Impacts of Three nC_{60} Dispersal Methods on *E. coli* Growth

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ABSTRACT

Fullerene may eventually end up in aquatic environments as stable aggregations (nC_{60}) mainly via two different routes, dispersing directly with mechanical force or solvent transfer. The dispersal methods and nC_{60} concentrations may impact *E. coli* growth and exhibit antibacterial activity. In order to compare the impacts of preparation methods on *E. coli* growth, three different dispersal methods and three range of nC_{60} concentrations were introduced into nutrient broth for *E. coli* growth in this study, respectively. Antibacterial activity was observed in the presence of THF/nC_{60} but tol/nC_{60} and aqu/nC_{60}, and increased with THF/nC_{60} dose. When the concentration of THF/nC_{60} was 0.01 mg/L, there is no apparent impact on *E. coli* growth. While in the first 6 hours with the concentration of 0.1 mg/L, the cell growth was inhibited. Comparatively when THF/nC_{60} was 1 mg/L, an initial cell death occurred while the growth rate of *E. coli* was also affected greatly.

Keywords: fullerene, dispersal method, nC_{60}, *E. coli*, toxicity

1 INTRODUCTION

Production and application of carbonaceous nanomaterials, such as fullerene (C_{60}), is increasing rapidly. Yearly production reached at least 10 tonnes in 2007 [1]. This implies that a substantial amount of C_{60} may end up in wastewater and finally enter aquatic environments, which has raised concerns over the potential risks to the environment. Recent studies have suggested that fullerenes may exhibit toxicity towards microorganisms [2-3], aquatic species [3-4], and human cells [4]. However, the mechanisms of fullerene toxicity are barely known because they are significantly associated with its physical properties in aquatic media, particularly particle size, polydispersity and morphology, related with different dispersal methods. Although recent researches have been focused on possible toxicity of aqueous C_{60} nanoparticles (nC_{60}), the adverse effect is still in debate [5-6].

Fullerene can form stable colloidal suspensions of nano-crystalline aggregates at concentrations of several parts per million (ppm) to 50 ppm in pure water when a properly dispersed, despite its virtual insolubility [7]. Fullerene colloids or aggregates consist of numerous C_{60} molecules carrying a negative surface charge which acts to stabilize them. The nC_{60} particle sizes usually vary from several to 200 nm [8-9]. The particle sizes are known to be sensitive to different fullerene powder dispersal methods [8-9]. nC_{60} are commonly prepared from a direct dispersal of fullerene powders in water through prolonged stirring [10] or an indirect dispersal via solvent exchange [8, 11]. Toluene or tetrahydrofuran (THF) is usually applied in the indirect dispersal of C_{60}. The preparations of nC_{60} through water directly and toluene or THF indirectly represent all possible dispersal routes of fullerene particles in aquatic environments. These different dispersal routes may result in different toxicity on microorganisms such as inactivation or inhibition. No studies on comparison of all the three preparation methods are found in the literature. Therefore, this study was to investigate the impacts of these three dispersal methods on the growth of *E. coli*. In order to minimize the effects of particle size, all types of nC_{60} were aging processed and formed in a mean particle size around 150 nm.

2 MATTERIALS AND METHODS

2.1 Production and Aging of nC_{60}

Fullerene powder was obtained from MER Corp. (>99.9%, sublimed). The water dispersal nC_{60} (aqu/nC_{60}) was conducted by mixing 400 mg C_{60} dry powder in 1 L ultra-pure water in an amber container, which was purged with pure N_2 gas and then sealed, followed by an extended stirring period of 20 days. The solvent dispersal was achieved through mixing of C_{60}-solvent solution with ultra-pure water, followed by removal of toluene or THF using sonication probe [8] or rotary evaporator [11], respectively. After stirring, sonication or rotary evaporation, the solution was filtrated through 0.45-μm cellulose acetate membrane filter paper (Millipore Corp.) and stored in dark at 4°C for 30 days.

2.2 nC_{60} Particle Size Analysis

Particle size distributions were assessed through dynamic light scattering (DLS) measurement using a Nano ZetaPlus instrument (90Plus/BI-MAS, Brookhaven Instruments Corporation, USA) at a laser beam wavelength

of 633 nm with a detection angle of 173º and a refractive index of 2.20. In each measurement, the temperature was controlled at 25°C. Each sample was analyzed at least three times, and each measurement was repeated five times. The intensity-weighted mean particle sizes of the nC_{60} agglomerates were found to be 150 nm in all dispersals after 30 days of storing.

