

Antibacterial Activity of Fullerene Water Suspensions (nC₆₀) Is Not Due to ROS-Mediated Damage

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ABSTRACT

The cytotoxic and antibacterial properties of nC₆₀, a buckminsterfullerene water suspension, have been attributed to photocatalytically generated reactive oxygen species (ROS). However, in this work, neither ROS production nor ROS-mediated damage is found in nC₆₀-exposed bacteria. Furthermore, the colorimetric methods used to evaluate ROS production and damage are confounded by interactions between nC₆₀ and the reagents, yielding false positives. Instead, we propose that nC₆₀ exerts ROS-independent oxidative stress, thus reconciling conflicting results in the literature.

Fullerenes have yet to find widespread commercial application, but their increasing availability and decreasing cost augurs the environmental dispersal of fullerene-containing products. The most environmentally relevant form of the prototypical fullerene, C₆₀ (i.e., buckminsterfullerene), is proposed to be the fullerene water suspension nC₆₀,^{1,2} which can be formed in water either through extensive stirring of C₆₀ powder or via an organic solvent. nC₆₀ can be toxic to mammalian cell lines,³⁻⁶ water fleas (*Daphnia magna*),⁷⁻⁹ several types of fish,⁸⁻¹⁰ and bacteria.^{2,11-13} However, the toxicity mechanisms have not been unequivocally discerned, which hinders the rational development of appropriate mitigation and remediation approaches.

The most researched and publicized mechanism for nC₆₀ toxicity for eukaryotic cells is the presumed (photocatalytic) production of reactive oxygen species (ROS).^{4,5,10,14,15} Fullerenes have been shown to cleave double-stranded DNA upon exposure to light,¹⁶ although this depends on the type of fullerene derivative.¹⁷ Markers of lipid peroxidation in fish brain tissue and human cell lines, a sign of ROS damage, were noted after exposure to nC₆₀,^{4,9,10} but another study

using bacterial phospholipid fatty acid analysis (PLFA) showed that, while lipid composition does change, there was no evidence of lipid peroxidation.¹⁸ The involvement of ROS in the death of mammalian cell lines has also been inferred using ROS-detecting dyes, such as the redox sensitive dihydroxyrhodamine (DHR), and ROS-damage detecting assays;^{5,19} this conflicts with other publications showing that nC₆₀ does not produce ROS.^{20,21}

In this study, we aim to clarify the role of ROS in the antibacterial activity of nC₆₀ and propose a hypothesis to rationalize the seemingly incongruous data in the literature. Using nC₆₀ prepared using tetrahydrofuran (THF) as a transitional solvent,² we look for ROS production and ROS-mediated damage in bacteria. The results show that (1) there is no ROS production nor ROS-mediated damage and (2) a number of the colorimetric assays for ROS production and ROS-mediated damage are interfered with by nC₆₀, leading to false positives. We posit an alternate hypothesis that nC₆₀ behaves as an oxidant and exerts ROS-independent oxidative stress.

Monitoring Photocatalytic ROS Production. The photocatalytic ROS production of nC₆₀ in the bacterial growth medium was evaluated using two methods: by measuring oxygen consumption by the ROS-trapping agent furfuryl alcohol (FFA)²¹ and by monitoring the color change of 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) due to reduction by superoxide (O₂^{•-}) to

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Table 1. Four Methods to Detect ROS Production Due to nC₆₀^a

treatment	detection method			
	FFA	XTT	hydroethidine	H ₂ DCFDA
	O ₂ consumption increase over control (%)	absorbance increase over control (%)	fluorescence increase over control (%)	fluorescence increase over control (%)
nC ₆₀ alone	19.1 ± 15	10.5 ± 16	187 ± 14 ^b	346 ± 12 ^{b,c}
<i>E. coli</i> + nC ₆₀	–	–	70 ± 12 ^b	268 ± 2 ^b
<i>E. coli</i> + H ₂ O ₂	–	–	–	75 ± 3 ^b
<i>B. subtilis</i> + nC ₆₀	–	–	46 ± 3 ^b	198 ± 9 ^b
<i>B. subtilis</i> + H ₂ O ₂	–	–	–	159 ± 11 ^b

^a Results are shown as a percent increase over a negative control after exposure to nC₆₀ with or without bacterial cells. ^b indicates a statistically significant difference at $\alpha=0.05$. ^c In the cell-free control of H₂DCFDA, an aliquot of dye was simply added to the dye-only control, so the increase in fluorescence of the dye-only control relative to the samples with cells can be partially attributed to a higher concentration of the dye.

