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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* DH5α 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_2O 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 um 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 at -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 37ºC in a shaking water bath (140 RPM) in Luria Bertani broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 L dH_2O, 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 Tris-HCl, 0.372 g EDTA, 100 mL H_2O, pH 8), denatured with denaturation solution (100 µL 2 M NaOH, 100 µL 10% SDS, 800 µL H_2O), and then renatured in KOAc solution (29.44 g KOAc, 11.5 mL glacial acetic acid, 88.5 mL H_2O). Plasmid DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1). Precipitation was carried out using 1/10 X volume sodium acetate (24.6 g NaOAc, 100 mL H_2O) and 2.5 X volume 100% ethanol, placed at -20ºC for 30 minutes. Precipitated solutions were centrifuged for 20 minutes at 4ºC, 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_2O. Concentration of plasmid was measured in a quartz cuvette using the Beckman spectrophotometer described above, and purity was assessed by running 1 µL from each tube on a 1% agarose gel at 87 V for 45minutes. The gel was stained in an ethidium bromide (EtBr) bath for 15 minutes (0.2 µg/mL) before being imaged using AlphaImager™ software (Alpha Innotech Corporation, California). Due to the presence of RNA within the plasmid preparation, the tubes of plasmid were treated with 20 µg of RNaseA at 37°C for 2 hours and 10 minutes. Each tube of treated plasmid was brought up to 200 µL total volume with sterile distilled water, and a phenol/chloroform extraction and NaOAc precipitation was carried out as described above.

Spectroscopy. Half of the pBR322 plasmid obtained was digested using 200 units EcoRI (New England BioLabs catalogue number: R0101S) at 37°C for 1 hour, and purified via extraction with phenol:chloroform:isoamyl alcohol and precipitation as described above for original plasmid purification. A small aliquot of digested plasmid was checked for full digestion by running this sample on a 1% agarose gel, and the concentration of linearized plasmid was assessed using spectroscopy, as described above. In duplicate, digested pBR322 was added to nano-C_{60} in a 7:1 molar ratio (bp:C_{60}) as previously described (1). This mixture was allowed to incubate at 4ºC for 24 hours prior to measurement of concentration. Digested plasmid for use in spectroscopy experiments was consecutively aliquoted into seven tubes for use to minimize spectroscopy error due to slight differences in concentration of plasmid. Two heat denatured controls were prepared by adding pBR322 DNA in equal concentration as used for the n-C_{60} binding, and were diluted with a volume of dH_2O equal to the volume of n-C_{60} suspension added to pBR322. Controls were denatured at 100ºC for 20 or 60 minutes on the day of the spectroscopy reading to ensure full denaturation, prior to being snap-cooled on ice to 4ºC for spectroscopy measurement. The EtBr control was prepared by adding 10 µl of 0.2 µg/mL EtBr to the aliquoted pBR322, and diluting to the same final concentration as the other experiments with sterile distilled water. For measurement of initial A_{260} of linear pBR322, two DNA controls were set up using equal volume of pBR322 as used for the other experiments, diluted to the same final concentration using sterile distilled water. Spectroscopy blanks were prepared as follows: for the pBR322 n-C_{60} measurements, a blank was prepared using sterile distilled water with the same amount of n-C_{60} as was added to the pBR322 plasmid; for the EtBr control, a 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 4ºC 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 37ºC 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 1/10th 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 minute. Heat shock was performed at 42ºC for 90 seconds, then samples were placed back on ice for 2 minutes. One mL of SOC medium (20 g bactro-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 37ºC 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_2O, pH 7) containing 15 µg/mL tetracycline, 100 µg/mL ampicillin, or no antibiotic (six plates total for each sample).

RESULTS

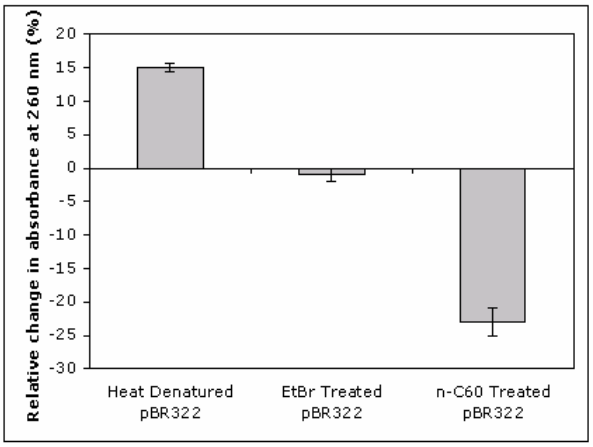

FIG 1. Relative effects of n-C_{60}, heat, and ethidium bromide treatment of pBR322 DNA on absorbance at 260nm. Results

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-C_{60} in water. The concentration of dissolved n-C_{60} was determined to be 1.14x 10^{-5} M, one third less than the value reported for saturation (1).

