Does Aqueous Fullerene Inhibit the Growth of *Saccharomyces cerevisiae* or *Escherichia coli*?  

Alex N. Hadduck,¹ Vihangi Hindagolla,¹ Alison E. Contreras,² Qilin Li,² and Alan T. Bakalinsky¹*  

Department of Food Science and Technology, Oregon State University, Corvallis, Oregon 97331-6602,¹ and  
Department of Civil and Environmental Engineering, Rice University, Houston, Texas 77005²  

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Studies reporting on potentially toxic interactions between aqueous fullerene nanoparticles (nC₆₀) and microorganisms have been contradictory. When known confounding factors were avoided, growth yields of *Saccharomyces cerevisiae* and *Escherichia coli* cultured in the presence and absence of independently prepared lots of underderivatized nC₆₀ were found not to be significantly different.

The increasing use of nanomaterials in industrial processes and commercial products is expected to lead to accumulation of these materials in the environment. Because the consequences of increased environmental exposure are unclear, it is important that studies be undertaken to determine potential risks (4). Both deleterious effects (5, 21, 26, 27, 30) and a lack of toxicity (7, 14, 18) have been reported for aqueous nanoparticles of underderivatized aqueous fullerene nanoparticles (nC₆₀). Only some of these conflicting observations have been rationalized (9). Accurately assessing doses of nanoparticles in cell culture systems can be problematic (24). The variety of methods used to prepare nC₆₀ also complicates interpretations of otherwise similar toxicological evaluations, because different preparation methods produce nC₆₀ particles with different physicochemical properties (2, 16).

With specific reference to microorganisms, conflicting data have also been previously reported (19). At least four factors confound assessments of toxicity. First, it is now recognized that tetrahydrofuran (THF) used in nC₆₀ preparation generates toxic derivatives (13, 22, 29). Unless these derivatives and trace THF are removed, observed toxicity cannot be ascribed to nC₆₀ alone. Reports from studies that found antibacterial activity by the use of THF-solubilized nC₆₀ prior to this discovery are thus difficult to interpret (6, 15–17). Second, in aqueous media, hydrophobic nC₆₀ particles tend to agglomerate as a function of the solution condition. For some microbiological media, this leads to precipitation of nC₆₀ alone and hence a reduction in the actual exposure dose. Binding of organic components in complex media to nC₆₀ particles can reduce nC₆₀ bioavailability or lead to agglomeration (15). Both effects would result in false-negative assessments of potential growth inhibition (3). Third, negative results reported from studies where C₆₀ powder was used directly without prior solubilization may reflect a lack of bioavailability (8, 20, 23, 25). Fourth, potential inhibitory effects toward one or few species in mixed cultures could be masked by other species when growth is assessed at the community level (8, 20, 25).

In light of these complications and a lack of studies done with fungi, which comprise a significant component of the soil microbial community, the toxicity of nC₆₀ towards the yeast *Saccharomyces cerevisiae* and *Escherichia coli* was assessed based on a simple growth endpoint under conditions where the aforementioned confounding factors were avoided. Pure microbial cultures were grown in minimal media to which carefully washed and characterized independent lots of nC₆₀ prepared using three different methods were added. At the single high dose used (about 30 μg/ml), no reduction in the cell yield of either *S. cerevisiae* or *E. coli* was observed. To our knowledge, this is the first report of a lack of microbial growth inhibition under conditions where factors known to generate false-negative results were avoided.

**Preparation and characterization of aqueous fullerene suspensions.** Aqueous nC₆₀ suspensions were prepared with sublimed C₆₀ powder (MER Corp., Tucson, AZ) (purity ≥ 99%) by three methods. The suspensions were termed tol-nC₆₀, THF-nC₆₀, and aq-nC₆₀, with the prefix indicating the solvent used in the preparation (toluene, THF, and water, respectively). Three parallel samples were prepared for each suspension type. A numerical suffix indicates the particular batch. C₆₀ concentrations were determined by total organic carbon measurements using a high-sensitivity TOC analyzer (Shimadzu Scientific Instruments, Columbia, MD). All nC₆₀ preparations were processed through a sterile filter with a 0.45-μm-pore-size membrane prior to use.