XTT-formazan.²² In the FFA method, glass vials containing 100 mM FFA (Sigma-Aldrich, St. Louis, MO) and 0.72 mg/L nC₆₀ in minimal Davis medium (MD),¹³ including a foil-wrapped negative control, were exposed to UV radiation (Licor 1800 spectroradiometer, total irradiance of 13.6 W/m² with a spectrum ranging from 310 to 400 nm) for 1–10 h at 22 °C in an EMS UV/Cryo chamber (Hatfield, PA). Consumption of oxygen, reflecting the amount of ROS (O₂^{•-} and ¹O₂) produced was measured using a ThermoOrion 830A polarographic oxygen sensor (Thermo Fisher Scientific, Inc., Waltham, MA). For the XTT method, samples containing 3.6 mg/L nC₆₀, 100 μ M XTT, and MD in 10 mL flasks were exposed to the UV source for 30 h and then monitored for a change in absorbance at 470 nm using an Ultrospec 2100pro UV/visible spectrophotometer (Amersham Biosciences, Pittsburgh, PA), which was indicative of superoxide production.

Both the FFA and the XTT method indicate that no ROS was produced by photoactivated nC₆₀. FFA consumed oxygen at similar rates when exposed to nC₆₀ (10.9 ± 1.6 mM O₂ consumed/min) and water (9.2 ± 0.6 mM O₂ consumed/min), leading to a statistically insignificant increase of 19.1 ± 15% (Table 1). When XTT was used specifically to detect superoxide radicals, samples with nC₆₀ increased insignificantly in absorbance by 10.5 ± 16%, or 2.1 ± 0.3 UA/min as compared to 1.9 ± 0.3 UA/min for the control. However, the absence of ROS production in cell-free systems does not preclude ROS-production in the presence of cells. In theory, ROS could be produced as a result of nC₆₀ contact with some bacterial electron carriers or as an immune response in the case of eukaryotes. Consequently, we investigated ROS production in the presence of bacteria.

Monitoring ROS Production in the Presence of Bacterial Cells. The bacteria *Escherichia coli* K12 (ATCC no. 25404) and *Bacillus subtilis* 168 (ATCC no. 31578) were maintained and grown on Luria–Bertani (LB) medium, but the experiments were performed in MD to avoid nC₆₀ coagulation and precipitation by salts. Two dyes, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA, Invitrogen, Carlsbad, CA) and hydroethidine (HEt, Invitrogen), were used per the manufacturer's instruction to measure ROS in and around the bacterial cells via increases in fluorescence as measured using a VersaFluor fluorimeter (BioRad, Hercules, CA). According to the manufacturer, HEt, which has blue fluorescence in the cytoplasm, is added at a final

concentration of 100 μ M to cells with or without 10 mg/L nC₆₀, and the dye is oxidized by superoxide to form an ethidium compound that fluoresces red when bound to DNA.²³ H₂DCFDA is nonfluorescent until it enters the cell, whereupon it is cleaved by an esterase, which improves cell retention and allows the compound to be oxidized (and fluoresce) by most ROS species. The protocol for H₂DCFDA involved incubating the cells with 200 μ M of dye for 30 min, then harvesting the exposed cells by centrifugation, resuspending them in LB such that the intracellular esterases could cleave the dye for 10 min, and finally exposing the cells to 10 mg/L nC₆₀ for another 15 min. Data were interpreted relative to nC₆₀-free controls with cells, except for the nC₆₀-alone samples, which were compared to a dye-only control.

In the presence of nC₆₀, both H₂DCFDA and HEt increased in fluorescence with or without cells (Table 1), suggesting ROS production and contradicting results obtained with the FFA and XTT ROS-detection methods described above. However, upon closer examination, there are inconsistencies in the behavior of H₂DCFDA and HEt in the presence of nC₆₀. H₂DCFDA fluoresced strongly in the presence of nC₆₀ without cells, but it is not supposed to fluoresce until after intracellular esterases cleave its acetate groups to free the active site for reaction with oxidizing species. The dye should not fluoresce without cells. Similarly, HEt should only fluoresce when bound to DNA in the cell, but it also fluoresced in the cell-free control. Additionally, HEt is presumed to be oxidized specifically by the superoxide radical to form a fluorescent 2-hydroxyethidium cation,²³ but the XTT results indicate no superoxide formation and the addition of superoxide dismutase (which degrades superoxide) did not significantly lower the fluorescence (data not shown).

