DNA binding capability of nano-C$_{60}$ to pBR322 and phenotypic analysis of potential binding effects

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Nano particles, particularly nano-C$_{60}$, have been part of a growing research initiative due to their unique properties. These particles are being investigated for medically relevant purposes, such as drug delivery. Disturbingly, part of this research has suggested the binding of these particles to DNA, which could be detrimental if used in vivo. In this study, we used spectroscopy to test the ability of nano-C$_{60}$ to bind to pBR322 plasmid, as well as the phenotypic effect of this binding on transformed E. coli DH5a cells. Although we showed that the nano-C$_{60}$ did interact with DNA, as measured by a significant decrease in absorbance at 260nm, no phenotypic effects of this change were measurable when the plasmid was introduced into the E. coli cells. As such, no conclusion could be drawn concerning the in vivo effects of nano-C$_{60}$ on cells; however, the presence of nano-C$_{60}$ was conclusively shown to alter the DNA in vitro.

Since its discovery in 1985, extensive research has been conducted into possible biomedical uses for the C$_{60}$ particle, alternatively known as buckminsterfullerene (6). Possible roles for its use in anti-viral, antioxidant, and neuroprotective therapies have been studied, as well as its possible use as a drug delivery system because of its hollow interior (3). Though it is somewhat insoluble in water, study on its use has been conducted on particles with added hydrophilic side-chains, in an effort to increase its solubility in water and therefore its utility in human systems (11). Though it seems like a very promising new molecule, more recently it has come to light that several problems may exist with the use of this molecule. The first issue is that it has been shown to be toxic to some animals in aquatic environments, such as large-mouth bass, Daphnia, and fathead minnow, and toxic effects have also been shown in human fibroblasts and rats (2,8,10,13). Though it has been shown that adding side chains to the C$_{60}$ can reduce its toxicity, spills or contamination of aquatic systems with untreated C$_{60}$ particles could be detrimental to the species living there (11). Another issue arising with its use is the in silico discovery that C$_{60}$ may be capable of binding to DNA (12). Experimental research shows the ability of C$_{60}$ particles to enter various subcellular compartments of the eukaryotic cell, most notably the nucleus where its access to DNA becomes probable (9). Previous studies (1,4) in the UBC Journal of Experimental Microbiology and Immunology have attempted to investigate the possible binding capability of C$_{60}$ to DNA, and the mutagenic effects that C$_{60}$ might have on Salmonella, but these studies failed to conclusively assess whether or not C$_{60}$ is capable of binding to DNA, and if so, whether or not this actually has an affect on the cell to utilize the DNA.

In this study, we intended to conclusively investigate the capacity of nano-C$_{60}$ to bind DNA in vitro, and the in vivo effects that binding may have had on the ability to utilize the DNA. Because in silico modeling has shown that C$_{60}$ may intercalate into the minor grooves of the DNA double helix to cause breakage of hydrogen bonds, we performed a modified spectroscopy experiment to determine the presence of C$_{60}$ binding to DNA under more optimal conditions so as to be measurable (1,12). An in vivo study of possible effects of bound C$_{60}$ was performed by transforming bacteria with pBR322 plasmid allowed to bind C$_{60}$, and testing the affects on the antibiotic resistance of the bacteria. Failure of the transformed cells to grow on antibiotic resistance medium to the same degree as control cells transformed with untreated pBR322 would indicate that the C$_{60}$ has either interfered with the ability of the resistance genes to be transcribed, interfered with the ability of the plasmid to replicate, or has caused mutagenesis of the plasmid DNA upon replication, all of which are undesirable effects in a human cell exposed to C$_{60}$.

MATERIALS AND METHODS

Preparation of nano-C$_{60}$ complex in water. Nano-C$_{60}$ (n-C$_{60}$) preparation was carried out as previously described with slight modification (1). Briefly, 2.5 mg of C$_{60}$(98 %, Aldrich, cat. # 483036) was added to 100 mL tetrahydrofuran (THF) (99%, Sigma, cat. # T5267) and stirred for four days to facilitate dissolving. On the fourth day, 20 mL additional THF was added to compensate for evaporation. Over a period of three days, 5 mL dH$_2$O was added until the added volume of the solution reached 15 mL. When the THF evaporated, the remaining solution was
filter sterilized using a 0.45 μm nylon syringe filter (Fisher, cat. # 09-719D).