The effects of nano-C_{60} on absorbance of double-stranded DNA. As expected, the heat control exhibited an increase in A_{260} 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 A_{260} was well within the range produced by the untreated DNA controls (Fig. 1). Surprisingly, n-C_{60} 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-C_{60} replicate measurements indicates that the A_{260} is significantly decreased in comparison to the untreated pBR322 DNA control.

TABLE 1. Growth of *E. coli* DH5α cells transformed with n-C_{60} treated or untreated pBR322, plated on LB agar containing tetracycline or ampicillin.

E. coli DH5α transformants	Colonies obtained on LB agar containing antibiotic:	
	Tetracycline	Ampicillin
n-C_{60} treated pBR322	34 ±5.8	66 ±8.1
Untreated pBR322	41 ±6.4	71 ±8.4

The effects of n-C_{60} 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-C_{60} 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-C_{60} treated and untreated pBR322. This trend reveals a low number of colonies on plates carrying *E. coli* DH5α with n-C_{60} 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-C_{60} presence was detected (Table I).

DISCUSSION

Following *in silico* observations (12) that n-C_{60} may be capable of binding DNA, Aw *et al.* undertook a series of experiments to test the DNA binding capacity of n-C_{60} *in vitro*, with variable results (1). In one of the experiments, it was hypothesized that the ability of n-C_{60} to intercalate into the bases of DNA would cause a separation of those bases and a concomitant increase in the A_{260} 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 A_{260} (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-C_{60} binding, and also tested whether or not the presence of a known intercalating agent would be sufficient to cause a measurable change in the A_{260} of DNA. When ethidium bromide was added to linearized pBR322 DNA, the A_{260} 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 A_{260} even if the n-C_{60} was bound to the linearized plasmid; however, actual experimental results demonstrate a significant *decrease* in the A_{260}. This appears to be indicative of a binding interaction, because free n-C_{60} in the solution should not cause a change in absorbance, as the spectrophotometer was blanked with n-C_{60} solution. We hypothesize that the observed decrease in absorbance is due to n-C_{60} 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-C_{60} treatment. In contrast to previous results with intact plasmid (1), large variation was not observed between n-C_{60} 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-C_{60} interaction with DNA.

Though it is both interesting and alarming to note that n-C_{60} 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-C_{60} to pBR322 may interfere with the utilization of this DNA in *E. coli* DH5α cells. Specifically, n-C_{60} 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-C_{60} 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-C_{60} 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-C_{60} treated pBR322 transformants relative to the untreated pBR322 controls for both antibiotics, as would be expected if n-C_{60} 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-C_{60} 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-C_{60} does not have an effect on pBR322 utilization. There are several possible mechanisms through which effects of n-C_{60} may have been masked. First, if the n-C_{60} 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-C_{60} in each cell should be reduced by 50%. This would allow less repression of the plasmid, and subsequent generations would dilute the n-C_{60} even further. In the end, because of the fast generation time of *E. coli*, a plate of transformants would dilute the n-C_{60} fast enough to facilitate formation of a visible colony from overnight incubation, as if the n-C_{60} had no effect. Second, potential mutagenic ability of n-C_{60} is of great concern, especially if the n-C_{60} persists in cells for long periods of time. While there weren't observable phenotypic effects of n-C_{60} presence, genotypic analysis was not performed in this experiment. If n-C_{60} 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-C_{60} 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-C_{60} to stay bound to the pBR322, and the stability of this complex is unknown. During the transformation protocol, the n-C_{60}-pBR322 complex was subjected to high $CaCl_2$ concentrations and 42°C heating conditions. These conditional changes may have been sufficient to cause release of the n-C_{60} from the DNA prior to entry into the cell. Additionally, because n-C_{60} 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-C_{60} was bound at the time of transformation, because of the similar colony counts obtained from n-C_{60} treated and untreated pBR322, it does not appear that the presence of n-C_{60} has an affect on transformation ability.