Apparently, the use of dyes to detect ROS is fraught with false positives due to the promiscuity of fluorescent dyes.²⁴ For example, HEt can be directly oxidized by heme proteins,²⁵ and pyocyanin was hypothesized to directly oxidize H₂DCF without a ROS intermediate.²⁶ As shown by the cell-free controls in this study, nC₆₀ oxidized the dyes directly, without need for superoxide production to stimulate HEt nor for the cleavage of the esterase group to allow H₂DCFDA to fluoresce. This leads to false positives of ROS production by nC₆₀, when in actuality nC₆₀ itself is probably behaving as the oxidant.

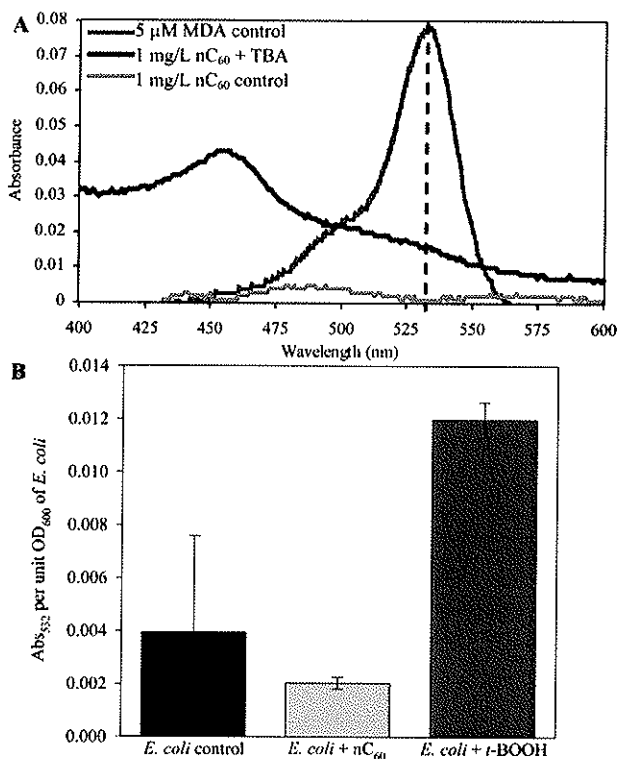


Figure 1. Assessment of lipid peroxidation in *E. coli* exposed to nC₆₀ through the formation of colored adducts between MDA and TBA. (A) In the absence of cells, nC₆₀ formed a colored product with TBA with increased absorption at 532 nm (dotted vertical line) as well as at other wavelengths. (B) After centrifuging the cells to remove most nC₆₀ particles, *E. coli* incubated with nC₆₀ showed no increase in absorbance at 532 nm compared to the negative control, whereas the positive *t*-BOOH control exhibited increased absorbance due to lipid peroxidation.

Searching for ROS-Mediated Damage: The Lipid Peroxidation Assay. Lipid peroxidation, a signature of ROS damage, can be detected by assaying for malondialdehyde (MDA), an oxidized product of polyunsaturated fatty acids for which there are established detection protocols. MDA forms an adduct with thiobarbituric acid (TBA), resulting in a pink product with increased absorbance at 532 nm.²⁷ This assay was utilized in the studies that encountered lipid peroxidation in various eukaryotic systems.^{4,5,9,10} To measure MDA in *E. coli*, several protocols were consulted.^{28–30} In the established protocol, the cells are incubated with the oxidizing agent of interest, the cell proteins are precipitated with trichloroacetic acid (TCA), and the supernatant is boiled with TBA. Following this protocol, the nC₆₀ particles in the cell suspension would be present during the TBA assay. To ascertain whether nC₆₀ interfered with the assay, nC₆₀ without cells was boiled with TBA as fully described below. A wavelength scan of the mixture was compared to TBA + 5 μM MDA (Sigma-Aldrich) as a positive control and 1 mg/L nC₆₀ with no TBA as a negative control (Figure 1A). The wavelength scan indicated that nC₆₀ formed a colored product with TBA that lead to increased absorption at 532 nm. The lipid peroxidation assay is predicated on the ability of TBA to form a colored adduct with MDA, but it is well-established that TBA can form adducts with other chemicals to yield

yellow, orange, and pink products.³¹ Because of the ability of nC₆₀ to interact with TBA and presumably oxidize it to yield a similar color with the same absorption as the TBA-MDA adduct, it is not possible to use this assay accurately, and previous reports of lipid peroxidation using this test should be re-evaluated.^{4,5,9,10}