Measurement of nano-C$_{60}$ concentration. The concentration of n-C$_{60}$ was measured using a two-step destabilization-extraction process as previously described (1). Briefly, 300 μL of nano-C$_{60}$ in water was destabilized by the addition of an equal volume of 2% NaCl solution (Fisher, cat. #S271-1), and this solution was extracted with 600 μL toluene (Sigma, cat. #244511). This mixture was vortexed for 10 minutes, then allowed to incubate at room temperature for phase separation for 5 days. Phases obtained were tested for the ability to freeze to -80°C, and the liquid phase was assumed to be the toluene phase. This phase was extracted to a new tube and was used for measurement via spectroscopy. Spectroscopy was carried out using a UV-visible light spectrophotometer (Beckman Coulter DU 530) in a quartz cuvette (Pyrocell, cat. #1007), using toluene as a blank.

Preparation of pBR322 plasmid DNA. pBR322 DNA was isolated from E. coli DH5α cells (MICB 421 frozen stock, Microbiology and Immunology Teaching lab, UBC) using the alkaline lysis technique, as previously described (1). E. coli cells were grown overnight at 37°C in a shaking water bath (140 RPM) in Luria Bertani broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 L dH$_2$O, pH 7) containing 10 μg/mL tetracycline and 50 μg/mL ampicillin. Overnight culture (6 mL total – 3 mL in two separate 1.5 mL microfuge tubes) was centrifuged to obtain a cell pellet. Each pellet was lysed in GET buffer (0.901 g glucose, 0.394 g 1.5 mL microfuge tubes) was centrifuged to obtain a cell pellet. Each pellet was lysed in GET buffer (0.901 g glucose, 0.394 g Tris-HCl, 0.372 g EDTA, 100 mL H$_2$O, pH 8), denatured with 10 μL 2 M NaOH, 100 μL 10% SDS, 800 μL H$_2$O, and then renatured in KOAc solution (29.44 g KOAc, 11.5 mL glacial acetic acid, 88.5 mL H$_2$O). Plasmid DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1). Precipitation was carried out using 1/10 X volume sodium acetate (4.6 g NaOAc, 100 μL H$_2$O) and 2.5 X volume 100% ethanol, placed at -20°C for 30 minutes. Precipitated solutions were centrifuged for 20 minutes at 4°C, and the pellets were washed once in 500 μL cold 70% EtOH. The pellets were allowed to air dry before being resuspended in sterile distilled H$_2$O. Concentration of plasmid was measured in a quartz cuvette using a UV-visible light spectrophotometer (Beckman Coulter DU 530) measurement via spectroscopy. Spectroscopy was carried out with the sample; for the heat denatured plasmids and the DNA controls, the spectroscopy blank was prepared by adding 10 μL of 0.2 μg/mL EtBr to distilled water, creating the same final solution volume and final EtBr concentration as was in the EtBr experimental sample; for the heat denatured plasmids and the DNA controls, the spectrophotometer was blanked with sterile distilled water. All spectroscopy measurements were carried out at 4°C to prevent DNA reannealing of heat controls, and variation due to temperature difference. Spectroscopy was carried out with the same equipment described above, measuring the A$_{260}$ of each sample.

