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Escherichia coli Inactivation by Water-Soluble, Ozonated C_{60} Derivative: Kinetics and Mechanisms

MIN CHO,[†] JOHN D. FORTNER,[‡] JOSEPH B. HUGHES,[†] AND JAE-HONG KIM*,[†]

School of Civil and Environmental Engineering, Georgia Institute of Technology, 200 Bobby Dodd Way, Atlanta, Georgia 30332, and Department of Chemistry, Rice University, Houston, Texas 77005

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Antibacterial and bactericidal effects of a C_{60} derivative prepared via excess ozonation of C_{60} aggregates in water were investigated using *Escherichia coli* as a representative microorganism. Ozonated C_{60} up to 10 mg/L did not cause significant growth inhibition nor inactivation of *E. coli* in the absence of either oxygen or light. However, it readily inactivated *E. coli* in the presence of both oxygen and light at much faster rate than parent C_{60} aggregates, suggesting involvement of photochemical generation of reactive oxygen species (ROS). Among ROS, hydroxyl radical was found as a primary agent for the cell inactivation. Little surface protein release and concurrent intercellular enzyme degradation during the course of *E. coli* inactivation, along with visual evidence obtained from transmission electron microscopic analysis, collectively indicated that the mechanism of *E. coli* inactivation by ozonated C_{60} involves efficient penetration of ozonated C_{60} into *E. coli* and subsequent photochemical production of hydroxyl radical within the cell cytoplasm. These findings suggest that oxidative transformation of C_{60} can lead to increased toxicity in addition to increased water solubility and reduced size, warranting further studies on other potential chemical derivatization possible in natural and engineered environments for an accurate assessment of environmental impact of this class of materials.

Introduction

Carbon fullerenes, despite extreme hydrophobicity, can be stabilized in water as nanoscale colloidal aggregates (often termed as nC_{60}) through various routes. For example, nC_{60} can be formed by first dissolving C_{60} in water-miscible, polar organic solvents such as tetrahydrofuran (THF) and mixing with water, subsequently removing the transfer solvent via distillation or dialysis (*1*). Alternatively, nC_{60} can be formed by dissolving C_{60} in a water-immiscible organic solvent such as toluene and applying ultrasound to gradually transfer C_{60} into water as the organic solvent is evaporated (*2*). Simply adding dry C_{60} to water and vigorously mixing for an extended period of time also leads to the formation of nC_{60} (*3, 4*). Such a facile formation of water stable suspensions has raised concerns for C_{60} as a potential water contaminant through routes previously considered unlikely based on exceedingly low molecular solubility (*5*).

Concerns have been elevated by studies reporting nC_{60} toxicity toward various cell lines including bacteria (*6*), aquatic organisms (*7*) and mammalian cells (*8*). However, more recent studies have proposed that the toxicological effects by nC_{60} may vary depending on the nC_{60} preparation method and the type of biological receptor (*9, 10*). Some also suggested that the toxicological effect of nC_{60}, in particular nC_{60} made using THF as transfer solvent (referred to as THF/nC_{60}), might be related to THF and THF derivatives (i.e., chemically modified during nC_{60} synthesis process) (*11*–*13*). For example, a recent study by Zhang et al. (*12*) reported that nC_{60} alone was only partially responsible for the inhibition of *Escherichia coli* growth, while the majority of the antimicrobial activity resulted from THF peroxide that formed during nC_{60} synthesis. In parallel, other studies (*14, 15*) have also suggested that the photochemical production of reactive oxygen species (ROS) is limited when C_{60} aggregates in the aqueous phase, which had previously been hypothesized as a causative agent for the cell damage by nC_{60} (*8, 16*).