In an attempt to remove the interfering nC₆₀ particles from the assay, a slight deviation was made from the established protocol. An exponentially growing culture of *E. coli* (OD₆₀₀ = 0.2 in 10 mL MD without glucose) was exposed in triplicate to either water as a negative control, 1 mM *tert*-butylhydroperoxide (*t*-BOOH) as a positive control, or nC₆₀ at 1 mg/L, and shaken for 1 h at 37 °C. The cells were harvested by centrifugation at 5000g for 10 min and resuspended in 0.5 mL MD without glucose; this step removes the majority of the nC₆₀ from the cell suspension. Then 1 mL of 10% (wt/vol) TCA was added to the 0.5 mL cell suspension, incubated at room temperature for 20 min, and then centrifuged at 11,000g for 35 min. then 0.5 mL of the supernatant was added to 0.5 mL of freshly prepared 1% 2-thiobarbituric acid (Sigma-Aldrich) in 0.1 M NaOH, boiled for 10 min, cooled to room temperature overnight, and assessed by spectrophotometer for absorbance at 532 nm (Figure 1B). Lipid peroxidation was only evident in the positive *t*-BOOH control; nC₆₀ did not result in lipid peroxidation according to this assay.

Searching for ROS-Mediated Damage: Protein Oxidation. Proteins, another cell component vulnerable to ROS damage, can be evaluated for the formation of carbonyl groups, hallmarks of protein oxidation, using an OxyBlot protein oxidation detection kit (Chemicon, Millipore, Temecula, CA). An exponential phase culture of *E. coli* was exposed to 2 mg/L nC₆₀ or water as a negative control at an OD₆₀₀ of 2.0 in 1 mL of MD for 1 h at 37 °C. The cells were sonicated at 12 W for 1 min in PBS with 50 mM dithiothreitol and 1 Protease Inhibitor Cocktail tablet (Roche Applied Science, Indianapolis, IN) per 10 mL. After centrifugation to remove cell debris, protein concentration was determined using a Bradford assay (BioRad, Hercules, CA), and the cell extract was assayed using the OxyBlot kit. In the protocol, carbonyl groups are derivatized to 2,4-dinitrophenylhydrazine (DNP), the derivatized proteins are loaded at an equal concentration onto a membrane, and the carbonyl groups are detected via an immunoassay using the chemiluminescent agent SuperSignal West Pico chemiluminescent substrate system (Pierce, Rockford, IL). The density of the dot blot images were analyzed using a Molecular Dynamics personal densitometer SI (Sunnyvale, CA) with ImageQuant 5.2 software.

The dot optical densities of the nC₆₀ exposed samples revealed no increase compared to the negative controls, indicating no increase in carbonyl groups in *E. coli* exposed to nC₆₀ (Figure 2). This indicates that there was no ROS-mediated damage of the cytoplasmic proteins; cell membrane proteins were not assayed as they were removed by centrifugation during the cell lysate preparation. If nC₆₀ produced ROS, these oxidative species should be able to enter the cell and induce protein oxidation.³² Other studies that noted lipid

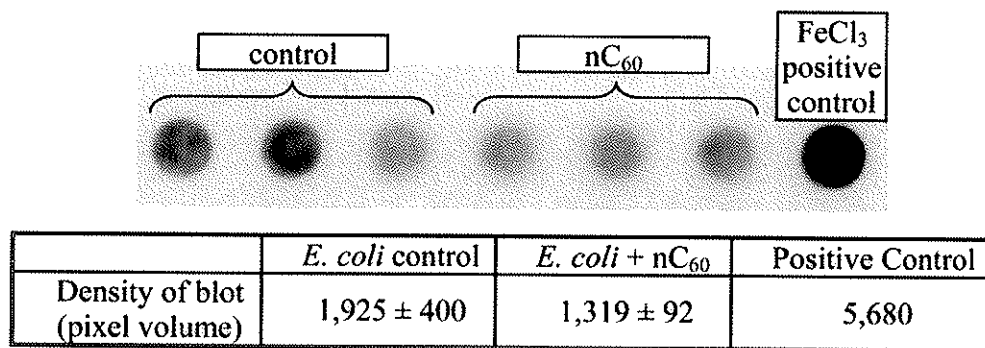


Figure 2. Densitometric analysis of a dot blot detecting carbonyl groups in *E. coli* exposed to nC₆₀ reveals no ROS damage to cytoplasmic proteins.