2.3 Characterization of Nanoparticle Structure and Morphology.

Transmission electron microscopy (TEM) (JEOL-2010F, JEOL USA Inc., Peabody, Massachusetts) was used to characterize the nanoparticles' structures and morphologies as well as to confirm the DLS particle size measurements.

2.4 Bacterial Growth Conditions and Toxicity Tests

The bacteria used in these studies were *Escherichia coli* K12 (ATCC11229). All three types of nC_{60} were introduced into a nutrient broth for *E. coli* growth, respectively, at the nC_{60} concentrations of 0, 0.01, 0.1, and 1 mg/l. Around 1×10^6 cells of *E. coli* was transferred to 50 ml of the broths, which were then incubated at 37℃ for 10 hours with a shaking of 150 strokes per min. Culturable cell amount, OD600 and polysaccharide in each broth were measured bi-hourly.

2.5 Statistical Analysis

The changes of OD600 at a specific concentration of nC_{60} prepared from different dispersal methods were plotted in the following figures. The growth curves of *E. coli* was fitted with a sigmoidal equation (Eq1) [12] (r^2>0.996).

$$Y = A_2 + \frac{A_1 - A_2}{1 + e^{(x - x_0)/dx}} \quad (1)$$

3 RESULTS AND DISCUSSION

3.1 Structure and Morphology of nC_{60}

TEM images of nC_{60} produced by all three dispersal methods were shown in figure 1. The rectangular and hexagonal shapes formed in the aqu/nC_{60} solution (figure 1a) and the larger ones tended to be more angular than smaller ones. The particle shapes of tol/nC_{60} (figure 1b) aggregates were close to spherical one. Comparatively, THF/nC_{60} (figure 1c) only contains triangular shape. Furthermore, the structure of THF/nC_{60} was much more uniform in terms of single nC_{60} aggregation. Different shapes appeared in all three types of nC_{60}, which may lead to various impacts on affinitive behaviors, adsorption and absorption of nC_{60} to *E. coli* and finally effects *E. coli* growth.

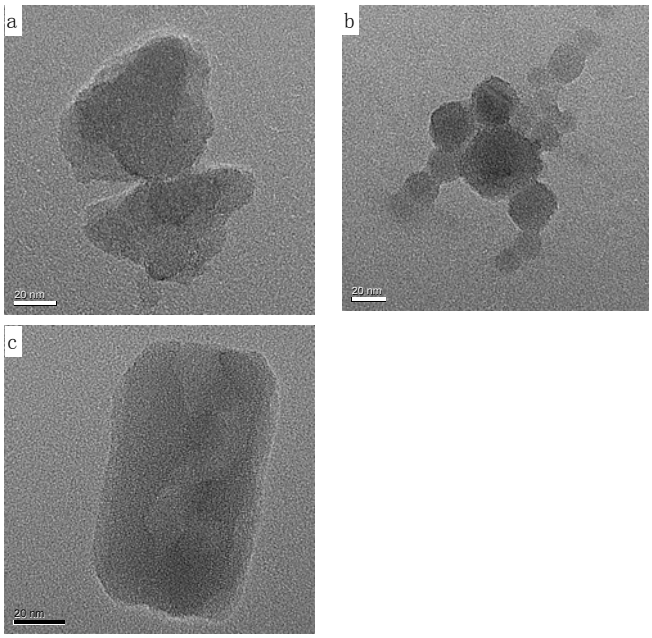

Figure 1: TEM images of all three types of nC_{60}.

3.2 Impact on *E. coli* Growth

E. coli inactivation was tracked by plating the bacteria at different times after exposure. Figure 2 shows OD600, cell amount and growth curve of *E. coli* at 1 mg/L all three types of nC_{60}. The results of OD600 and cell amounts coincidently showed that *E. coli* growth would not be influenced with the presence of tol/nC_{60} or aqu/nC_{60}, which appears stable growth as the control one. While, the increasing of OD600 and cell amounts almost stopped and even appeared decreasing trend at first 4 hours with exposure to THF/nC_{60}. It indicated that the nC_{60} prepared from both the water dispersal (aqu/nC_{60}) and the toluene dispersal (tol/nC_{60}) did not inhibit the growth, however the nC_{60} prepared from the THF dispersal (THF/nC_{60}) inhibited the growth significantly, confirming that the THF dispersal method produces toxic nC_{60}.

