***In vivo* toxicity of nano-C₆₀ aggregates complex with atrazine to aquatic organisms**

YAN XiaoMin, ZHA JinMiao, SHI BaoYou, WANG DongSheng, WANG ZiJian & TANG HongXiao*

State Key Laboratory for Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

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The present study aims at investigating the potential impacts of the nC₆₀ complex with atrazine on the development of Japanese medaka (*Oryzias latipes*) embryos and chronic toxicity to *D. magna*. Although exposure to nC₆₀ below 8 mg/L had no effects on the hatching rate of medaka embryos, it increased the deformity of hatched larvae. Moreover, the presence of nC₆₀ significantly increased the hatching time of the embryos exposed to atrazine. 14-day exposure of *D. magna* to nC₆₀ significantly reduced offspring production even at a concentration as low as 0.5 mg/L. This indicates that nC₆₀ is able to reduce the ability of *D. magna* to produce offspring, and therefore would have an impact on population. For atrazine, no significant difference in offspring production was observed. The nC₆₀ complex with atrazine was also found to significantly decrease the reproduction of *D. magna*, which was similar to the result of exposure to nC₆₀. These results suggest that nC₆₀ would have potential risks to aquatic organisms. Therefore, much attention should be paid to their associations with other contaminants for fullerene's risk assessment.

Fullerenes, complex nanotoxicity, nanoparticles, aquatic organisms

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Buckminsterfullerene (C₆₀) has been one of the most extensively studied nanomaterials since it was discovered in 1985 [1], due to its unique physicochemical properties and numerous applications in areas, such as material science and biomedicine [2,3]. With market production valued at 4.7 trillion \$ by 2016 in the whole world, a large amount of C₆₀ will be ultimately released into the natural system, which would induce potential impacts on the environment [4]. Yet, up to now, there is still lack of information regarding its environmental risks. Timely assessment of the potential impacts will therefore serve as important building blocks for regulating this material.

Due to the unusually high hydrophobicity, C₆₀ is almost insoluble in water [5], which hinders its interaction with biological systems, and studies have shown that C₆₀ alone had no toxicity effects on cells *in vitro* [6] and rats [7]. However,

water stable nano-aggregates of C₆₀ (termed nC₆₀) can be formed from pristine C₆₀ in certain conditions, for example, by solvent exchanging (such as tetrahydrofuran and toluene) [8], hand-grinding [9], and prolonged stirring in water [10]. As it may be a most environmentally relevant form of C₆₀ [11], nC₆₀ has recently caused much concern about its toxicity to humans and the environment, though its concentration is usually less than 10 mg/L in aquatic environment [12,13]. Although the composition and surface chemistry of nC₆₀ are not well understood, studies have shown that nC₆₀ are toxic to different biological systems, such as cells [14–16], bacteria [11,17,18], daphnia [19,20], and fishes [20–22]. Reactive oxygen species are considered as a significant factor contributing to the toxicity of nC₆₀ [22–24]. On the contrary, some studies showed that nC₆₀ did not have potential toxicity [9,25–27], and could even act as free radical scavenger [7]. Therefore, due to different prepared methods and organisms used, there is still no consensus until now in the

*Corresponding author (email: tanghx@rcees.ac.cn)

literature regarding the toxicity of nC_{60} .

Owing to its high specific surface area, nC_{60} can adsorb and enrich environmental contaminants on the surface [10,28], thus forming a combined pollution system. Both of the toxicity effects from the nanomaterials and from the adsorbed contaminants and complex effects may be produced from such complex systems. Moreover, nC_{60} may act as contaminants-carrier to the organisms. However, very little information is available in relation to nC_{60} complex with contaminants. Baun et al. [29] found that the addition of nC_{60} changed the toxicity and bioaccumulation of contaminants, and such complex effects are contaminant-dependant. It was also found that the presence of TiO_2 -nanoparticles enhanced the bioaccumulation of cadmium in the carp [30]. Therefore, investigating the toxicity of nanomaterials complex with contaminants is also of great importance for assessing the environmental risks of nanomaterials.

As there is difference in the response of different organisms to contaminants, the potential toxicity effects of nC_{60} complex with contaminants may be dependent on the test organisms and endpoints. The present study aims at investigating the potential impacts of the nC_{60} complex with atrazine, a typical contaminant in the environment, on the development of Japanese medaka (*Oryzias latipes*) embryos and chronic toxicity to *D. magna*.