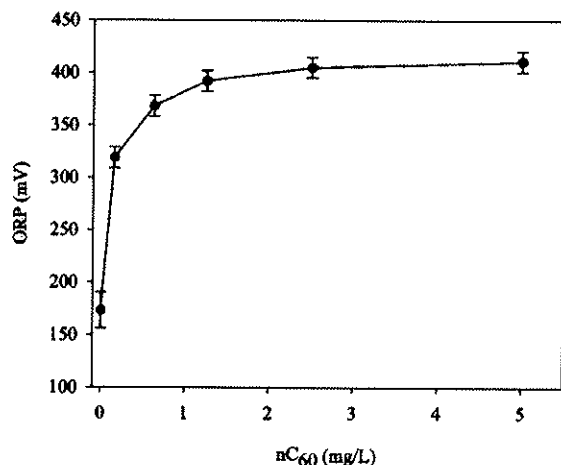


Figure 3. Oxidation–reduction potential (ORP) of nC₆₀ increases with increasing concentration. The overall values suggest that nC₆₀ is an oxidant.

peroxidation and other evidence of ROS-mediated damage have also failed to detect protein oxidation.^{4,10} This assay focused on carbonyl groups as indicators of ROS-mediated damage, so the absence of carbonyl groups does not negate other potential forms of oxidative stress.³³

Evidence of nC₆₀ as an Oxidant. Past research has provided evidence of nC₆₀ exerting oxidative damage in eukaryotic cells but no solid evidence of nC₆₀ producing ROS. nC₆₀ could behave as a direct oxidant, thus leading to misleading results for various oxidative-damage assays and interference with redox sensitive dyes. There is evidence of other fullerenes mediating redox reactions.^{34–36} To investigate this hypothesis, the oxidation-reduction potentials (ORP) of nC₆₀ suspensions were measured using an ORP (Redox) combination electrode with an UltraBasic benchtop pH meter (Denver Instrument, Denver, CO). The samples were sparged with nitrogen while the probe was allowed to equilibrate for at least 10 min prior to reading; a positive ORP value (mV) indicates an oxidizing compound. The ORP of nC₆₀ at various concentrations in water, shown in Figure 3, indicates that nC₆₀ is an oxidant. Besides direct readings of ORP, the ability of nC₆₀ to oxidize ROS-detecting dyes is another indication of its oxidizing capacity (Table 1).

Reconciling the Data: A Hypothesis of the nC₆₀ Antibacterial Mechanism. There are several pieces of apparently conflicting data in the literature and in this study. First, ROS-detection dyes, like the H₂DCFDA and HET in this study and DCFDA and DHR in other studies, indicate that ROS are produced by nC₆₀.^{5,19} However, in this study, we demonstrate that nC₆₀ interferes with the reliability of those ROS-detecting dyes, with H₂DCFDA fluorescing without esterase-mediated cleavage of the diacetate group and HET fluorescing outside the cell. Other methods to detect ROS using FFA or XTT in this and previous studies have revealed no ROS production.²⁰ Another piece of confusing data comes from using the standard TBA-MDA method to identify lipid peroxidation specific to ROS damage. This study showed that nC₆₀ interferes with the proper functioning of this assay, leading us to question the involvement of ROS in other studies that used this method.^{4,5,10} The lack of lipid peroxidation (Figure 1B) is corroborated by a phospholipid fatty acid analysis (PLFA) of *B. subtilis* and *Pseudomonas putida* cell membranes, which found changes in membrane composition but no oxidized lipids.¹⁸ Finally, an ROS-independent toxicity mechanism is indicated by the lack of protein oxidation in this and other studies,¹⁰ and the persistence of antibacterial properties in the absence of light and oxygen and thus photocatalytic ROS production.^{11,13} Thus, ROS are not a significant cause of the toxicity of nC₆₀.³⁷

