction solution. These results indicate that the DNA conformation stability and the surface properties of C₆₀ are important factors regulating DNA degradation. We propose that C₆₀ can bind DNA, decrease DNA conformation stability, and induce the formation of reactive oxygen species and DNA cleavage at room temperature. Our results provide a possible explanation for the genotoxicity of C₆₀, which should be considered in future use of this particular nano-material.

C₆₀, DNA degradation, reactive oxygen species, genotoxicity

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Since the discovery of fullerene (C₆₀) in 1985, the unique physicochemical and mechanical properties of this novel carbon allotrope have attracted extensive attention, and the practical applications of fullerene as a biological and pharmacological agent have been studied [1,2]. In addition, various biological activities such as antiviral, antioxidant and chemotactic activities of fullerene derivatives have been investigated [3–5].

In recent years, increasing numbers of publications and reports indicated that the production and use of nanoparticles may be deleterious to humans and to the environment [6–8]. The earlier studies showed that in several tests the

acute toxicity of C₆₀ is low [3]. However, more recent research suggested that C₆₀ could induce oxidative stress in the brain of juvenile largemouth bass and the lipid peroxidation in some freshwater and marine species. Some lipid metabolism genes involved in adverse effects were found to be regulated by C₆₀ [9–11]. Furthermore, it has been shown that C₆₀ can impair the cell membrane by generating reactive oxygen species (ROS), and that the antibacterial activity and the genotoxicity of C₆₀ were also related to ROS generation [12–14].

The results of atomistic molecular dynamics simulations and genotoxicity tests showed that C₆₀ could strongly bind to nucleotides and further affect the structure, stability and biological functions of DNA molecules [15,16]. It has also

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been found that photoactivated C_{60} could induce DNA cleavage by generating superoxide radical anions ($O_2^{\cdot-}$) [17]. The photoactivation of C_{60} is required for ROS generation; however, the light conditions *in vivo* are quite different from the DNA cleavage assay that was processed in a tube with longer and more intensive irradiation [9,12–14]. Therefore, more evidence is required to support the hypothesis that ROS generation is induced by C_{60} in cells.

In this study, the DNA electrophoresis assay was performed to assess the genotoxicity of C_{60} . We found that C_{60} could degrade pBR322 plasmid DNA at room temperature without photoactivation. The degradation correlated with the increase in incubation temperature, reaction time or concentration of C_{60} . These results are different from those of DNA cleavage induced by photoactivated C_{60} in a previous study [17]. Furthermore, we detected the formation of $O_2^{\cdot-}$ in the C_{60} solution using the Griess reaction. $O_2^{\cdot-}$ generation and the DNA cleavage induced by C_{60} were inhibited by superoxide dismutase (SOD). The DNA degradation was significantly inhibited by acid amide chemicals and by increased ionic strength of the reaction solution. Our results indicate that C_{60} could be used to induce DNA cleavage under appropriate conditions.

1 Materials and methods

1.1 Water-dispersible C_{60} preparation

C_{60} (99.5%; Alfa Aesar, Ward Hill, MA, USA) was suspended in water following the method of Yamakoshi [18]. First, 8 mg C_{60} were dissolved in 10 mL toluene and mixed adequately until it was completely dissolved. Then 20 mL $CHCl_3$ containing 1 g polyvinylpyrrolidone (PVP, 99%, average molecular weight 40000, Amresco, Solon, OH, USA) were added into the toluene solution and mixed for 4 h. The solvent was evaporated by a vacuum evaporator for 1 h at 40°C. The brown residue was re-dissolved in 20 mL sterilized Milli-Q water. The final concentration of C_{60} was 400 $\mu\text{g/mL}$. The C_{60} solution was stored at 4°C in the dark.

1.2 Transmission electron microscopy measurement

Specimens were prepared by placing a drop of C_{60} suspension on a Formvar-membrane-coated copper grid, which was then air-dried at room temperature. The C_{60} size and morphology were determined with a transmission electron microscope (TEM, JEM-100CXII, JEOL Ltd) operated at 100 kV.