1 Materials and methods

1.1 Materials

Fullerene C_{60} (purity 99.9%) was obtained from Puyang Yongxin Corp., Henan Province. Nitro blue tetrazolium (NBT) and NADH were purchased from Sigma. Other reagents were all above analytical reagent.

1.2 Producing and characterizing nC_{60}

nC_{60} was produced by solvent exchanging after sonicating C_{60} solution in toluene with water [8]. A solution of 1 g/L C_{60} in toluene was prepared, and 25 mL of the solution was added to 500 mL deionized water. This layered mixture was then sonicated for more than 5 h until all the toluene was evaporated. This brown suspension was then filtered through a 0.45 μm membrane. The filtrate was termed as nC_{60} .

The morphology and particle size were observed by transmission electron microscopy (TEM) and dynamic light scattering (DLS) device (BI-200SM, Brookhaven Instruments Corp.). TEM was taken on H-7500 transmission electron microscopy (H-7500, Hitachi). TEM grids were prepared by naturally evaporating one drop of nC_{60} solution onto a 300 mesh grid. Surface charge of nC_{60} was measured through Zeta potential (Zetasizer 2000, Malvern, U.K.).

1.3 Atrazine adsorption on nC_{60}

Atrazine adsorption was conducted with nC_{60} by batch reactors, using glass vials with Teflon-septum caps. Ten milliliter nC_{60} suspensions were pipetted into 12-mL glass vials, then two milliliter solutions with different initial atrazine concentrations were added into the vials. Sample vials were closed tightly with caps. Another control vial was set up in the same way as the sample vials except that no nC_{60} was added. These vials were stirred for 5 d (150 r/min, 25°C). Then the samples were filtered with 0.02 μm inorganic filter membrane (Anodisc13, Whatman Inc.), and the concentration of atrazine in the filtrate was measured by HPLC (Waters Co., American) [31].

1.4 Exposure of Japanese medaka embryos to nC_{60} complex with atrazine

Japanese medaka is reared as mentioned by Zha et al.[32]. Different concentrations of only nC_{60} or atrazine were prepared in dilution water (2.5 mmol/L CaCl_2 , 0.4 mmol/L MgSO_4 , 0.77 mmol/L NaHCO_3 , 0.077 mmol/L KCl, pH 7.8). Fertilized medaka embryos were selected to expose to nC_{60} or atrazine. Twenty eggs were transferred into each well of the 6-well multiplates containing 8 mL test solution. There were 6 replicates per concentration. The multiplates were stored in an incubator at 25°C under a 16:8 (h) light:dark photoperiod. The test solutions were renewed every day until the embryos were hatched. Numbers of live, dead and deforming larvae were recorded daily. Embryos cultured in dilution water were used as the control.

Exposure of medaka embryos to the complex of nC_{60} with atrazine was performed at different atrazine concentrations at 0.5, 1.2, 3.7, 7.3 and 14.2 mg/L, respectively, and at constant nC_{60} concentration 3 mg/L. The exposure process was the same as the aforementioned.

1.5 Exposure of *Daphnia magna* to nC_{60} complex with atrazine

Daphnias were exposed to test samples in dilution water using standard protocols [33]. One <24 h neonatal daphnia per group was exposed to 30 mL test solution in dilution water in 50 mL glass beaker and fed daily. Life-cycle assay was performed for 14 d. The test solution was changed every two days, and molting and reproduction were monitored daily. There were 8 replicates per concentration. The beakers were stored in an incubator at 25°C under a 16:8 (h) light:dark photoperiod. The complex was prepared with different atrazine concentrations and with constant nC_{60} concentration 2 mg/L.

1.6 Detection of superoxide radical anion (NBT method) *ex vivo*

Whether generation of $O_2^{\cdot-}$ was induced by nC_{60} under light irradiation was tested by the NBT method [34]. Briefly, to a solution of 6 mg/L nC_{60} (2 mL), 2.4 mmol/L NBT (0.4 mL), 50 mmol/L NADH (0.4 mL), 1 mmol/L EDTA (0.4 mL), and 0.2 mol/L phosphate buffer (0.8 mL, pH 6.8) were added and mixed well. The reaction mixture was irradiated with sunlight (13:30~16:00, Oct. 16, 2007, Beijing), and then 0.5 mL mixture was taken out and diluted with 40 mmol/L phosphate buffer (2.0 mL) for the measurement of OD_{560} .

