Aqueous fullerene aggregates (\(nC_{60}\)) generate minimal reactive oxygen species and are of low toxicity in fish: a revision of previous reports
Theodore B Henry\(^1,2,3\), Elijah J Petersen\(^4\) and Robert N Compton\(^5,6\)

This review aims to clarify inconsistencies in previous reports regarding the potential for aqueous aggregates of fullerenes (\(nC_{60}\)) to generate reactive oxygen species (ROS) and cause toxicity in fish. Methods for evaluation of ROS production and toxicity of aqueous \(nC_{60}\) have evolved over time and limitations in initial studies have led to unintentional erroneous reports of \(nC_{60}\) ROS generation and toxicity. Some of these reports continue to lead to misconceptions of the environmental effects of \(C_{60}\). Critical review of the evidence (2007–2011) indicates that aqueous \(nC_{60}\) have minimal potential to produce ROS and that oxidative stress in fish is not induced by environmentally relevant exposure to \(C_{60}\). Future studies should acknowledge that current evidence indicates low toxicity of \(nC_{60}\) and refrain from citing articles that attribute toxicity in fish to \(nC_{60}\) based on methods shown to be compromised by experimental artifacts. Despite low toxicity of \(nC_{60}\) in fish, an emerging environmental issue is that \(nC_{60}\) can affect environmental fate, transport, and bioavailability of co-contaminants in aquatic environments in a similar manner to that observed for other anthropogenic particulates (e.g., microplastics).

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Current Opinion in Biotechnology 2011, 22:533–537
This review comes from a themed issue on Nanobiotechnology
Edited by Florian Hollfelder and Gary Sayler

Available online 28th June 2011

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DOI 10.1016/j.copbio.2011.05.511

Potential for \(C_{60}\) to generate reactive oxygen species (ROS) in water

Generation of ROS by \(C_{60}\) is influenced by the medium, functionalization of \(C_{60}\), state of \(C_{60}\) aggregation, and presence and type of illumination. Delocalized \(\pi\) double bonds of the fullerene cage can absorb energy from light and produce a triplet excited state sufficiently long lived for high efficiency transfer of energy to molecular oxygen and formation of reactive singlet oxygen [11]. In water, the lifetime of singlet oxygen is only nano or micro-seconds, but this is sufficient to induce formation of other
ROS species [12] that are also highly reactive with biological molecules. However, C₆₀ is extremely insoluble in water [13] and detection of ROS has been inconsistent for aqueous preparations of C₆₀ [termed nC₆₀; diameter tens to hundreds of nm (and some exceed nanodimensions >100 nm)] [14]. If functional groups are added to C₆₀ [e.g., C₆₀(OH)], the aqueous fullerenes can produce ROS [15], but this review is focused on un-derivatized C₆₀.

ROS generation by aqueous nC₆₀ has been related to preparation method, presence of associated substances in water, and exposure to light. Preparation methods for aqueous nC₆₀ have been reviewed extensively [8] and can be divided into three categories as follows: solvent extraction (nC₆₀(solvent) [16]); use of micellar solutions (nC₆₀(micelle) [17]); and water stirred (nC₆₀(stirred)) [18]. ROS have been detected in aqueous nC₆₀(preparations [notably tetrahydrofuran (THF)]; e.g. [19,20,21]; however, these reports have been confounded by evidence that solvent can reside between individual C₆₀ molecules within nC₆₀(solvent) [22], and that degradation products of solvents (e.g., THF) can remain in the water [23,24,25]. Zhang et al. [26] demonstrated that nC₆₀(solvent/THF) preparations contained oxidizing agents (THF degradation products) that explained ROS activity and that vigorous washing of nC₆₀(solvent/O₂) preparation was necessary to eliminate ROS activity. Results of experiments that did not appropriately control for solvent effects should no longer be used as evidence that aqueous nC₆₀ can produce ROS.

Potential for ROS generation in aqueous nC₆₀ preparations that are not compromised by solvents depends on aggregate structure and interactions among C₆₀ molecules. Aqueous nC₆₀ preparations absorb light at wavelengths (near 450 nm [15,27]) expected to excite C₆₀ to the triplet state; however, lack of photoreactivity and minimal generation of ROS have now been consistently reported for aqueous nC₆₀(solvent) and nC₆₀(stirred) preparations [15,28,29,30,26]. An exception is the studies by Hou and Jafvert [32,33] that report detectable ROS production after longer periods of solar irradiation, but investigators note that ROS is ‘drastically’ less than expected for C₆₀. Selfquenching (interactions among C₆₀ within nC₆₀) is suggested to resolve higher C₆₀ energy states induced by light absorption [29,32], a process influenced by aggregate size (based on theoretical models [34]), and also expected with bulk C₆₀ [27]. Overall, present evidence indicates that ROS production by aqueous nC₆₀ is minimal.