Although a number of studies have reported the toxicological effects of nC_{60}, little is known about the chemically modified (derivatized) forms of C_{60}, in particular those that may form due to chemical reactions in natural and/or engineered systems. A recent study has reported that nC_{60} is readily oxidized by ozone, a commonly employed disinfectant for water treatment and a reactive atmospheric constituent, to form highly oxidized, water-soluble molecular products (~29 oxygen added to each C_{60} molecule with functionalities including hydroxyl and hemiketal moieties) (*17*). In addition, numerous forms of C_{60} with similar hydrophilic functionalities have been synthesized to increase aqueous solubility, including fullerenol, a commercially available polyhydroxylated C_{60}, among others (*18*–*21*).

The addition of functional groups via covalent bonding to C_{60} cage has been reported to generally decrease its intrinsic photochemical reactivity of individual C_{60} molecules due to disruption in global conjugation (*17, 22*). However, as derivatized C_{60} still can produce a measurable amount of ROS (for example, fullerenol *23, 24*), chemical transformation of nC_{60} into molecularly soluble, oxidized forms may actually result in increased overall aqueous reactivity compared to the parent material. It is also noted that the number of C_{60} molecules available for the reaction is significantly larger in molecularly dispersed form compared to nC_{60} (since only a fraction is located at the surface) when the comparison is based on the same mass. Furthermore, the decrease in material size and altered chemistry (*17*) may make C_{60} penetration through the cell membrane possible, leading to direct contact with cytoplasmic constituents. Despite these considerations, a previous work has suggested that C_{60} could become less toxic with increased hydroxyl derivatization (*8*), although few related studies exist. In this study, we evaluate the kinetics and mechanism of inactivation of *E. coli* by a well characterized, water-soluble C_{60} derivative, prepared through ozonation of nC_{60} (referred to herein as ozonated C_{60}). Results indicate that ozonated C_{60} readily inactivates *E. coli*, at a much faster rate compared to parent nC_{60}, primarily due to facile penetration into the cell and subsequent generation of hydroxyl radical (within the cell) in the presence of light.

* Corresponding author e-mail: jaehong.kim@ce.gatech.edu; phone: 404-894-2216; fax: 404- 385-7087.
[†] Georgia Institute of Technology.
[‡] Rice University.

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Experimental Section

Ozonated C_{60} Preparation. Ozonated C_{60} was prepared according to the method described by Fortner et al. (*17*). The details of preparation method are provided in Supporting Information, SI (Text S1). The property of ozonated C_{60} has been fully characterized in our past study (*17*) which indicated C_{60} cage structure with symmetrical, oxygen based functionalities as repeating hemiketal and hydroxyl moieties. The generic formula for the molecule is $C_{60}(O)_x(OH)_y$ where $x + y = \sim 29$. Among 29 oxygenated carbons, approximately 11 were monooxygenated, while the remaining 18 were dioxygenated. Note that the level of ozonation used to prepare ozonated C_{60} (overall ozone exposure at 150 mg/L-min as *CT*, i.e., the concentration of ozone (*C*) × contact time (*T*)) is much greater than that typically used in water disinfection, but produces the product without intermediates with varying levels of oxygen addition which otherwise might complicate the interpretation of results (*17*).

Photochemical Experiments. Photochemical experiments were carried out using a 50 mL cylindrical quartz reactor which held 30 mL of phosphate buffer solution (PBS, 10 mM) at pH 7.1 containing ozonated C_{60} (10 mg/L) under intense mixing by a magnetic stirrer. The reactor was placed at the center of the black cube chamber equipped with four 18-W black light blue lamps (Philips Co.). The emission spectrum of the BLB lamp at the active wavelength of 350−420 nm was measured using a Spectrapro-500 spectrophotometer (Acton Research Detection System). The incident light intensity was measured by ferrioxalate actinometry (*25*) as 1.2×10^{-6} Einstein/L-s. Experimental temperature was controlled at 23 ± 1 °C by air cooling. Experimental procedures to measure ROS production by ozonated C_{60} are provided in SI Text S2.