The results of Figure 3 obtained with different THF/nC_{60} concentrations. The higher concentration of THF/nC_{60} (more than 0.1 mg/L) showed a strong growth inhabitation on *E. coli*. When the concentration of nC_{60} was 0.01 mg/L, the cell growth trend was similar to the control one, which indicated that the activity of *E. coli* would not be influenced by extremely low concentrations. While, in the first 6 hours with the concentration of 0.1 mg/l, the cell growth was inhibited; however, the cell amount exponentially increased after this period. This revealed that the initial inhibition did not impact the cell growth eventually, when the concentration was kept at 0.1 mg/L. It also indicated that the toxicity of nC_{60} on *E. coli* is a short term behavior. After all the nC_{60} adsorbed onto *E. coli*, the toxicity effects will be disappeared and the health cell still has growth ability and can not be influenced.

Comparatively, when the concentration of THF/nC_{60} was as high as 1 mg/L, the decrease in the cell amount in the first 4 hours occurred but no exponential growth in the subsequent time was observed. This indicated that a initial cell killing occurred while the growth rate of *E. coli* was also affected greatly. Therefore, the toxicity of THF/nC_{60} to *E. coli* was dose-related.

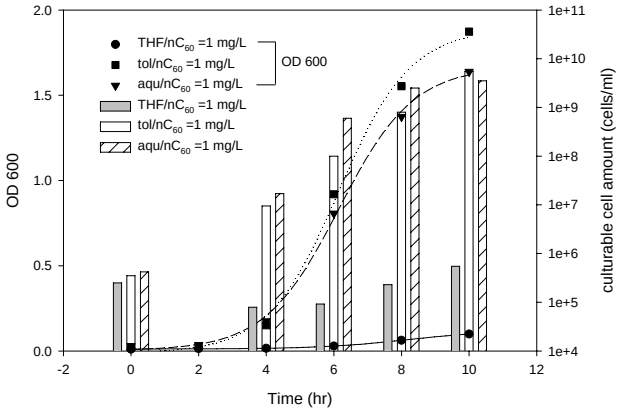

Figure 2: OD600, cell amount and growth curve of *E. coli* at 1 mg/L of all three types of nC_{60}.

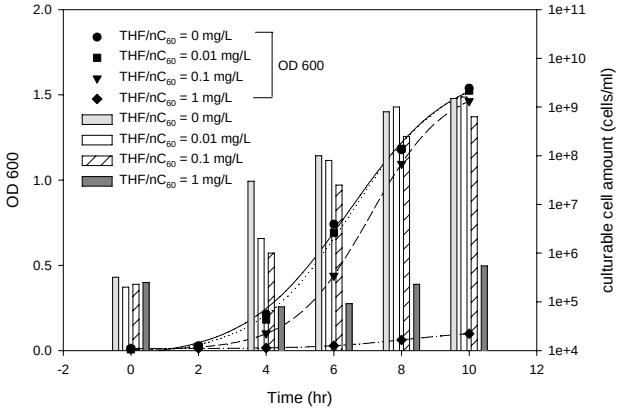

Figure 3: OD600, cell amount and growth curve of *E.coli* at different THF/nC_{60} concentrations.

Furthermore, Table 1 shows the *E. coli* growth curve fitted with a sigmoidal equation, which indicated growth (A_2) and exponential growth time (X_0) were led by different disposal methods and nC_{60} concentrations. A_2 and X_0 were close to the control one with addition of 1 mg/L of tol/nC_{60}, 1 mg/L of aqu/nC_{60} or 0.01 mg/L THF/nC_{60}, which were around 1.6 and 6 respectively. This further certificated that aqu/nC_{60}, tol/nC_{60} and trace level of THF/nC_{60} are no toxicity effects on *E. coli*. While, the extremely low A_2 (0.12) and high X_0 (8.15) indicated that the growth inhibition and exponential growth was delayed by 1 mg/L of THF/nC_{60}. The growth inhibition and exponential growth delay decreased with the exposure to 0.1 and 0.01 mg/L of THF/nC_{60}. The growth curve further verified the results shown in Figures 2 and 3.

type	concentration	X_0	A_2	Equation
tol/nC_{60}	1 mg/L	6.15	1.89	$Y = 1.89 + \frac{-0.01-1.89}{1+e^{(x-6.15)dx}}$
aqu/nC_{60}		6.15	1.67	$Y = 1.67 + \frac{0.003-1.67}{1+e^{(x-6.15)dx}}$
THF/nC_{60}		8.15	0.12	$Y = 0.12 + \frac{-0.01-0.12}{1+e^{(x-8.15)dx}}$
	0.1 mg/L	7.06	1.57	$Y = 1.57 + \frac{0.003-1.57}{1+e^{(x-7.06)dx}}$
	0.01 mg/L	6.50	1.64	$Y = 1.64 + \frac{-0.04-1.64}{1+e^{(x-6.50)dx}}$
	0 mg/L	6.43	1.67	$Y = 1.67 + \frac{-0.04-1.67}{1+e^{(x-6.43)dx}}$

Table 1: Statistical analysis of *E. coli* growth

3.3 Decreasing of Substrate

Figure 4 shows the decrease of polysaccharide as the substrate to *E. coli*, which verified the growth inhibition and the cell killing with the exposure to THF/nC_{60}.