pBR322 transformation of E. coli DH5α. Of the pBR322 plasmid saved from above, half was treated with n-C$_{60}$ (as described above), and the other half remained untreated. E. coli DH5α cells were grown overnight at 37°C in a shaking water bath (140 RPM) in Luria Bertani broth. The cells were made competent as follows: 3 mL of overnight culture was centrifuged at 2500 rpm for 20 minutes; Pellet was resuspended in ½ culture volume of ice cold 50 mM CaCl$_2$; Culture was then centrifuged at 2500 rpm for 20 minutes, then resuspended in 1/10$^4$ of the culture volume in ice cold 50 mM CaCl$_2$; Cells were transformed via CaCl$_2$ transformation. Forty μl aliquots of E.coli DH5α cells were added to three pre-chilled 15 mL test tubes. Five μl of pBR322 DNA, pBR322 incubated with n-C$_{60}$, or sterile distilled water were added to the cells and gently mixed by pipette, then the cells were chilled on ice for 30 minutes. Heat shock was performed at 42°C for 90 seconds, then samples were placed back on ice for 2 minutes. One mL of SOC medium (20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl and 10 mL 250 mM KCl) was added to each tube, and tubes were incubated at 37°C for 2.5 hours. From each tube, 50 μL and 200 μL aliquots were plated on Luria Bertani agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar, 1 L dH$_2$O, pH 7) containing 15 μg/mL tetracycline, 100 μg/mL ampicillin, or no antibiotic (six plates total for each sample).

RESULTS

![Graph](https://via.placeholder.com/150)

**FIG 1.** Relative effects of n-C$_{60}$, heat, and ethidium bromide treatment of pBR322 DNA on absorbance at 260nm. Results
Displayed are calculated from the average of the replicates for each treatment condition, as compared to the average of the replicates from the untreated pBR322 control. Error was calculated as the difference observed between the replicates.

**Concentration of nano-C₆₀ in water.** The concentration of dissolved n-C₆₀ was determined to be 1.14 x 10⁻⁵ M, one third less than the value reported for saturation (1).

**The effects of nano-C₆₀ on absorbance of double-stranded DNA.** As expected, the heat control exhibited an increase in A₂₆₀ of 16% relative to the double-stranded DNA control (Fig. 1). This result was obtained by taking an average of the measured absorbance for the heat denatured controls at 20 minutes and 60 minutes, as these values did not show a large amount of variation, indicating that full denaturation of the DNA likely occurred. Unexpectedly, when the plasmid was treated with ethidium bromide, a known intercalating agent, the A₂₆₀ was well within the range produced by the untreated DNA controls (Fig. 1). Surprisingly, n-C₆₀ treatment of pBR322 DNA caused a reduction in absorbance of 23% relative to the untreated DNA control, which is opposite to the predicted effect (Fig. 1). The minor error associated with the n-C₆₀ replicate measurements indicates that the A₂₆₀ is significantly decreased in comparison to the untreated pBR322 DNA control.

**TABLE 1. Growth of E. coli DH5α cells transformed with n-C₆₀ treated or untreated pBR322, plated on LB agar containing tetracycline or ampicillin.**

<table>
<thead>
<tr>
<th>E. coli DH5α transfectants</th>
<th>Colonies obtained on LB agar containing antibiotic</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Tetracycline</td>
</tr>
<tr>
<td>n-C₆₀ treated pBR322</td>
<td>34 ± 5.8</td>
</tr>
<tr>
<td>Untreated pBR322</td>
<td>41 ± 6.4</td>
</tr>
</tbody>
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**The effects of n-C₆₀ on pBR322 utilization in E.coli DH5α cells.** Confluent growth was obtained on all agar plates lacking antibiotic for samples and controls, so the variation in initial concentration of plated transformants could not be assessed. As expected, the E. coli DH5α control “transformed” with water did not yield any colonies on plates containing either ampicillin or tetracycline. For the E. coli transformed with n-C₆₀ treated or untreated pBR322, the 200µL volume plated yielded sufficient colony numbers to be within the countable range (Table I). Results for ampicillin and tetracycline containing LB agar plates show the same trend for relative numbers of transformants with n-C₆₀ treated and untreated pBR322. This trend reveals a low number of colonies on plates carrying E. coli DH5α with n-C₆₀ treated plasmid; however, the error associated with the measurements and the lack of initial concentration comparison from LB lacking antibiotics indicates that the results are not significantly different from each other, so no significant effect of n-C₆₀ presence was detected (Table I).