1.3 DNA cleavage assay

A supercoiled plasmid DNA (pBR322, 200 $\mu\text{g/mL}$, Sino-American Biotechnical Company) was diluted in sterilized Milli-Q water to 20 $\mu\text{g/mL}$. The DNA solution, C_{60} solution and other chemical solutions were mixed well in Milli-Q

water and incubated in a water bath at different temperatures (4, 10, 20, 30 and 37°C). The final volume of incubation solution was 20 μL and the assays were all conducted in the dark. After incubation, an aliquot (7 μL) of the solution was mixed with bromophenol blue-glycerol solution (1 μL) and was analyzed by agarose gel electrophoresis (1% agarose in Tris-acetate-EDTA buffer, 100 V, 30 min). The gel was stained with ethidium bromide (Fluka, Ronkonkoma, NY, USA), and visualized and photographed by the UVP gel imaging system. To evaluate the possible mechanism of the DNA cleavage induced by C_{60} , different kinds of organic chemicals such as formamide (100% ion-free, Amresco), acrylamide, urea, thiourea and inorganic salt (NaCl, Amresco) were added to the assay solution. The reactions were incubated in a polymerase chain reaction (PCR) machine (TC-312, Techne, Cambridge, UK) using the following program: 94°C for 1 min, 55°C for 5 min and 10°C for 5 min. Immediately after the reaction, the DNA solution was analyzed by gel electrophoresis.

1.4 Detection of $O_2^{\cdot-}$ (hydroxylamine-Griess reaction)

$O_2^{\cdot-}$ generation was detected by a previously reported hydroxylamine-Griess reaction method with some minor modifications [19]. Briefly, $O_2^{\cdot-}$ reacted with vitriolic hydroxylamine to generate nitrous acid; the $NO_2^{\cdot}$ reacted with the Griess reagent (*p*-aminobenzene sulfonic acid and 2-aminonaphthalene) to generate pink azocompounds, which can be detected by a UV-VIS spectrophotometer (SP-2000UV, Shanghai Spectrum Instruments Company, China) at 550 nm. The $O_2^{\cdot-}$ detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used in this study. Different concentrations of C_{60} or PVP (100 μL) solutions were mixed with 20 μL hydroxylamine sulfate (0.6 mmol/L) and 100 μL reaction buffer (62.4 mmol/L sodium borate and 42.8 mmol/L monopotassium phosphate, pH 8.2). The mixtures were incubated at different temperatures, and then mixed with 200 μL visualization reagent (0.43 mg/mL *p*-aminobenzene sulfonic acid, 0.048 mg/mL 2-aminonaphthalene and 26.7% acetic acid) at room temperature for 10 min. The solutions were centrifuged at 12000 *g* for 3 min to remove C_{60} from the solution. The generation of $O_2^{\cdot-}$ was assessed by measuring absorption of the final clear solutions at 550 nm. The incubation was conducted in the dark.

2 Results

2.1 Size and shape of the C_{60} particle

TEM imaging was used to analyze the size and morphology of C_{60} particle. The typical size and shape of C_{60} particles observed in the C_{60} suspension are illustrated in Figure 1. The TEM image indicates that the C_{60} spherical aggregates range from 15 nm to 45 nm in diameter. The size and shape

Figure 1 TEM image of C_{60} suspension.

are consistent with a previous report [14].

2.2 DNA cleavage assay

First, the pBR322 supercoiled DNA was employed to explore the DNA cleavage activity of C_{60} . Surprisingly, C_{60} cleaved the DNA without irradiation. The pBR322 supercoiled DNA was incubated with different concentrations of C_{60} at 37°C for 12 h in the dark. With increased concentrations of C_{60} the supercoiled DNA was gradually cleaved to linear DNA, nick DNA and finally smear DNA (Figure 2(a)). Because the reaction was conducted in the dark, and photoactivation is believed to be required for C_{60} -induced DNA cleavage, we hypothesized that thermal energy is important for the reaction. To confirm this unusual property of C_{60} , we conducted the DNA cleavage assay at different temperatures or incubation durations without light irradiation. The results clearly showed that C_{60} could not cleave DNA at low temperatures such as 4 and 10°C (Figure 2(b)). Even when the incubation time was prolonged to 24 h, no cleavage product could be observed (data not shown). When the reaction was carried out at 20°C for 12 h, the supercoiled form DNA was partially cleaved to the nicked form. When the reaction was carried out at 30 or 37°C, DNA was completely cleaved by 80 $\mu\text{g/mL}$ C_{60} (Figure 2(b)). These results indicate that DNA cleavage induced by C_{60} was temperature dependent. On the contrary, no DNA cleavage was observed in all control groups where 1% PVP solution was evaluated (Figure 2(c)). When incubating DNA with 80 $\mu\text{g/mL}$ C_{60} for different periods of time (2–11 h), DNA was gradually cleaved with increasing incubation time. At 6 h incubation, an obvious DNA cleavage induced by C_{60} could be detected (Figure 2(d)). These results strongly suggest that C_{60} can induce DNA cleavage in a temperature-dependent manner without the requirement of photoactivation.