**Tol-nC₆₀.** Three 10-ml solutions of C₆₀ in toluene at 2 g/liter were filtered through 0.45-μm-pore-size nylon filters (Millipore, Billerica, MA) and added to 100 ml of ultrapure water. Toluene was evaporated by continuous sonication at 100 W using a cell disruptor probe (Sonics and Materials, Inc., Newtown, CT), and the resulting aqueous suspensions were passed through 0.45-μm-pore-size sterile membrane filters and stored at 4°C in the dark. Residual toluene concentrations measured by gas chromatography and mass spectrometry (GC/MS) were less than 0.2 ppm.

**THF-nC₆₀.** THF-nC₆₀ samples were prepared following a protocol that removes residual THF and toxic byproducts (29). Samples were washed repeatedly using ultrapure water in an Amicon stirred cell (Millipore, Billerica, MA) equipped with an ultrafiltration membrane (YM-10; Millipore, Billerica,
MA). Residual THF concentrations measured by GC/MS were 1.8, 6.4, and 0.5 ppm in THF-nC60 1, THF-nC60 2, and THF-nC60 3, respectively.

Aq-nC60. Aliquots of dry C60 powder (50 mg) were mixed with 200 ml of ultrapure water in autoclaved 500-ml glass bottles and vigorously stirred in the dark for 28 days. The resultant suspensions were filtered through 0.45-μm-pore-size sterile membrane filters and concentrated using centrifugal concentrators (Centricon YM-10; Millipore, Billerica, MA).

Particle sizes and electrophoretic mobilities (EPM) of nC60 particles were determined using a Zeta-sizer Nano (Zen 3600; Malvern Instruments, Worcestershire, United Kingdom) at 25°C. All samples were prepared in triplicate at 3 mg/liter in the corresponding background solutions used in the toxicity tests. Measurements were performed over a 24-h period immediately following sample preparation to monitor changes in particle size and EPM. At each time point, samples were measured at least five times for particle size and 10 times for EPM. The refractive index of nC60 was set at 2.20 for the particle size measurements.

Figure 1 summarizes the mean particle size and EPM measurements for all nC60 suspensions. All suspensions were very stable in deionized water, with insignificant changes in particle size over the period of the study (data not shown). In general, tol-nC60 particles were the smallest and aq-nC60 particles were the largest (Fig. 1A), which is consistent with previous findings (2). All suspensions were highly negatively charged in deionized water (Fig. 1B). The THF-nC60 samples had the highest negative EPM, while that of the aq-nC60 was the lowest, as in previous reports (2). There was little variation among the replicate preparations, except for aq-nC60 3, which exhibited notably lower negative EPM compared to the other two aq-nC60 samples. The direct mixing method used to prepare aq-nC60 was the least reproducible.

When mixed with growth media (defined below), the negative EPM of all samples was reduced due to the high salt concentrations (Fig. 1B). The reduction was greater in yeast nitrogen base without amino acids (Difco) that was supplemented with 2% glucose, 20 μg/ml hisidine, 30 μg/ml each of leucine and lysine, and 10 μg/ml of uracil (henceforth referred to as YNB) than in a reduced-phosphate minimal medium (1; henceforth referred to as MD) because of the higher total ionic strength (142 mM in YNB versus 48 mM in MD) and divalent cation concentration in YNB. As a result, particle aggregation occurred, as indicated by the larger particle sizes after 24 h compared to those in deionized water. Particle aggregation was notably greater in YNB than in MD, consistent with the lower negative EPM in YNB. Aggregation of nC60 depended on the sample type. Aggregation in YNB was much greater for THF-nC60 than for the other two types, even though the EPM was similar to or more negative than those of tol-nC60 and aq-nC60. This suggests that the reduction in electrostatic repulsion was not the only cause of particle aggregation; the surface chemistries of the various nC60 types may differ from one another. Despite the aggregation, there was no notable precipitation of nC60 over a 48-h period.