Antibacterial Activity. Minimal inhibitory concentration (MIC) of ozonated C_{60} on *E. coli* growth was evaluated as a measure of antibacterial activity (*26*). Modified Minimal Davis (MD) media was prepared by decreasing the potassium phosphate concentration in standard Davis media by 90%. The medium was made anaerobic by bubbling pure nitrogen gas under heat and placing it into nitrogen-filled tubes during cooling. MD media tubes containing various concentration of ozonated C_{60} (0.03, 0.1, 0.25, 0.5, 1.0, 2.0 mg/L) were inoculated with *E. coli* (ATCC DH5α) which had been grown on Luria−Bertani (LB) broth overnight.

Inactivation Experiments. Kinetics of *E. coli* inactivation by ozonated C_{60} was evaluated using the method by Cho et al. (*27*). First, *E. coli* (ATCC 8739) was inoculated in a Tryptic Soy Broth and grown at 37 °C for 18 h. *E. coli* stock solution (1×10^{10} cfu/mL) was then prepared by washing with 20 mM PBS, centrifuging the suspension at 1000*g*, and resuspending the pellet in PBS. Other stock solutions of *E. coli* QC779 mutant (i.e., superoxide dismutase (SOD) gene was deleted) and *E. coli* GC4468 (i.e. wild type of *E. coli* QC779) were prepared following the same procedure except that LB broth was used for culture. The *E. coli* concentration was determined by the spread plate method with incubation using nutrient agar at 37 °C for 24 h. A 1-mL aliquot was withdrawn from each sample and was diluted to 1/1, 1/10, and 1/100. Three replicate plates were prepared for each dilution. Inactivation experiments were performed using the photoreactor described above. Selected experiments were performed under an anaerobic condition by bubbling with ultrapure nitrogen gas. *t*-Butanol (*t*BuOH, 30 mM) and *L*-histidine (20 mM) were used to scavenge $^1O_2$ and OH (*14, 28*), respectively. An additional control experiment was performed using TiO_2 photocatalyst (P25, Degussa Co.). TiO_2 was coated at the thickness of 20 μm on the inner surface (30 mm × 30 mm) of a quartz reactor which contained 40 mL of PBS (10 mM).

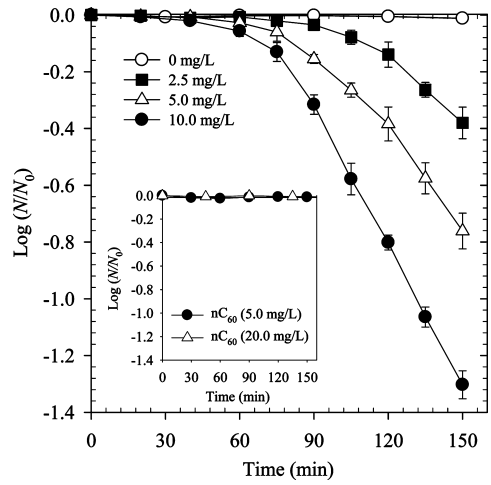


FIGURE 1. Kinetics of *E. coli* inactivation by ozonated C_{60} applied at different concentrations. Inset: Kinetics of *E. coli* inactivation by 5.0 and 10 mg/L nC_{60} at the same experimental condition.

An SDS−PAGE (sodium dodecyl sulfate−polyacrylamide gel electrophoresis) assay was performed following the method by Laemmli (*29*). The ONPG (*o*-nitrophenyl-β-D-galactopyranoside) assay was performed according to Ching et al. (*30*). Physical damage to *E. coli* by ozonated C_{60} treatment was also examined using a transmission electron microscope (TEM) following the procedure by Khadre and Yousef (*31*). Details of these methods are provided in SI Text S3.