**DISCUSSION**

Following in silico observations (12) that n-C₆₀ may be capable of binding DNA, Aw et al. undertook a series of experiments to test the DNA binding capacity of n-C₆₀ in vitro, with variable results (1). In one of the experiments, it was hypothesized that the ability of n-C₆₀ to intercalate into the bases of DNA would cause a separation of those bases and a concomitant increase in the A₂₆₀ of the DNA (1). Though it was shown that denaturation of the DNA causing a full separation of the bases indeed produces an increase in A₂₆₀ (1), the fundamental component of this hypothesis was not tested. Namely, it was not tested whether intercalation itself actually causes significant enough separation so as to be measurable via spectroscopic change. In this study, we repeated the spectroscopy experiments using more favourable conditions to allow optimal n-C₆₀ binding, and also tested whether or not the presence of a known intercalating agent would be sufficient to cause a measurable change in the A₂₆₀ of DNA. When ethidium bromide was added to linearized pBR322 DNA, the A₂₆₀ was well within the range produced by the unaltered pBR322 controls (Fig. 1). This indicates that intercalation does not induce a large enough separation to cause a change in absorbance, so we would not expect to see a change in A₂₆₀ even if the n-C₆₀ was bound to the linearized plasmid; however, actual experimental results demonstrate a significant decrease in the A₂₆₀. This appears to be indicative of a binding interaction, because free n-C₆₀ in the solution should not cause a change in absorbance, as the spectrophotometer was blanked with n-C₆₀ solution. We hypothesize that the observed decrease in absorbance is due to n-C₆₀ induced formation of a more condensed DNA structure. A recent study using IR spectroscopy to examine DNA in human cells reported that in cells with inactivated nuclei, containing condensed chromatin, no IR absorbance could be obtained from the DNA (7). The demonstration that absorbance can be decreased due to the condensation of structure provides support for
our hypothesis - though our absorbance was not decreased so far as to be undetectable, an increasing compactness of DNA structure should couple to a decrease in absorbance, as was observed with n-C60 treatment. In contrast to previous results with intact plasmid (1), large variation was not observed between n-C60 treated, linearized pBR322 replicates, or between linearized pBR322 controls denatured for different amount of times, which is to be expected as full denaturation should occur very quickly. This indicates that the results obtained via the modified protocol with linearized plasmid used in this experiment are more reliable, and can conclusively demonstrate n-C60 interaction with DNA.

Though it is both interesting and alarming to note that n-C60 can bind to (or at least interact with) DNA in vitro, the full impact of this interaction can not be realized without the use of in vivo studies. We hypothesized that binding of n-C60 to pBR322 may interfere with the utilization of this DNA in E. coli DH5α cells. Specifically, n-C60 could interfere with transcription or replication of the DNA. Alternatively, as it is common knowledge that the intercalating agent ethidium bromide can cause DNA mutagenesis, n-C60 binding to pBR322 may also cause mutation of the DNA. As this plasmid contains two antibiotic resistance genes, one for ampicillin and the other for tetracycline, we attempted to test this hypothesis by transforming E. coli DH5α cells with n-C60 treated pBR322 and determining of the ability of the transformants to grow in the presence of either antibiotic. Results from this experiment did show a slightly lower colony count on plates with the n-C60 treated pBR322 transformants relative to the untreated pBR322 controls for both antibiotics, as would be expected if n-C60 has an adverse effect on plasmid utilization; however, the difference observed was not great enough to be above the expected error rate observed with the procedure. As such, it cannot be concluded that n-C60 has a significant effect on the ability of the DH5α cells to successfully use the plasmid to confer antibiotic resistance. Nevertheless, it also cannot be concluded that n-C60 does not have an effect on pBR322 utilization. There are several possible mechanisms through which effects of n-C60 may have been masked. First, if the n-C60 causes impairment of transcription or translation but still allows a lower amount to occur, the transformed cells would still be able to exhibit antibiotic resistance and replicate at a slightly slower rate. Due to segregation of the affected DNA into the two daughter cells, effective concentration of n-C60 in each cell should be reduced by 50%. This would allow less repression of the plasmid, and subsequent generations would dilute the n-C60 even further. In the end, because of the fast generation time of E. coli, a plate of transformants would dilute the n-C60 fast enough to facilitate formation of a visible colony from overnight incubation, as if the n-C60 had no effect. Second, potential mutagenic ability of n-C60 is of great concern, especially if the n-C60 persists in cells for long periods of time. While there weren't observable phenotypic effects of n-C60 presence, genotypic analysis was not performed in this experiment. If n-C60 is a mutagen, the mutation rate may be sufficiently low to allow the replicative production of pBR322 plasmid with functional antibiotic genes. It would only take the production of one plasmid capable of conferring antibiotic resistance to keep the progeny cell alive after replication. Because of the dilution effect discussed above, after a few generations the presence of one cell still containing a functional antibiotic resistance gene on the plasmid would be sufficient to cause the generation of a colony and no observable phenotypic effect. Third, because confluent growth was obtained on all LB agar plates lacking antibiotics, it cannot be ruled out that the variation in the number of transformants was due to a small variation in the initial concentration of cells used in the transformation protocol. Finally, the actual presence of n-C60 within the DH5α cells cannot be ascertained. It was not tested whether or not the conditions of transformation had an effect on the ability of the n-C60 to stay bound to the pBR322, and the stability of this complex is unknown. During the transformation protocol, the n-C60-pBR322 complex was subjected to high CaCl2 concentrations and 42°C heating conditions. These conditional changes may have been sufficient to cause release of the n-C60 from the DNA prior to entry into the cell. Additionally, because n-C60 is hydrophobic, it is not known whether or not it will preferentially associate with the hydrophobic interior of the lipid bilayer instead of remaining bound to the DNA while entering the cell. Though it cannot be confirmed that the n-C60 was bound at the time of transformation, because of the similar colony counts obtained from n-C60 treated and untreated pBR322, it does not appear that the presence of n-C60 has an affect on transformation ability.