1.7 Statistical analysis

Statistical comparisons were made using SPSS 13.0 software, by ANOVA to determine significant differences with $P < 0.05$.

2 Results and discussion

2.1 Characterization of nC_{60}

Brown emulsion was immediately formed after the C_{60} solution was sonicated in toluene with water. Through evaporating toluene during this process, the dissolution of C_{60} in toluene would be gradually saturated, and then nano- C_{60} aggregates (termed nC_{60}) were formed. The TEM images of nC_{60} show mainly spheres, and the size of the single sphere particle was less than 100 nm (Figure 1(a)). The size distribution of the hydrodynamic diameters determined by DLS was mainly from 80 to 180 nm, while centering at 100~120 nm (Figure 1(b)), and the calculated average hydrodynamic diameter was (124 ± 10.1) nm. These show that C_{60} nanoparticles could be prepared in water by sonicating its toluene solution with water. The surface charge of nC_{60} was characterized by negative zeta potential (-21 mV). Because of the negative surface of nC_{60} , the electric repulsion became the main factor which could make the nC_{60} suspension stable in water. However, when the solution conditions were changed, such as the increase of the ionic strength or addition of coagulants, nC_{60} nanoparticles would precipitate from the solution [4], which would decrease the interactions of nC_{60} with the biology system.

UV-vis spectra of nC_{60} are shown in Figure 1(c). Characteristic peak of hydrated C_{60} in solution was found at 343 nm, while the characteristic peak of C_{60} in toluene was at 332 nm, thus red shift occurred. Moreover, there was additional broad absorption in the wavelength region from 400 to 500 nm with nC_{60} , which did not appear in C_{60} solution in toluene [11]. The absorption in this region might be due to charge transfer between the delocalized π electron of C_{60} and the oxygen group of H_2O .

2.2 Atrazine adsorption on nC_{60}

The adsorption of atrazine on nC_{60} is shown in Figure 2. Within the test concentrations, a linear isotherm adsorption equation ($q_e = K_d C_e$) was observed to well describe the adsorption curve, where K_d (mL/g) is the solid-water distribution coefficient. The K_d value of atrazine to nC_{60} was $10^{3.46}$ mL/g by linear fitting, which was close to that of naphthalene to nC_{60} [10]. According to the K_d value, the distribution

Figure 1 Characterization of nC_{60} . (a) TEM of nC_{60} ; (b) size distribution of nC_{60} by DLS; (c) UV-vis of nC_{60} in water and C_{60} in toluene.

Figure 2 Adsorption of atrazine on nC_{60} .

of atrazine in the solid-solution system could also be calculated. For example, when atrazine and nC₆₀ concentrations were 1 and 10 mg/L, respectively, calculation results show that most of atrazine was in the solution, and only 3% of atrazine was adsorbed on nC₆₀.

2.3 Effect of nC₆₀ and its complex with atrazine to medaka embryos' development

The sensitivity to toxic compounds is species-dependent, and is also related to the life stages of organisms. In the early development stage, fish is very sensitive to toxic chemicals. The development of medaka embryos exposed to nC₆₀ is shown in Figure 3. The percent of hatching was below 80% when nC₆₀ concentration was higher than 4 mg/L, while the control group was 88.3% (Figure 3(a)). However, there was no significant difference between the control group and nC₆₀ group ($P > 0.05$). Interestingly, the hatching time decreased significantly when the embryos were exposed to nC₆₀ in the concentration range of 0.5–2 mg/L ($P < 0.05$). For example, the hatching time in the control group was 9.49 d, while that of nC₆₀ group at the concentration of 0.5 mg/L was 8.53 h (Figure 3(b)). However, the percentage of deformity of hatched larvae increased to 12.5% when the embryos were exposed to nC₆₀ at the highest concentration 8 mg/L, while there were no deformed larvae in the control group (Figure 3(c)). Then the exposure of these hatched larvae to nC₆₀ continued at the same concentrations as that for the exposure of the embryos for 20 days, and no obvious abnormal physiological effects and death were observed, which shows that nC₆₀ also had no acute toxicity to larvae medaka. Because of the presence of ions in dilution water, nC₆₀ could be aggregated during the exposure and adhere to the outer layer of chorion of medaka embryos.