Appreciable amounts of ROS can be generated by micellar solutions of aqueous nC₆₀(micelle) depending on arrangement of C₆₀ molecules and associated substances. Numerous studies have reported ROS generation in nC₆₀(micelle) preparations [15,28,29,35,36,27,17], and ROS production can occur when a surfactant (e.g., Triton X100, TX) is applied at above the critical micelle concentration (cmc) [27]. Above the cmc, C₆₀ may behave as if dissolved in an organic solvent; however, when nC₆₀ aggregates are present within micelles, selfquenching is likely (as described above) and ROS are minimal [15,27,34]. Environmental relevance of nC₆₀(micelle) is debatable, but behavior of C₆₀ within micelles may be similar to what could happen if C₆₀ is able to reside within a lipid bi-layer of a cell membrane [15].

### Clarification of C₆₀ toxicity in fish

Oxidative stress has been reported in fish exposed to aqueous nC₆₀(solvent) preparations, but recent studies indicate that some results must be revised. Oxidative stress reported in nC₆₀(solvent/THF) exposures that did not control for solvent effects [e.g. [19,37,38]] is no longer an appropriate evidence of nC₆₀ toxicity because effects have been convincingly linked to THF decomposition products (Figure 1) rather than nC₆₀ [23,24,25,26]. Investigations with nC₆₀(solvent/dimethyl sulfoxide(DMSO)) in zebrafish and Japanese medaka Oryzias latipes reported significant embryo mortality and deformity [39,31], induction of antioxidant defense genes [40], and induction of GSH [31]; and similar effects have been reported for nC₆₀(solvent/toluene) in Japanese medaka embryos [31]. However, although solvents are known to reside within...
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Figure 2

<table>
<thead>
<tr>
<th>Concentration (µmol/g protein)</th>
<th>Gill</th>
<th>Liver</th>
<th>Brain</th>
<th>Gill</th>
<th>Liver</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>TBARS</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

Total glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) in the gills, liver, and brain of juvenile rainbow trout \(Oncorhynchus mykiss\) fed 500 mg \(C_{60}/kg\) food (shaded bars) compared to control fed fish (light bars) after 6 weeks exposure. No significant differences in GSH or TBARS were detected and there were also no significant effects on any other toxicological endpoint measured (including: survival, growth, haematology, tissue ion concentration, histopathology, osmoregulation, or biochemistry). No dietary toxicity of \(C_{60}\) was observed; details in Fraser et al. [45].

\(nC_{60}\) [22], the effects of residual solvents on these fish embryo toxicity test results are not well understood. Perhaps solvent generated aqueous \(nC_{60}\) will become industrially important such that \(nC_{60}\) exposure in fish becomes environmentally relevant, but it is inappropriate to attribute toxicity to \(C_{60}\) until effects of the solvent are more adequately understood. Preparation of \(nC_{60}\) without solvents (i.e., \(nC_{60}(stirred)\)) is recognized as most environmentally relevant and numerous studies have now investigated toxicity of these preparations [e.g., [41,23,42,31]].

Oxidative stress has been reported in fish exposed to aqueous \(nC_{60}(stirred)\), but critical review indicates that these results are more likely a consequence of the assay technique rather than \(nC_{60}\). Indications of oxidative stress (enzyme induction, lipid peroxidation) in fish attributed to \(nC_{60}(stirred)\) was reported in one study [37], but the same investigators reported no effect on these endpoints in a separate study [41]. Chronic (32 d) exposure to \(nC_{60}(stirred)\) had a subtle reduction in growth of goldfish \(C. auratus\) and some changes in antioxidant enzyme activity, but effects were not related to \(C_{60}\) concentration (0.04, 0.2, 1.0 mg/L) [43]. Some results of oxidative stress reported in the literature are likely to be false positives. Shinohara et al. [44*] demonstrated lipid peroxidation assays are vulnerable to false positives when \(nC_{60}\) is present, and when conditions (light intensity) were properly controlled, no effects were observed. Similar false positives could explain inconsistencies in oxidative stress indicators reported in Zhu et al. [43] and Blickley and McClellan-Green [42]. It is noteworthy that 72-h exposure to 6 mg/L \(nC_{60}(stirred)\) did not cause significant changes in global gene expression in larval zebrafish \(D. rerio\) assessed by the Affymetrix GeneChip\textsuperscript{®} Zebrafish Genome array (\(\approx 15,000\) gene transcripts [23]). The only dietary exposure (500 mg \(C_{60}/kg\) food) in fish (juvenile rainbow trout \(O. mykiss\)) did not report any oxidative stress or other toxicological effects of \(C_{60}\) during or after 6-week exposure (Figure 2) [45*]. Taken together these studies indicate \(nC_{60}(stirred)\) is of minimal toxicity in fish for the endpoints that have been assessed.