**Microorganisms, media, and growth assays.** Saccharomyces cerevisiae BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and a number of cell wall mutants in the BY4742 genetic background and E. coli DH5α were used to assess the growth-inhibitory activity of fullerene. The yeast mutants (Open Biosystems, Inc.) have been previously described (28) (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html). E. coli DH5α was chosen specifically because it has been used in previous studies of nC60 toxicity (6, 17, 29). S. cerevisiae was chosen as a model microbial eukaryote and constituent of the soil microbial community. S. cerevisiae was grown in YNB. E. coli was grown in MD, with a 90% reduction in phosphate as described previously (18), consisting of 0.9 g of potassium phosphate, 1 g of ammonium sulfate, 0.5 g of sodium citrate dihydrate, 0.1 g of magnesium sulfate heptahydrate, and 2 g of glucose per liter (pH 7). Liquid media were sterilized by filtration through 0.45-μm-pore-size membrane filters.

Yeast cells were subjected to aerobic preculturing for 24 h at 30°C at 200 rpm in 1 ml of YNB, centrifuged (12,000 × g for 20 s), washed twice in distilled water, resuspended in 1 ml of
byproducts in the THF-nC<sub>60</sub> preparation used in earlier studies (6, 17) cannot be ruled out as a cause of the reported changes in the viability of subpopulations of cells, it is possible that exposure to nC<sub>60</sub> could have slowed growth of or irreversibly damaged some cells without affecting the maximum attainable population size. It was recently discovered that physical contact was required in order for single and multwall carbon nanotubes to damage E. coli and other bacteria (10, 12). Forced physical contact between E. coli and an aq-nC<sub>60</sub>-coated filter was reported to kill about 60% of nC<sub>60</sub>-exposed cells (11). Unfortunately, making a rational comparison of cell-particle contact in the assay used in the present study to that in the forced contact assay is difficult.

**Yeast cell wall mutants are not sensitive to nC<sub>60</sub>.** We speculated that growth inhibition would depend on fullerene uptake or association with cells and therefore assayed 48 yeast deletion mutants with known defects in cell wall biosynthesis or organization or with greater sensitivity or resistance to dyes that bind wall components (calcofluor white and Congo red) to determine whether they might be more susceptible. Growth of the deletion mutants was assayed as described above except that the 24-h inoculum was diluted 100-fold and only one lot of nC<sub>60</sub> (31 μg/ml of tol-nC<sub>60</sub>) was tested. None of the observed modest differences in cell yield between the 48 strains grown in the absence versus the presence of tol-nC<sub>60</sub> were significant at the P < 0.05 level (data not shown; Wilcoxon–Mann-Whitney 2-tailed test [XLSTAT 2009.1.02]). The 48 mutants tested are listed here according to the systematic names of the deleted genes: YAL059w, YBL001c, YBL006c, YBL007c, YBL043w, YBL061c, YBL101c, YBR005w, YBR023c, YBR067c, YBR076w, YBR078w, YCL005w, YDR125c, YDR245w, YDR446w, YER083c, YER093c, YGR189c, YGR229c, YHR030c, YHR132c, YHR142w, YHR181w, YIL146c, YIL201w, YJR075w, YJR106w, YJR137c, YKL090w, YKL190w, YKR076w, YLR110c, YLR300w, YLR332w, YLR342w, YLR390w, YLR425w, YLR436c, YLR443w, YMR238w, YMR307w, YOR008c, YOR092w, YPL089c, and YPL180w. We conclude that loss of these particular cell wall-related functions does not make S. cerevisiae more sensitive to nC<sub>60</sub>-mediated growth inhibition.

**Assessment of growth-inhibitory activity of nC<sub>60</sub>.** Inhibition of yeast or E. coli growth was assessed by comparing cell yields (A<sub>600</sub>) in the presence and absence of nC<sub>60</sub> (Table 1). No reduction in the cell yield of yeast or E. coli was observed for any of the 9 nC<sub>60</sub> lots tested. While we are not aware of published data on the response of the widely used model eukaryote S. cerevisiae to fullerene, the E. coli results are inconsistent with two previous studies in which growth inhibition of the same E. coli strain in MD was observed at concentrations as low as 0.4 mg/liter of THF/nC<sub>60</sub> (6, 17). However, as noted above and in references 13, 22, and 29, residual THF and toxic byproducts in the THF-nC<sub>60</sub> preparation used in earlier studies (6, 17) cannot be ruled out as a cause of the reported toxicity. In contrast, the THF-nC<sub>60</sub> used in the present study was washed as previously recommended (29). On the other hand, growth inhibition of Bacillus subtilis in MD has been reported from studies using nC<sub>60</sub> preparations made without THF (16). Because our assay did not measure growth rates or changes in the viability of subpopulations of cells, it is possible that exposure to nC<sub>60</sub> could have slowed growth of or irreversibly damaged some cells without affecting the maximum attainable population size. It was recently discovered that physical contact was required in order for single and multimauer carbon nanotubes to damage E. coli and other bacteria (10, 12).