There are several indicators from this and previous studies that nC₆₀ exerts ROS-independent oxidative stress. ROS-detecting systems, such as H₂DCFDA and HET, that depend on reduction/oxidation of the dye would not function properly as reporter molecules if nC₆₀ itself is an oxidant. The FFA method of ROS-detection circumvents possible interference by nC₆₀ by measuring oxygen consumption in the medium; XTT undergoes a *reduction* by superoxide, so it too is impervious to false positives via oxidation. Both of these methods indicate that nC₆₀ does not produce ROS, but the behavior of the H₂DCFDA and HET indicate that oxidants are present (Table 1). nC₆₀ itself is an oxidant (Figure 3), and this property could lead to the toxicity observed. Future research needs include looking for non-ROS-mediated oxidative damage of the membrane proteins and lipids as well as other supporting evidence for this hypothesis to improve our understanding of the antibacterial mechanism of nC₆₀. This

understanding could facilitate better control over nC₆₀ toxicity in both applications and responses to accidental discharge into the environment.

Previous analyses proposing that nC₆₀ exerts toxicity via ROS-production may have been confounded by direct oxidation of the dyes by nC₆₀, yielding false positives, and may need to be revisited. In the past, carbon nanotubes have been reported to interfere with a widely used (MTT) dye-based cell viability assay,³⁸ resulting in conflicting results similar to those reported in this work. These papers stress the need for caution and extensive controls when using established assays with novel compounds, particularly nanomaterials. Overall, understanding the drawbacks and potential hazards associated with nanomaterials like fullerenes should result in more cost-efficient and savvy manufacture and use of the materials as well as decreasing negative side effects and public backlash.