Because $O_2^{\cdot -}$ induced by C_{60} was considered to be the major cause for DNA cleavage [17], in this study, the ROS

Figure 2 Gel electrophoresis results of the DNA cleavage assay. (a) DNA cleavage induced by different concentrations of C_{60} . pBR322 was incubated with C_{60} for 12 h at 37°C in the dark. (b) C_{60} induced DNA cleavage at different temperatures. pBR322 was incubated with C_{60} for 12 h at different temperatures. (c) Effects of PVP on C_{60} -induced DNA cleavage. pBR322 was incubated with 1% PVP for 12 h at different temperatures. (d) C_{60} was incubated with pBR322 for different times in the dark at 37°C. Reaction conditions are indicated. All experiments were repeated at least five times.

scavenger SOD was added to the incubation solution to assess the possible mechanism. SOD and C_{60} together with the plasmid DNA were incubated at 37°C in the dark for 8 h.

The results show that DNA cleavage induced by C_{60} was significantly inhibited by 0.5 U/ μ L SOD, but not by bovine serum albumin (BSA) as a control protein (Figure 3). Although most of the supercoiled DNA was transformed to nicked DNA in the SOD treated group, the amount of plasmid DNA was not obviously decreased compared with that of the control. The SOD treatment results indicate that ROS induced DNA cleavage in this experiment system.

Figure 3 C_{60} -induced DNA cleavage was inhibited by SOD. Plasmid DNA (pBR322, 20 μ g/mL) was incubated with C_{60} , SOD or BSA at 37°C for 8 h in the dark. Experiments were repeated at least 5 times.

2.3 Detection of $O_2^{\cdot-}$

The above results revealed that SOD could significantly inhibit DNA cleavage induced by C_{60} . A chemical method was used to detect the generation of $O_2^{\cdot-}$ in the incubation solution under different experiment conditions. First, C_{60} was incubated in the dark for 40 min at different temperatures, and then the generation of superoxide radical anions in the solution was examined. C_{60} was removed by centrifugation to avoid the interference of C_{60} itself. The results show that the absorbance of the solution significantly increased with the increase in temperature (Figure 4(a)). The PVP solution failed to induce the generation of $O_2^{\cdot-}$. The generation of $O_2^{\cdot-}$ induced by C_{60} was also temperature-dependent. To further verify the temperature-dependence result, C_{60} was incubated in the dark for different periods of time at 4 or 37°C, and the generation of $O_2^{\cdot-}$ was detected. The results revealed that the generation of $O_2^{\cdot-}$ at 37°C significantly increased with the prolongation of incubation time (Figure 4(b)). However, C_{60} could not induce $O_2^{\cdot-}$ generation at 4°C, even with a C_{60} concentration of 125 μ g/mL (Figure 4(c)). To eliminate the generation of $O_2^{\cdot-}$ induced by C_{60} , 0.5 U/ μ L SOD were added to the reaction solution, which clearly,

Figure 4 Detection of $O_2^{\cdot-}$ by the hydroxylamine-Griess reaction. (a) $O_2^{\cdot-}$ generation induced by PVP or C_{60} at different temperatures. (b) $O_2^{\cdot-}$ generation induced by C_{60} after different periods of time. 62.5 μ g/mL C_{60} were incubated at 4 or 37°C for 5, 10, 15, 20, 25, 30, 35, 40 min. (c) $O_2^{\cdot-}$ generation induced by different concentrations of C_{60} (6.25, 12.5, 25, 31.25, 46.87, 62.5, 93.75, 125 μ g/mL) at 4 or 37°C for 40 min. (d) $O_2^{\cdot-}$ generation induced by C_{60} was significantly inhibited by SOD. The incubation was carried out at 37°C for 40 min in the dark. All experiments were performed in the dark and data are represented as the average values $\pm$ standard deviations of four individual experiments.

quenched $O_2^{\cdot -}$ generation (Figure 4(d)). As a ROS scavenger, SOD inhibited DNA cleavage and quenched $O_2^{\cdot -}$ induced by C_{60} . Based on the DNA cleavage and $O_2^{\cdot -}$ generation results, we propose that the induction of ROS, specifically $O_2^{\cdot -}$, might be the major cause for DNA cleavage induced by C_{60} .