There was, however, much discrepancy between our results and other researcher's results about the development of fish embryos exposed to nC₆₀. Zhu et al. found that nC₆₀ prepared with THF could significantly delay the development of zebrafish embryos, and decrease survival and hatching rate [35]. Moreover, the addition of antioxidant could be effective to reduce the toxicity of nC₆₀. Usenko et al. used microarrays analysis to examine the global gene expression after zebrafish embryos were exposed to nC₆₀ and observed that genes associated with a response to stress were up-regulated [36]. Additionally, the cytotoxicity mechanisms of THF/nC₆₀ was considered to be the production of reactive oxygen species such as O₂^{•-} [14,15,23,24]. These findings show that the toxicity of nC₆₀ would be related to oxygen stress.

Under sunlight irradiation, nC₆₀ could not produce O₂^{•-} *ex vivo* detected by NBT (Figure 4). The slight increase of absorption is due to the production of purple formazan by the oxidation of the reduction of NADH by NBT under sunlight. These results indicated that nC₆₀ prepared with toluene was not able to produce O₂^{•-} under sunlight, which was in contradiction to THF/nC₆₀. Some studies have shown

Figure 3 Effect of nC₆₀ on Japanese medaka embryos hatching. (a) Percent of hatching; (b) hatching time; (c) percent of deformity (* $P < 0.05$).

Figure 4 Photoinduced O₂^{•-} by nC₆₀ solution under sunlight detected by NBT method.

that new reactive species are produced from THF during the preparation of THF/nC₆₀, such as γ -butyrolactone, tetrahydro-2-furanol, and tetrahydro-2-(tetrahydrofuran-2-ylperoxy) funan [37,38]. These matters are reactive oxygen species, and may be the main compositions attributing to the toxicity of THF/nC₆₀ system. Therefore, the acute toxicity of THF/nC₆₀ may not be originated from nC₆₀ itself, but from these reactive byproducts of THF produced during the preparing of nC₆₀. Also, the acute toxicity of THF/nC₆₀ to zebrafish embryos would be mainly induced by these reactive

species [35].

Spurlin et al. observed that nC_{60} only accumulated on the surface of lipid bilayers, but did not penetrate into the hydrocarbon tails of the bilayers by AFM [39]. However, the presence of nC_{60} would alter membrane physiological properties, such as lipid composition, phase transition temperature, and fluidity of bacteria membrane [40]. Therefore, due to the lipid bilayers structure of the outer layers of medaka embryos, the hydrophilic nC_{60} could not penetrate into the inter layers, but mainly adsorbed onto the outer layers of embryos, which, however, would affect the exchange of substances from the lipid bilayers and the membrane characteristics, and even cause membrane stress [41]. The decrease of hatching percentage and increase of the number of deformed larvae might be related to the adherence of nC_{60} on the embryos.

Therefore, the negative effects of nC_{60} on medaka embryos did not result from the production of reactive oxygen species, but from the disruption of membrane properties after nC_{60} adhered to chorion.

The effects of nC_{60} complex with atrazine on the development of medaka embryos are shown in Figure 5. The toxicity of atrazine to medaka embryos showed significant dosage-effect relationship. With increasing atrazine concentration, embryos' hatching rate decreased, hatching time prolonged, and the abnormal rate increased. For example, the hatching rate was 66.7% at the concentration of 2.47 mg/L, while it was only 4.2% at 28.7 mg/L. Compared with the toxicity of atrazine alone, the presence of nC_{60} to atrazine did not significantly affect the hatching rate of embryos during the exposure to atrazine. It was also calculated from Figure 2 that the presence of nC_{60} at low concentrations had very little influence on the bioavailability of atrazine. Although the presence of nC_{60} to atrazine had no significant effect on the hatching rate of embryos, the complex significantly delayed the hatching time compared with only nC_{60} or atrazine, for instance, the hatching time was 11.97 d in the exposure to the complex in which the concentration of atrazine was 1.19 mg/L, while it was 9.75 d in the exposure to atrazine alone at 1.04 mg/L. Therefore, the presence of nC_{60} to atrazine would enhance the toxicity of atrazine to medaka embryos.