**Ability of \(nC_{60}\) to affect the environmental fate and bioavailability of co-contaminants**

Emerging concerns about release of \(C_{60}\) into the environment include interactions between aqueous \(nC_{60}\) and other substances (e.g., toxicants, termed here ‘co-contaminants’) and consequent effects on co-contaminant fate, transport, and bioavailability. Changes in environmental behavior of co-contaminants by \(nC_{60}\) could be similar to that recognized as an important component of the presence of other anthropogenic particulates, such as microplastics, in aquatic environments [46]. Co-contaminants can accumulate in aqueous \(nC_{60}\) and accumulation appears to be related to physicochemistry of both \(nC_{60}\) and the co-contaminant [47,48]. Some co-contaminants appear to associate strongly within \(nC_{60}\) and there is some evidence that co-contaminants [e.g., 17α-ethinylestradiol (EE2)] adsorb to aggregate surfaces before absorption within \(C_{60}\) and then become considerably more difficult to disassociate [48,49*].

Effects of \(nC_{60}\)-co-contaminant associations on co-contaminant bioavailability are largely unknown. Only one study has effectively tested environmentally relevant bioavailability of a co-contaminant (EE2) associated with \(nC_{60}(stirred)\) and demonstrated by assessment of vitellogenin gene (\(vtg\)) expression that \(nC_{60}\) reduced bioavailability of EE2 in fish [49*]. The association between EE2 and \(C_{60}\) led to a greater propensity for aggregates to sediment out of the water column over time, and supports the hypothesis that accumulation of settled aggregates by filter feeding and sediment dwelling organisms as a first step into the aquatic food chain [50]. Filter feeding invertebrates can accumulate \(C_{60}\) with associated EE2 [51]; however, the EE2 did not become bioavailable (\(vtg\) not induced) when invertebrates were fed to fish suggesting that aggregate integrity and \(nC_{60}\)-EE2 association were sufficiently robust to withstand fish digestive processes [51]. Co-contaminants held within \(nC_{60}\) may be less vulnerable to degradation processes and lead to enhanced persistence and transport of co-contaminants in the environment although perhaps with decreased co-contaminant bioavailability.

**Conclusions**

Techniques for evaluation of ROS production and toxicity of aqueous preparations of \(nC_{60}\) have evolved
over time, and current understanding of fullerene toxicity must recognize that limitations in some initial techniques have led to unintentional erroneous reports of C60 ROS generation and toxicity. Minimal ROS production by aqueous C60 based on current evidence and revisions of early reports of oxidative stress induced by C60 in fish leads to the conclusion that C60 is of minimal toxicity in fish when appropriate experimental controls have been employed to eliminate artefacts (i.e., solvent effects and controlling light). An emerging environmental issue is that C60 may have important effects on environmental fate, transport, and bioavailability of co-contaminants in aquatic environments similar to that observed for microplastics.

Acknowledgments

Certain commercial equipment, instruments, and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the NIST nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest


2. Gottschalk F, Sonderer T, Scholz RW, Nowack B: Broader societal issues of nanotechnology. 1. Roco MC: have been highlighted as: Papers of particular interest, published within the period of review, References and recommended reading


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The potential for aqueous nC60 to generate ROS under conditions of illumination relevant to the solar spectrum were investigated over significant exposure durations (15 h). ROS generation determined by furfuryl alcohol as an indicator demonstrated ROS production by aqueous nC60. Increases in nC60 resulted in declines in singlet oxygen accumulation that were attributed to increased light attenuation and increased quenching by nC60.


The potential for C60 to cause lipid peroxidation (LPO) in brain tissue, reported in other studies, was investigated to determine if experimental artifacts could explain the previous observations. Homogenized brain tissues were exposed to C60 and LPO was evaluated under different conditions of laboratory illumination. Results demonstrate that laboratory illumination could explain LPO in brain homogenates and emphasize the importance of controlling laboratory conditions during toxicity evaluations of NPs. In vivo exposure to aqueous nC60 and subsequent evaluation of brain LPO indicated no translocation of C60 to brains of fish.


Dietary exposure to carbon nanotubes and C60 (500 mg/kg food) in fish was conducted to determine if differences in shape of these NPs could be related to toxicity. Results indicate no overt toxicity or evidence of shape effects from these NPs. Dietary exposure is recognized as a particularly relevant route for fish, but lack of toxicity after six-week exposure to rather high levels of these NPs suggests they are of minimal toxicity.


Previous studies have demonstrated that aqueous nC60 can associate with co-contaminants; however, this study is the first to evaluate effects of nC60 on environmentally relevant bioavailability of co-contaminants. Bioavailability of EE2 was assessed by measuring induction of vitellogenin genes in zebrafish, and results indicate that EE2 associates with nC60 and becomes unavailable to fish. Retention of EE2 (or other co-contaminants) within nC60 could prevent degradation and thereby enhance persistence and transport of co-contaminants in the environment.