2.4 Inhibition of C_{60} -induced DNA cleavage

Computer simulation results have previously indicated that C_{60} could bind to DNA and significantly decrease the stability of the DNA conformation [15]. To evaluate the ability of C_{60} to induce DNA cleavage, the DNA denaturation and renaturation process was examined in a thermal cycle machine. By heating at 94°C for 1 min, the plasmid DNA was denatured, then cooled at 50°C for 5 min, the plasmid DNA was partially renatured. This simple and rapid method could provide valuable information about the interaction between C_{60} and DNA conformation stability. The plasmid DNA was incubated with different concentrations of C_{60} before the treatment. The results showed that the quantity of supercoiled DNA was constant after heat treatment, but the nicked DNA was transformed to another form of DNA (Figure 5(a)). This new form of DNA (around 1500 bp) was named heat-treated DNA because it was generated after the denaturation to renaturation procedure. The same form of DNA band was also detected in previous results (Figure 2(b), Lane 2; Figure 2(c)). One possible explanation is that

this special form of DNA is the partial renaturation product of nicked DNA. With the increase in C_{60} concentration, three forms of DNA were cleaved gradually (Figure 5(a)). As a control, PVP could not induce DNA cleavage in all concentrations tested (Figure 5(b)). Based on the results of different DNA cleavage assays, it is clear that C_{60} could cleave DNA without photoactivation.

The ability of formamide and monovalent ions to alter DNA stability has been well investigated, and they have been widely used as additives in PCR and other nucleic acid research [20–22]. To understand their roles in C_{60} -induced DNA degradation, four different types of organic chemicals that include an amide group, formamide, acrylamide, urea and thiourea, were used. The results show that DNA degradation induced by C_{60} was significantly inhibited by increasing the concentration of organic chemical in the reaction solution (Figure 6(a) and (b)). Based on the degree of cleavage of the three forms of DNA and the amount of acid amide compounds added to the solution, the order of inhibitory activity of these chemicals was: thiourea > urea > formamide > acrylamide.

The effect of ionic strength on C_{60} -induced DNA cleavage was also studied. The results show that the DNA cleavage induced by C_{60} was significantly inhibited by increasing the ionic strength of the reaction solution (Figure 7). The effects of formamide and NaCl were evaluated by incubation with DNA and C_{60} at 37°C for 12 h in the dark. Both formamide and Na^+ inhibited DNA cleavage (data not shown).

Figure 5 Effects of C_{60} on DNA cleavage in the process of DNA denaturation and renaturation. (a) DNA cleavage induced by different concentrations of C_{60} . pBR322 (20 µg/mL) was incubated with C_{60} for 1 min at 94°C in the dark. (b) pBR322 (20 µg/mL) was incubated with different concentrations of PVP for 1 min at 94°C in the dark. All experiments were repeated at least 5 times.

Figure 6 Effects of acid amide compounds on DNA cleavage in the DNA denaturation and renaturation processes. (a) pBR322 (20 µg/mL) was incubated with C_{60} and acrylamide or formamide for 1 min at 94°C in the dark. (b) pBR322 (20 µg/mL) was incubated with C_{60} and urea or thiourea for 1 min at 94°C in the dark. The direction of electrophoresis is from top to bottom. All experiments were repeated at least 5 times.

Figure 7 Effects of NaCl on DNA cleavage in the DNA denaturation and renaturation process. pBR322 (20 $\mu\text{g/mL}$) was incubated with C_{60} and NaCl for 1 min at 94°C in the dark. All experiments were repeated at least 5 times.

3 Discussion

It is well known that photoactivated C_{60} can induce several types of radicals via different pathways [17,23,24]. Several *in vivo* and *in vitro* tests have confirmed that C_{60} can induce a variety of irreversible adverse effects, especially DNA damage [16,17,25,26]. Our study shows that C_{60} can cleave pBR322 plasmid DNA at room temperature without photoactivation. The generation of $\text{O}_2^{\cdot-}$ in the solution was also confirmed by the Griess reaction. Moreover, we show that SOD could inhibit DNA cleavage and quench $\text{O}_2^{\cdot-}$. These results indicate that the ROS induced by heat-activated C_{60} might be the major cause of DNA cleavage. Photoactivated C_{60} induced ROS generation through the energy transfer mechanism or the electron transfer reaction [17]. According to electron spin resonance spectra of water-soluble C_{60} , the radical species was hypothesized to be a carbon-centered free radical, which was potentially responsible for oxidative damage [27]. The yielding nC_{60} radical can form organic radicals by donating electrons, and then pass the electrons to oxygen to form ROS [24]. In addition, Lyon et al. [28] demonstrated that water-soluble C_{60} as a direct oxidant could cause antibacterial activity and the oxidative damage is not due to the generation of ROS.