Results and Discussion

The MIC test results indicate that ozonated C_{60} with the concentration as high as 10 mg/L did not inhibit *E. coli* growth (i.e., at 37 °C in modified Minimal David media in the dark, under anaerobic condition). Consistent with this result, *E. coli* was not inactivated when exposed to 5 and 20 mg/L ozonated C_{60} for 3 h during experiments performed in the dark and in the absence of oxygen (results not shown). Even with UVA irradiation, only a slight level of inactivation was observed (0.14 log for 150 min by 10 mg/L of ozonated C_{60}) in the absence of oxygen. A control test performed in the absence of ozonated C_{60} confirmed that *E. coli* was not inactivated solely by UVA irradiation. A minor level of inactivation observed might have been caused by residual oxygen that was not completely removed during N_2 purging for 1 h (see discussion below). These results collectively suggested that ozonated C_{60} itself is neither directly antibacterial nor bactericidal to *E. coli* under the conditions investigated.

However, a significant level of *E. coli* inactivation was observed when *E. coli* was exposed to ozonated C_{60} in the presence of dissolved oxygen (ambient level) and under UVA irradiation (Figure 1). As the ozonated C_{60} concentration was increased from 0 to 10 mg/L, the inactivation upon 150 min of exposure increased from 0 to 1.3 log, exhibiting the linear relationship between the concentration and log inactivation achieved ($R^2 = 0.97$). Considering the fact that ozonated C_{60} did not inactivate *E. coli* in the absence of oxygen and without light, the observed result suggested that the inactivation could have been caused by the photocatalytic activity of ozonated C_{60}. Previous studies suggested that ROS, such as $^1O_2$ (*32*), $O_2^{\bullet -}$ (*28*), and $^\bullet OH$ (*33*), could be generated by C_{60} derivatives including fullerenol (*14, 16, 24*). Results shown in Figure S1 provide evidence that these ROS can be also photochemically produced by ozonated C_{60}.

Among ROS produced by ozonated C_{60}, $^1O_2$ was found to have negligible role on *E. coli* inactivation. When excess $^1O_2$

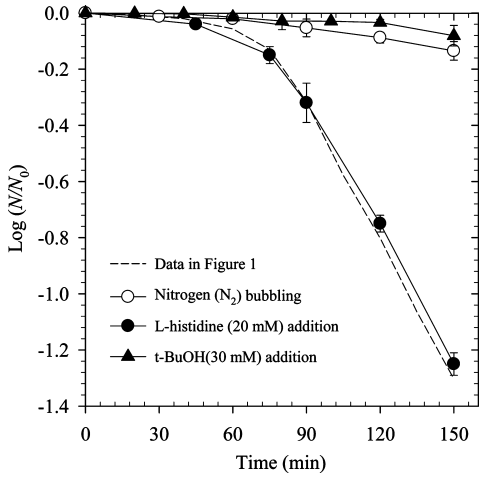


FIGURE 2. Effects of N_2 bubbling and addition of $^1O_2$ scavenger (20 mM L-histidine) and •OH scavenger (30 mM *t*-BuOH) on the kinetics of *E. coli* inactivation compared to the kinetics presented in Figure 1 (ozonated C_{60} concentration = 10 mg/L).

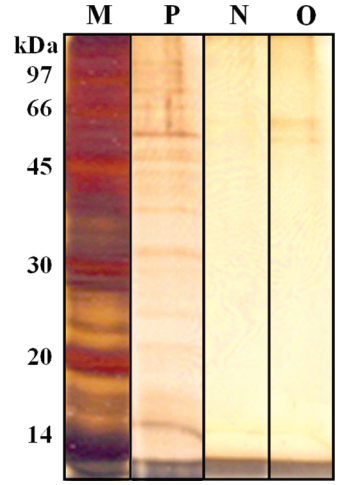


FIGURE 3. Results of SDS−PAGE assay for the proteins extracted from *E. coli* upon 130 min of exposure to 10 mg/L of ozonated C_{60} (M: marker; P: 1 log inactivation by $O_3 + H_2O_2$; N: 0.1 log inactivation by ozonated C_{60} under N_2 purging; and O: 1 log inactivation by ozonated C_{60} in the presence of oxygen).