In summary, contrary to the expected results, it was demonstrated that n-C_{60} treatment of DNA generates a reduction in the A_{260} 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-C_{60} associated with pBR322 disrupts the utility or integrity of the DNA. Though other toxic effects have been associated with n-C_{60} (8,10,11,13), knowledge of potential effects of n-C_{60} 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-C_{60} on DNA an improved transformation experiment could be carried out, ensuring the presence of n-C_{60} in the cell. This could be accomplished by first testing the effect on binding of each variable the DNA-n-C_{60} 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 A_{260}, which would indicate decreased binding of n-C_{60}. The protocol causing the least amount of variation in A_{260} could then be used to repeat the transformation experiment. Furthermore, we were not completely sure as to whether nano-C_{60} 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-C_{60} 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-C_{60}.

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REFERENCES

1. **Aw, C., C. Greenwood, A. Kong, and D. Siu.** 2006. Investigating Possible Interactions Between Nano-C_{60} and Plasmid pBR322 DNA. J. Exp. Microbiol. Immunol. **9**:68-74.
2. **Bosi, S., L. Feruglio, T. Da Ros, G. Spalluto, B. Gregoretti, M. Terdoslavich, G. Decorti, S. Passamonti, S. Moro and M. Prato.** 2004. Hemolytic effects of water-soluble fullerene derivatives. J. Med. Chem. **47**:6711-6715.
3. **Bradford, C. B., A. G. Fernando, C. Bi-Xing, N. K. Austin, R. W. Stephen and F. E. Bernard.** 2000. X-ray crystal structure of an anti-buckminsterfullerene antibody Fab fragment: biomolecular recognition of C_{60}. Immunology. **97**:12193-12197.
4. **Chu, S., G. Eom., K. Q. Little, and M. Peacock.** 2006. The Effect of Colloidal n- C_{60} on DNA Mutagenesis and SOS DNA Repair Mechanism of *Salmonella typhimurium* strains TA1538 and TA1535. J. Exp. Microbiol. Immunol. **9**:61-67.
5. **Hinze, E.** 2004. Microbiology 323 Microbiological Techniques II Laboratory Manual. The University of British Columbia, Vancouver, B.C.
6. **Kroto, H. W. , J. R. Heath, S. C. O'Brien, R. F. Curl and R. E. Smalley.** 1985. C_{60}: Buckministerfullerene. Nature. **318**:162-163.
7. **Muhlenhoff, B., M. Romeo, M. Diem, and B. R. Wood.** 2005. Mie-Type Scattering and Non-Beer-Lambert Absorption Behaviour of Human Cells in Infrared Microscopy. Biophysical Journal. **88**:3635-3640
8. **Oberdorster, E**. 2004. Manufactured nanomaterials (Fullerenes, C_{60}) induce oxidative stress in the brain of juvenile largemouth bass. Environ. Health Perspect. **112**:1058-1062.
9. **Porter, A. E., K. Muller, J. Skepper, P. Midgley, M. Welland.** 2006. Uptake of C_{60} by human monocyte macrophages, its localization and implications for toxicity: studied by high resolution electron microscopy and electron tomography. Acta Biomaterialia. **2**:409-19.
10. **Sayes, C. M., A. M. Gobin, K. D. Ausman, J. Mendez, J. L. West, V. L. Colvin**. 2005. Nano-C_{60} cytotoxicity is due to lipid peroxidation. Biomaterials. **36**:7587-95.
11. **Sayes, C. M., J. Fortner, D. Lyon, A Boyd, K. Ausman, Y. Tao, B. Sitharaman, L. Wilson, J. West and V. L. Colvin.** 2004. The differential cytotoxicity of water soluble fullerenes. Nano Letters. **4**:1881.
12. **Zhao, X., A. Striolo and P. T. Cummings.** 2005. C_{60} binds to and deforms nucleotides. Biophys. J. **89**:3856-3862.
13. **Zhu, S., E. Oberdorster, and M. L. Haasch.** 2006. Toxicity of an engineered nanoparticle (fullerene, C_{60}) in two aquatic species, Daphnia and fathead minnow. Mar. Environ. Res. **62**(S1):S5-S9.
14. **Zupko, W.** Transformation of competent DH5α, San Francisco State University: http://www.sfusd.k12.ca.us/programs /sf_base/protocols/lab102d_dh5a_transformation.pdf.