Because the light condition of the conventional toxicity test is quite different from the DNA cleavage assay, and because C_{60} can induce the generation of $\text{O}_2^{\cdot-}$ in a cell-free aqueous solution regardless of the ambient light exposure [12], the generation of ROS in the cell induced by C_{60} under physiological conditions requires further investigation. Our results reveal that C_{60} could induce DNA cleavage without light irradiation and the induction was temperature dependent. With heat treatment for only 1 min at 94°C, 40 $\mu\text{g/mL}$ C_{60} could cleave DNA completely. Incubated at 37°C for 6 h, the DNA was fully broken down by 80 $\mu\text{g/mL}$ C_{60} . By decreasing the incubation temperature, DNA cleavage was entirely eliminated. These results indicate that C_{60} may react with biomolecules through electron- and energy-transfer under proper catalysis conditions. Similar to photo energy, thermal energy is a very important catalysis factor in the

cleavage reaction, thus a possible mechanism is that C_{60} is an excellent electron acceptor in the ground state and the excited C_{60} transfers the electron to O_2 to generate $\text{O}_2^{\cdot-}$ or other types of ROS by absorbing thermal energy and electrons. A previous report showed that C_{60} can react with PVP to form highly stable charge transfer complexes, but whether the complex formation was the crucial step during DNA cleavage requires further evaluation [29].

Another interesting result was that when formamide or acid amide compounds was added to the reaction solution, and the DNA degradation induced by C_{60} was inhibited. Previous computer simulation results have revealed that the binding site of C_{60} is the minor groove of nucleotides or the hydrophobic ends of the DNA strand. The major interaction energy between two molecules is contributed by hydrophobic interactions [15]. A former report proposed that the stability of DNA conformation could be disturbed because the bases could be amidated. The C–N bond of formamide has a substantial double-bond character, and the bidentate formamide paired with the bases can gain additional binding support from cooperative π -electron charge transfer interactions [30]. The order of inhibition resulting from acid amide chemicals showed that chemicals with higher positive potentials could inhibit C_{60} -induced DNA cleavage more effectively.

Moreover, while increasing the ionic strength of the reaction solution, the degradation was eliminated, indicating that DNA conformation stability and C_{60} surface characteristics are additional important factors in DNA degradation. The nucleic acid conformation stability is related to the salt concentration and cation valence [31]. It is generally believed that the stability of DNA could be increased by enhancing the ionic strength appropriately. Because certain cation concentrations can neutralize the negative charge of phosphates of double strand DNA and screen the electrostatic repulsions of DNA strands, the DNA structure and conformation can be stabilized by ions. The stability of DNA enforced by ions should contribute to the prevention of C_{60} -induced adverse effects. As mentioned in the electron-transfer theory, the electronic coupling between the donor and acceptor states dominates the absolute rate constant, and the separation distance and nature of the intervening spacer can change the electronic coupling [24]. C_{60} possesses ample negative potentials in an aqueous solution. The increase of the ionic strength in solution can decrease the negative potentials of C_{60} via electrostatic interactions between the particles and positive ions [7]. For acid amide compounds, due to the p- π conjugation effect of the C=O bond and the C–N bond in the amide group, acid amide chemicals possesses positive potentials which make electrostatic interactions with the negative charge on the surface of C_{60} . Thus, C_{60} can act as a strong electron receptor, and the cation or acid amide compounds can assemble a special ion atmosphere surrounding it. Therefore, the reaction carried out on the surface of the C_{60} molecule could be inhibited or

shielded.

On the other hand, the dissolution state of C_{60} is of great importance in determining its activity [7,32]. When C_{60} was introduced into water solution by solvent exchange or the supplement of co-solvent, the particles accepted more electrons and possessed a negative surface charge to maintain stability, hence the elevation of reduction potential leads to redox features of C_{60} in water solution [24]. The counterions may neutralize the surface charge of aqueous C_{60} , which can lead to the instability of colloid C_{60} , changing the size and solubility of C_{60} [33]. This may be another possible explanation for the activation inhibition by increasing ionic strength in the solution.

Conflicting conclusions have been drawn on the toxicity of nanoparticles based on different assay methods [13,34–40]. Our results revealed that C_{60} induced DNA cleavage without photoactivation, while the incubation temperature, ionic strength and acid amide compounds interfered with DNA cleavage. Therefore, DNA damage could only be detected under certain conditions, which may explain the conflicting conclusions compared with different experiment systems.

4 Conclusions

C_{60} can induce DNA cleavage without photoactivation. The DNA cleavage assay and the detection of $O_2^{\cdot -}$ indicate that the generation of $O_2^{\cdot -}$ could be the major cause of DNA cleavage. Increasing ionic strength of the reaction solution or adding acid amide compounds eliminated the DNA cleavage. Our results provide a possible explanation for the genotoxicity of C_{60} , which should be considered in future applications of C_{60} in antimicrobial medicine and even cancer therapy.

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