scavenger, 20 mM L-histidine, was used, the kinetics of inactivation did not show any measurable change (Figure 2). Note that *E. coli* was exposed to 1.3×10^{-12} M of $^1O_2$ cumulatively for 150 min by 10 mg/L of ozonated C_{60} in Figure 1. Even when a much larger amount of $^1O_2$ (6.7×10^{-12} M over 180 min, produced using 5 μM of rose bengal (*34*)) was cumulatively exposed to *E. coli* in a control experiment, no measurable level of *E. coli* inactivation was observed. The role of O_2^{-}• on *E. coli* inactivation was also found to be negligible. When ozonated C_{60} was applied to both *E. coli* QC779 (i.e., mutated *E. coli* with genes for SOD generation are deleted) and *E. coli* GC4468 (i.e., wild type of *E. coli* QC 779) with UVA irradiation in the presence of oxygen, no measurable difference was observed. In both cases, approximately 0.95 log inactivation was observed upon 150 min of exposure to 10 mg/L of ozonated C_{60}. If O_2^{-}• were a major agent for inactivation of *E. coli* by ozonated C_{60}, a greater level of *E. coli* QC779 inactivation should have been observed compared to *E. coli* GC4468 (*35*). In contrast, *E. coli* inactivation in this case was found to be primarily due to •OH, which has been reported to efficiently inactivate *E. coli* with the second-order rate constant of 0.0016 L/mg-min (*28*). When excess •OH scavenger, 30 mM *t*-BuOH, was used, only a very minor level of *E. coli* inactivation was observed, i.e., approximately 0.1 log in 150 min exposure (Figure 2). This response was similar to that observed in the absence of oxygen.

It is interesting to note that these inactivation kinetics were characterized by the presence of a lag phase in which little or no inactivation occurred during the initial exposure to ozonated C_{60}, followed by a first-order decay of viability (Figures 1 and 2). Similar presence of the initial lag phase in *E. coli* inactivation kinetics during chemical disinfectant treatment has been reported in the past (*28, 36*). The outer surface of *E. coli* consists of thick and rigid structures, including a cell wall, membrane, and cortex (*37, 38*) These structures provide a primary protective barrier against the attack by chemical disinfectant. The time required for an oxidant such as •OH (as well as other disinfectants such as free chlorine) to disrupt the cell surface components and to penetrate into the cytoplasm has been attributed as the reason for the presence of the initial lag phase (*39*).

The results obtained from SDS−PAGE assay (Figure 3) suggested that the mechanism of inactivation by ozonated C_{60} could be different from a case whereby •OH is generated in the bulk solution, reacting with *E. coli* surface components during the initial phase of inactivation. When •OH in the solution reacts with the *E. coli* cell wall components such as protein, lipids, and carbohydrates (*40*), cell permeability normally increases, eventually causing cell death (*41, 42*). Consequently, as the inactivation proceeds via surface denaturation, proteins from the cell wall are released to the solution. For example, extraction of proteins under exposure to excess •OH from the bulk solution (i.e., •OH generated by O_3/H_2O_2 process, 0.1 mg/L of ozone and 1 mg/L of H_2O_2) is evident as several bands representative of cellular surface proteins appear in SDS−PAGE (*43*) (Figure 3). However, when ozonated C_{60} was applied both in the absence and presence of oxygen to achieve the same level of inactivation observed for O_3/H_2O_2, these protein bands were not observed.

The concentration of •OH produced by 10 mg/L of ozonated C_{60} in the presence of *E. coli* cells under UVA irradiation was measured using *p*CBA as a probe compound. Assuming steady-state production of •OH, the concentration of •OH was approximately 4.96×10^{-15} M (*44*) (Figure S2). This translates into a •OH *CT* (i.e., the product of radical concentration and contact time) needed for a 1 log inactivation of *E. coli* as 1.1×10^{-8} mg/L-min (*28*), if the •OH in the solution, as measured by *p*CBA, were solely responsible for *E. coli* inactivation. As a comparison, this value is ap-