### TABLE 1. Cell yields of E. coli and S. cerevisiae grown in the presence of nC<sub>60</sub>

<table>
<thead>
<tr>
<th>nC&lt;sub&gt;60&lt;/sub&gt; lot</th>
<th>Control</th>
<th>Treated (26 μg/ml)</th>
<th>Control</th>
<th>Treated (31 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tol-nC&lt;sub&gt;60&lt;/sub&gt;&lt;sub&gt;b&lt;/sub&gt;</td>
<td>0.247 ± 0.027</td>
<td>0.238 ± 0.030</td>
<td>1.963 ± 0.111</td>
<td>1.940 ± 0.042</td>
</tr>
<tr>
<td>Tol-nC&lt;sub&gt;60&lt;/sub&gt;&lt;sub&gt;c&lt;/sub&gt;</td>
<td>0.084 ± 0.013</td>
<td>0.095 ± 0.024</td>
<td>1.907 ± 0.016</td>
<td>2.068 ± 0.094</td>
</tr>
<tr>
<td>Tol-nC&lt;sub&gt;60&lt;/sub&gt;&lt;sub&gt;d&lt;/sub&gt;</td>
<td>0.084 ± 0.013</td>
<td>0.085 ± 0.011</td>
<td>2.092 ± 0.022</td>
<td>2.187 ± 0.185</td>
</tr>
<tr>
<td>Aq-nC&lt;sub&gt;60&lt;/sub&gt;&lt;sub&gt;e&lt;/sub&gt;</td>
<td>0.103 ± 0.008</td>
<td>0.108 ± 0.010</td>
<td>1.699 ± 0.019</td>
<td>1.683 ± 0.085</td>
</tr>
<tr>
<td>Aq-C&lt;sub&gt;60&lt;/sub&gt;&lt;sub&gt;f&lt;/sub&gt;</td>
<td>0.103 ± 0.008</td>
<td>0.106 ± 0.007</td>
<td>1.699 ± 0.019</td>
<td>1.888 ± 0.140</td>
</tr>
<tr>
<td>THF-nC&lt;sub&gt;60&lt;/sub&gt;</td>
<td>0.107 ± 0.010</td>
<td>0.135 ± 0.004</td>
<td>1.656 ± 0.042</td>
<td>1.652 ± 0.044</td>
</tr>
<tr>
<td>THF-nC&lt;sub&gt;60&lt;/sub&gt;&lt;sub&gt;g&lt;/sub&gt;</td>
<td>0.107 ± 0.010</td>
<td>0.138 ± 0.008</td>
<td>1.656 ± 0.042</td>
<td>1.725 ± 0.120</td>
</tr>
<tr>
<td>THF-nC&lt;sub&gt;60&lt;/sub&gt;&lt;sub&gt;h&lt;/sub&gt;</td>
<td>0.107 ± 0.010</td>
<td>0.143 ± 0.005</td>
<td>1.656 ± 0.042</td>
<td>1.744 ± 0.071</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are means ± standard deviations for 3 replicates. Treated yeast and E. coli cells were grown in YNB and MD, respectively, containing nC<sub>60</sub>. Control cells were grown in media lacking nC<sub>60</sub>. No differences in yield between the control and treated cultures for any nC<sub>60</sub> lot were significant at P < 0.05 (Wilcoxon–Mann–Whitney 2-tailed test; XLSTAT 2009.1.02).

<sup>b</sup> The growth yield of both control and treated E. coli cells was unexpectedly higher in the experiments performed to evaluate Tol-nC<sub>60</sub>1 than in those performed with Tol-nC<sub>60</sub>2 or Tol-nC<sub>60</sub>3. Because the batch of medium used with control and Tol-nC<sub>60</sub>3-treated cells was prepared independently from the single batch of medium used to evaluate both Tol-nC<sub>60</sub>2 and Tol-nC<sub>60</sub>3, we presume that batch differences in medium formulations may account for this difference in growth yield.
REFERENCES