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References

- Brant, J.; Lecoanet, H.; Wiesner, M. R. *J. Nanopart. Res.* **2005**, *7*, 545.
- Fortner, J. D.; Lyon, D. Y.; Sayes, C. M.; Boyd, A. M.; Falkner, J. C.; Hotze, E. M.; Alemany, L. B.; Tao, Y. J.; Guo, W.; Ausman, K. D.; Colvin, V. L.; Hughes, J. B. *Environ. Sci. Technol.* **2005**, *39*, 4307.
- Sayes, C. M.; Fortner, J. D.; Guo, W.; Lyon, D.; Boyd, A. M.; Ausman, K. C.; Tao, Y. J.; Sitharaman, B.; Wilson, L. J.; Hughes, J. B.; West, J. L.; Colvin, V. L. *Nano Lett.* **2004**, *4*, 1881.
- Sayes, C. M.; Gobin, A. M.; Ausman, A. M.; Mendez, J.; West, J. L.; Colvin, V. L. *Biomaterials* **2005**, *26*, 7587.
- Isakovic, A.; Markovic, Z.; Todorovic-Markovic, B.; Nikolic, N.; Vranjes-Djuric, S.; Mirkovic, M.; Dramicanin, M.; Harhaji, L.; Raicevic, N.; Nikolic, Z.; Trajkovic, V. *Toxicol. Sci.* **2006**, *91*, 173.
- Tsao, N.; Luh, T.; Chou, C.; Chang, T.; Wu, J.; Liu, C.; Lei, H. *J. Antimicrob. Chemother.* **2002**, *49*, 641.
- Lovern, S. B.; Klaper, R. *Environ. Toxicol. Chem.* **2006**, *25*, 1132.
- Oberdörster, E.; Zhu, S.; Blickley, T. M.; McClellan-Green, P.; Haasch, M. L. *Carbon* **2006**, *44*, 1112.
- Zhu, S.; Oberdörster, E.; Haasch, M. L. *Mar. Environ. Res.* **2006**, *62* (Suppl.), S5.
- Oberdörster, E. *Environ. Health Perspect.* **2004**, *112*, 1058.
- Lyon, D. Y.; Adams, L. K.; Falkner, J. C.; Alvarez, P. J. *J. Environ. Sci. Technol.* **2006**, *40*, 4360.
- Lyon, D. Y.; Brown, D. A.; Alvarez, P. J. *J. Water Sci. Technol.* **2007**, submitted.
- Lyon, D. J.; Fortner, J. D.; Sayes, C. M.; Colvin, V. L.; Hughes, J. B. *Environ. Toxicol. Chem.* **2005**, *24*, 2757–2762.
- Sera, N.; Tokiwa, H.; Miyata, N. *Carcinogenesis* **1996**, *17*, 2163.
- Kai, Y.; Komazawa, A.; Miyajima, A.; Miyata, N.; Yamakoshi, Y. *Fullerene Nanotube Carbon Nanostruct.* **2003**, *11*, 79.
- Takenaka, S.; Yamashita, K.; Takagi, M.; Hata, T.; Tsuge, O. *Chem. Lett.* **1999**, 321.
- Babynin, E. V.; Nuretdinov, I. A.; Gubskaya, V. P.; Barabanshchikov, B. I. *Genet. Microorg.* **2002**, *38*, 453.
- Fang, J.; Lyon, D. Y.; Wiesner, M. R.; Dong, J.; Alvarez, P. J. *J. Environ. Sci. Technol.* **2007**, *41*, 2636.
- Markovic, Z.; Todorovic-Markovic, B.; Kleut, D.; Nikolic, N.; Vranjes-Djuric, S.; Misirkic, M.; Vucicevic, L.; Janjetovic, K.; Isakovic, A.; Harhaji, L.; Babic-Stojic, B.; Dramicanin, M.; Trajkovic, V. *Biomaterials* **2007**, *28*, 5347.
- Lee, J.; Fortner, J. D.; Hughes, J. B.; Kim, J. H. *Environ. Sci. Technol.* **2007**, *41*, 2529.
- Pickering, K. D.; Wiesner, M. R. *Environ. Sci. Technol.* **2005**, *39*, 1359.
- Okado-Matsumoto, A.; Fridovich, I. *Anal. Biochem.* **2001**, *298*, 337.
- Zielonka, J.; Sarna, T.; Roberts, J. E.; Wishart, J. F.; Kalyanaraman, B. *Arch. Biochem. Biophys.* **2006**, *456*, 39.
- Patsoukis, N.; Papapostolou, I.; Georgiou, C. D. *Anal. Bioanal. Chem.* **2005**, *381*, 1065.
- Papapostolou, I.; Patsoukis, N.; Georgiou, C. D. *Anal. Biochem.* **2004**, *332*, 290.
- O'Malley, Y. Q.; Reszka, K. J.; Britigan, B. E. *Free Radical Biol. Med.* **2004**, *36*, 90.
- Esterbauer, H.; Cheeseman, K. H. *Methods Enzymol.* **1990**, *186*, 407.
- Maness, P. C.; Smolinski, S.; Blake, D. M.; Huang, Z.; Wolfrum, E. J.; Jacoby, W. A. *Appl. Environ. Microbiol.* **1999**, *65*, 4094.
- Yoon, S. J.; Park, J. E.; Yang, J. H.; Park, J. W. *J. Biochem. Mol. Biol.* **2002**, *35*, 297.
- Kono, Y.; Shibata, H.; Adachi, K.; Tanaka, K. *Arch. Biochem. Biophys.* **1994**, *311*, 153.
- Kosugi, H.; Kato, T.; Kikugawa, K. *Anal. Biochem.* **1987**, *165*, 456.
- Sigler, K.; Chaloupka, J.; Brozmanova, J.; Stadler, N.; Hofer, M. *Folia Microbiol.* **1999**, *44*, 587.
- Dukan, S.; Belkin, S.; Touati, D. *Arch. Biochem. Biophys.* **1999**, *367*, 311.
- Patolsky, F.; Tao, G.; Katz, E.; Willner, I. *J. Electroanal. Chem.* **1998**, *454*, 9.
- Hamano, T.; Mashino, T.; Hirobe, M. *J. Chem. Soc., Chem. Commun.* **1995**, 1537.
- Foley, S.; Curtis, A. D. M.; Hirsch, A.; Brettreich, M.; Pelegrin, A.; Seta, P.; Larroque, C. *Fullerene Nanotube Carbon Nanostruct.* **2002**, *10*, 49.
- Harhaji, L.; Isakovic, A.; Raicevic, N.; Markovic, Z.; Todorovic-Markovic, B.; Nikolic, N.; Vranjes-Djuric, S.; Markovic, I.; Trajkovic, V. *Eur. J. Pharmacol.* **2007**.
- Worle-Knirsc, J. M.; Pulskamp, K.; Krug, H. F. *Nano Lett.* **2006**, *6*, 1261.

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