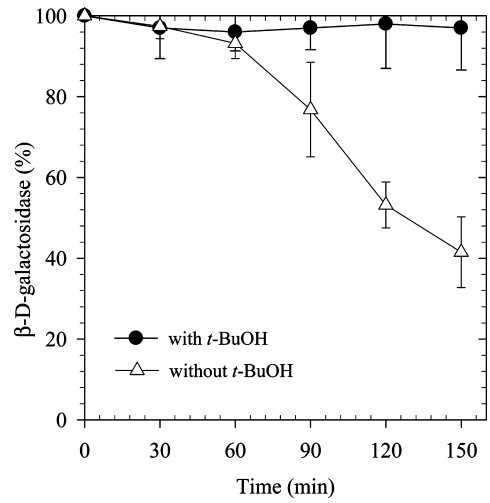
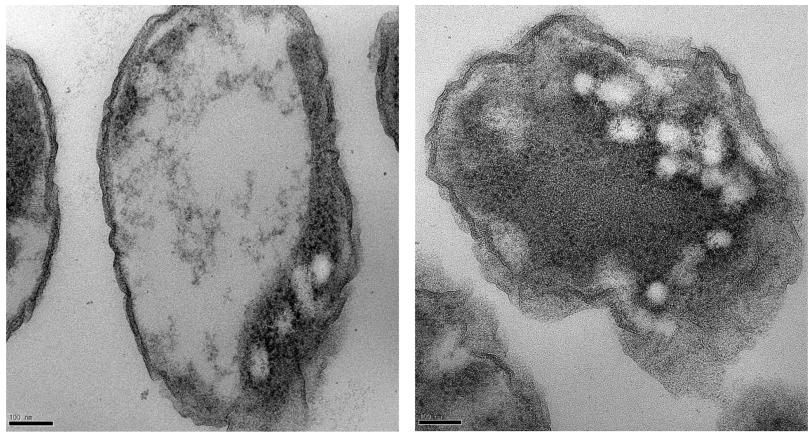


FIGURE 4. Degradation of β-D-galactosidase during *E. coli* inactivation by 10 mg/L ozonated C_{60} with and without 30 mM *t*-BuOH.



(a) (b)

FIGURE 5. Representative TEM images of *E. coli* after 1 log inactivation by (a) ozonated C_{60} and (b) TiO_2 (scale bar represents 100 nm).

proximately 540 times less than the •OH *CT* value of 6.0 × 10^{-6} mg/L-min determined from a separate experiment performed using TiO_2 photocatalyst (*28*) to achieve the same level of *E. coli* inactivation. This comparison indicates that •OH generated in the solution by ozonated C_{60} (perhaps by electron donation from extracellular materials or from cell surface components) was too small to induce the same degree of surface damage to the *E. coli* as that generated by TiO_2.

Alternatively, results from the ONPG assay provide evidence that •OH attack by ozonated C_{60} involves intercellular damage. Figure 4 shows that as much as 60% of intercellular enzyme, β-D-galactosidase, released from *E. coli* after cell lysis, was degraded upon exposure to 10 mg/L of ozonated C_{60} for 150 min (which corresponds to 1.3 log inactivation). For 0.5 log inactivation, approximately 30% of β-D-galactosidase was degraded. In contrast, when 0.5 log inactivation was achieved by TiO_2 photocatalyst, degradation of intracellular enzyme was not observed (data not shown), once again suggesting difference in the mode of radical attack between these two cases. When *t*-BuOH was added during the inactivation, β-D-galactosidase was not degraded, further indicating that the enzyme degradation was caused by •OH.

Figure 5a shows the TEM images of *E. coli* treated with ozonated C_{60} (1 log inactivation in the presence of oxygen). White regions in the cytoplasm, which were absent in intact *E. coli*, developed indicating oxidative damage to the cell. In marked contrast, when *E. coli* was inactivated at the same level (1 log) by TiO_2, considerable damage to the cell wall structure was observed. This TEM analysis clearly distinguishes the internal •OH damage caused by ozonated C_{60} that penetrates into *E. coli* from external damage by bulk •OH produced by TiO_2. Similar visual observations have been made in the past regarding cell damage to *E. coli* due to chemical disinfectants that penetrate into the cell and damage on cytoplasmic constituents (*45*).