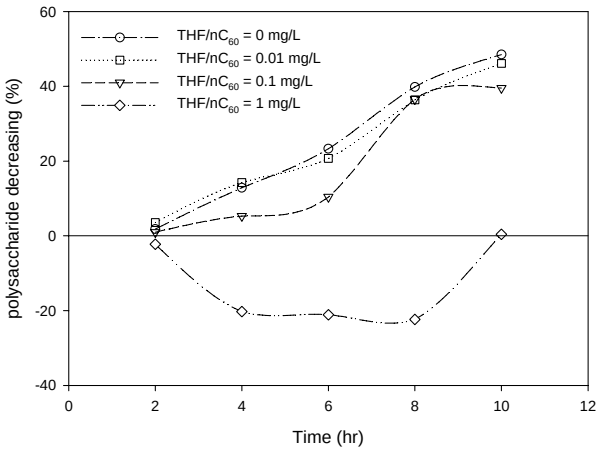

Figure 4: Polysaccharide decreasing rate.

The polysaccharide decreasing rates with 0.01 mg/L and 0 mg/L of THF/nC_{60} exposures appeared in the same increasing trend, which indicated that the substrates were taken up gradually by *E. coli* for growth. The polysaccharide decreasing rate was much smaller with exposure to 0.1 mg/L THF/nC_{60} than that to 0.01 mg/L and 0 mg/L THF/nC_{60} in the initial 6 hours. This further verified the initial inhibition of low concentration of THF/nC_{60} on *E. coli*. The negative decrease or the increase of polysaccharide with the exposure to 1 mg/L THF/nC_{60} verified the initial cell death caused by THF/nC_{60}. After the cell dead, there will be some polysaccharide release from the dead cells, which re-dissolves into water phase and increases the amount of polysaccharide in nutrients. Therefore, in the first 4 hours, great amount of *E. coli* cells were killed with exposure to 1 mg/L THF/nC_{60}. From 4 to 8 hours of exposure, polysaccharide concentration and the

cell amount slightly increased. This indicated that from 4 to 8 hours, the amount of dead cell decreased a lot and *E. coli* slightly recovered and grown up.

3.4 Half Maximal Effective Concentration

The dose-response curve is a common tool used in toxicology to determine the half maximal effective concentration at which 50% of the bacteria exhibits a response (EC_{50}), which is loss of cell viability. Figure 5 shows the EC_{50} calculation result. For THF/nC_{60}, the EC_{50} is around 0.54 mg/L with the correlation coefficient of 0.9998, which is lower than the EC_{50} for the disinfectant triclosan at 5 mg/L [13], indicating that nC_{60} is a more powerful antibiotic.

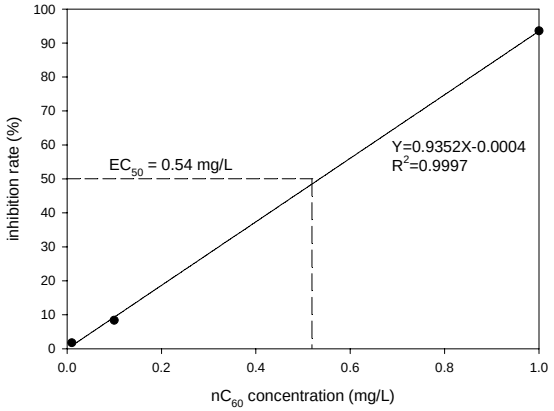

Figure 5: Inhibition rate of THF/nC_{60}.

4 CONCLUTION

nC_{60} is not a strong broad spectrum antibacterial agent, which only appears its toxicity under a special condition or using a specific dispersal method.

- Different shapes appeared in all three types of nC_{60}.
- Antibacterial activity was observed in the presence of THF/nC_{60} but tol/nC_{60} and aqu/nC_{60}.
- The toxicity of THF/nC_{60} to *E. coli* was dose-related. More than 0.1 mg/L THF/nC_{60} may not only inhibit the growth of *E. coli* but also cause the cell death.
- For THF/nC_{60} to *E. coli*, the EC_{50} is around 0.54 mg/L.

Further researches on the environmental impacts of nC_{60} in the early stages on nanotechnology development are recommended to enhance risk assessment.

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