2.4 Effect of nC_{60} and its complex with atrazine on *D. magna*

Acute toxicity of nC_{60} to *D. magna* was not observed at 48 h (results not shown). The chronic toxicity of nC_{60} and its complex with atrazine to *D. magna* was examined by 14-day life-cycle assay (Figure 6). It was found that the cumulative number of *D. magna* offspring significantly decreased even at the concentration as low as 0.5 mg/L during 14-day exposure to nC_{60} (Figure 6(a)). The number of offspring in the control group and in the nC_{60} group at the concentration of 0.5 and 5 mg/L was 80 ± 18.9 , 59.1 ± 23.5

Figure 5 Effect of atrazine with or without nC_{60} on Japanese medaka embryos hatching. (a) Percent of hatching; (b) hatching time; (c) percent of deformity ($nC_{60}=3$ mg/L, * $P<0.05$).

and 39.4 ± 19.4 , respectively, which indicated that nC_{60} was able to reduce the ability of *D. magna* to produce offspring, and therefore would have an impact on the population-level, although nC_{60} had no acute toxicity to *D. magna*. These results are in accordance with the findings of Oberdörster et al. using Aqu/nC_{60} [20]. Moreover, Lovern et al. found that *D. magna*'s exposure to nC_{60} at the lowest observable effect concentration increased the hopping frequency and appendage movement of *D. magna* using a high-speed camera [42]. The changes of these behavior parameters would increase the risk of predation and decrease the productivity. Therefore, the release of C_{60} to the environment would lead to a series of consequences to the aquatic ecosystem.

Exposure to atrazine increased the number of offspring although not significantly ($P > 0.05$). The nC_{60} complex with atrazine was observed to significantly decrease the

Figure 6 Cumulative number of *D. magna* offspring exposed to nC₆₀ (a) and nC₆₀ complex with atrazine (b) over 14-day life-cycle assay (* $P < 0.05$), where A1, A2 and A3 represent the concentration of atrazine at 1, 2 and 5 mg/L, respectively, SA1, SA2 and SA3 represent the complex of nC₆₀ at the constant concentration of 2 mg/L with atrazine at the concentration of 1, 2 and 5 mg/L, respectively.

reproduction of *D. magna*, which was similar to the effect of nC₆₀ at the concentration of 2 mg/L. There was no significant difference in reproduction between the nC₆₀ group and the group of nC₆₀ complex with atrazine by statistical analysis ($P > 0.3$).

The effects of nC₆₀ complex on organisms were contaminant-dependant. Baun et al. investigated the toxicity of nC₆₀ complex with different contaminants to algae and *D. magna* and found that the presence of nC₆₀ increased the toxicity of phenanthrene, but decreased the toxicity of pentachlorophenol, and had no significant influence on the toxicity of atrazine and methyl parathion [29]. Usenko et al. found that nC₆₀ enhanced the toxicity of H₂O₂ to zebrafish embryos [36]. Therefore, it is necessary to study the toxicity effects of nC₆₀ with more contaminants with different toxicity mechanisms, which is also important to the evaluation of environmental risks of C₆₀. The toxicity mechanism of the complex system of nanomaterials with contaminants is also one of the important aspects of nanomaterial's toxicity investigations.

Biological responses to nC₆₀ are related to the test organisms and selected endpoints. Oberdörster et al. found that exposure to Aqu/nC₆₀ induced chronic toxicity to *D. magna*, but not to *Hyalella azteca* and *Copepods*, and significantly reduced the peroxisomal lipid protein PMP70 in fathead minnow, but not medaka [20]. Additionally, exposure to nC₆₀ increased total GSH in the liver tissue of *Fundulus*

heteroclitus, but had no significant effects on LPO-bio-marker for oxygen stress in the tissue [21]. Therefore, further investigations on the biological effects of nC₆₀ are needed.

Additionally, the preparation methods of nC₆₀ also have great influence on the toxicity of nC₆₀, due to different sizes, morphology and surface chemistry. It is therefore important to make a consentaneous method for the production of nC₆₀ for the comparison of the results in the literature. Also, detailed characterization of nC₆₀ contributes to reveal the toxicity mechanisms.

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