In summary, contrary to the expected results, it was demonstrated that n-C60 treatment of DNA generates a reduction in the A260 of the DNA, which could possibly be explained by a condensation of the DNA structure. Transformation experiments performed failed to elucidate whether the presence of n-C60 associated with pBR322 disrupts the utility or integrity of the DNA. Though other toxic effects have been associated with n-C60 (8,10,11,13), knowledge of potential effects of n-C60 association
with DNA is definitely essential if this molecule is to be used for industrial applications.

FUTURE EXPERIMENTS

To demonstrate the in vivo effects of n-C₆₀ on DNA an improved transformation experiment could be carried out, ensuring the presence of n-C₆₀ in the cell. This could be accomplished by first testing the effect on binding of each variable the DNA-n-C₆₀ complex is to be exposed to. This may include temperature changes, changes in ionic strength, or electric charge (if electroporation is to be used). The effect could be measured by sequentially subjecting the complex to each variable introduced during the transformation, and immediately measuring the absorbance to detect an increase in A₂₆₀, which would indicate decreased binding of n-C₆₀. The protocol causing the least amount of variation in A₂₆₀ could then be used to repeat the transformation experiment. Furthermore, we were not completely sure as to whether nano-C₆₀ particles actually entered the cell during transformation, and therefore, future experiments should be aimed at optimizing this aspect of the experiment. It is recommended that the E. coli cells be flooded with a solution of saturated nano-C₆₀ particles at the time of the transformation to help increase the odds that the particles will be taken up by the cell. In addition, it is important to include a countable dilution on LB agar not containing an antibiotic, as similarity in the number of colonies on this plate would lend credibility to smaller changes, as the changes are less likely to be due to a small difference in starting concentration. Including a range of nano-C₆₀-treated plasmids may also help to ascertain as to whether the similarity in response seen in Table 1 is due to a limitation in the competent cells rather than a similarity of the effects of nano-C₆₀.

ACKNOWLEDGMENTS

We would like to thank the Department of Microbiology and Immunology at the University of British Columbia for the funding for this work. We would like to thank Jennifer Sibley and Karen Smith and for their encouragement, technical support and guidance during our procedures. We would like to thank all of the helpful staff in the media room for dealing with our last minute media and glassware emergencies. Lastly, we would like to express a special gratitude to Dr. William Ramey for his extensive wealth of knowledge and never ending encouragement, even during the most dire moments of the research process. His valuable guidance allowed for our project to be possible.

REFERENCES