Additional experiment was performed by exposing *E. coli* to ozonated C_{60} for 60 min in the absence of UVA during which no inactivation occurred. When UVA irradiation was initiated after ozonated C_{60} had presumably diffused inside, the shoulder was significantly decreased from approximately 60 to 34 min with little change in the postshoulder kinetics (Figure 6). The above results collectively suggest that ozonated C_{60} penetrates into *E. coli* and •OH was generated within the cytoplasm causing internal damage and consequently the cell death. The presence of lag phase in this case is likely to be related to the time required for ozonated C_{60} to penetrate into the cell wall.

In summary, the response of *E. coli* to ozonated C_{60} was clearly different from that of the parent material, nC_{60}. In the presence of both light and oxygen, ozonated C_{60} efficiently

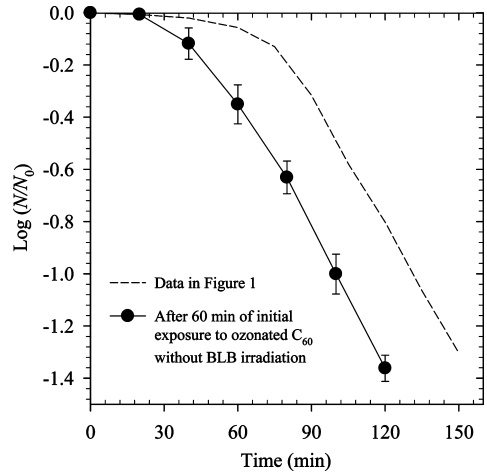


FIGURE 6. Inactivation kinetics of *E. coli* after initially exposing to 10 mg/L of ozonated C_{60} for 60 min prior to experiments.

inactivated *E. coli*, compared to nC_{60} at concentrations of up to 20 mg/L which did not cause any *E. coli* death within 150 min (Figure 1, inset). As mentioned above, this result was expected as photochemical reactivity is limited and cell wall penetration becomes unlikely when C_{60} forms aggregates. It should be noted that 150 min exposure is a much shorter duration than previous assays evaluating toxicity of nC_{60} to *E. coli* (*10*). However, we did observe an MIC of approximately 2.0 mg/L for nC_{60}, although ozonated C_{60} was not antibacterial at or below 10 mg/L. These MIC experiments were conducted under dark, anaerobic conditions. The exact mechanism of nC_{60}'s intrinsic toxicity to *E. coli* (i.e., not involving its catalytic activity) is currently unknown.

C_{60} is considered only as a hypothetical contaminant that may occur due to unintended discharge into environmental systems of which the possibility is largely undefined. In such an event, nC_{60} and perhaps other carbon based engineered nanomaterials may be subject to various oxidative environments in both natural and engineered systems. This study suggests that ozonated C_{60} is more toxic toward *E. coli* compared to nC_{60} due to a mechanism which is not likely with nC_{60}—i.e., molecular penetration into the *E. coli* cell and corresponding ROS production which damages inner cell components. Combined with increased water solubility and reduced size (from colloidal aggregates to molecules), these findings suggest that systematic studies regarding the potential transformation of (similar) anthropogenic nanomaterials is necessary to accurately assess material life cycles, thus potential impact on environmental systems as well as

human health. Note that even though this study employed extreme ozonation conditions (i.e., very high *CT*), ozone disinfection processes designed to comply with current drinking water treatment regulations would transform a significant fraction of nC_{60} to molecularly soluble ozonated C_{60} according to Fortner et al. (*17*). Furthermore, while we focus specifically on ozonated C_{60} in this study, other aqueous based reaction and transformation of C_{60} should be considered such as UV irradiation and advanced oxidation processes employing radicals as nonselective oxidants, among others.

Acknowledgments

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Supporting Information Available

This material is available free of charge via the Internet at http://pubs.acs.org.

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