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Fullerene Water Suspension (nC_{60}) Exerts Antibacterial Effects via ROS-Independent Protein Oxidation

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Buckminsterfullerene (C_{60}) can form water suspensions (nC_{60}) that exert toxic effects. While reactive oxygen species (ROS) generation has been implicated as the mechanism for mammalian cytotoxicity, we propose that nC_{60} exerts ROS-independent oxidative stress in bacteria, with evidence of protein oxidation, changes in cell membrane potential, and interruption of cellular respiration. This mechanism requires direct contact between the nanoparticle and the bacterial cell and differs from previously reported nanomaterial antibacterial mechanisms that involve ROS generation (metal oxides) or leaching of toxic elements (nanosilver).

Introduction

The potential benefits of nanotechnology are shadowed by the uncertainty of its impact on living systems and the environment. The advent of engineered nanomaterials in commercial and industrial applications raises concern about the potential health impacts and environmental safety of these products (*1*). The fullerene C_{60} is an example of a nanomaterial that was initially considered benign but has since shown potential toxicity in both prokaryotic and eukaryotic systems (*2*−*4*). Initially, the hydrophobicity of C_{60} (water solubility $<10^{-9}$ mg/L) was thought to limit its interactions with biological systems (*5*), and oral administration, skin application, or injection of C_{60} in rats and other cell systems revealed no acute toxicity (*6*−*9*). However, once C_{60} is introduced into water (either via solvents or by extensive stirring) it forms stable nanoscale suspended aggregates known as fullerene water suspensions (FWS) or nC_{60} (*10*−*13*) and becomes biologically active. nC_{60} is highly toxic to eukaryotic cell lines (*2, 14*), *Daphnia magna* (*15, 16*), and fish (*3, 17*). The nC_{60} particles can also accumulate in eukaryotic cells and adhere to lipids (*18, 19*), and it has antibacterial properties against different bacterial species (*20*).

The preparation of nC_{60} frequently involves the use of tetrahydrofuran (THF) to stabilize C_{60} in water, and the THF is subsequently removed by evaporation as described by Fortner et al. (*13*). The use of THF as an intermediary solvent has raised concern as a confounding factor in toxicity studies (*16, 21*−*23*). The type of solvent used to make nC_{60} does affect the properties of the nC_{60} formed (*24*). However, concerns that toxicity is mainly due to residual THF are allayed by the fact that nC_{60} prepared without THF by long-term stirring of C_{60} powder in water exhibits toxicity (*16, 17, 25, 26*). Furthermore, a negative control without C_{60} (i.e., THF added to the water and then evaporated) or controls with THF at 15 mg/L had no antibacterial properties (data not shown), indicating that THF was not directly involved in toxicity at the concentrations it was present. THF could contribute to toxicity via peroxide formation, but this was unlikely because water stabilizes THF and decreases its ability to form peroxides. Also, peroxides are short-lived while nC_{60} retains its toxicity for over two years (*20*).

The toxicity mechanism of nC_{60} has generally been attributed to its ability to generate reactive oxygen species (ROS) and singlet oxygen when photosensitized (*27, 28*). These ROS can trigger various types of cell damage, such as lipid peroxidation, various forms of DNA damage, and protein oxidation, and these effects can be used as indicators of ROS production (*29*). Several studies with eukaryotic systems used the presence of these damage hallmarks to conclude that nC_{60} exerts oxidative stress resulting in cytotoxicity (*3, 14, 17, 24, 30*). However, we recently showed that assays for ROS and its damage may be confounded by nC_{60} itself. Specifically, nC_{60} can interfere both with dyes that detect the presence of lipid peroxidation and dyes that detect ROS (*31*), resulting in false positives. A few studies also noted that nC_{60} induced toxic effects without ROS; a low concentration of nC_{60} induced ROS-independent anti-proliferative effects in tumor cells (*32*) and a study using phospholipid fatty acid analysis (PLFA) showed no evidence of lipid peroxidation in bacteria exposed to nC_{60} (*33*). Another abiotic study showed no ROS production by nC_{60} using detection dyes and ROS-trapping agents (*34*). Furthermore, nC_{60} retains its antibacterial properties in the absence of light and oxygen, both of which are required for fullerene ROS production (*4, 25*).

Microorganisms are the foundation of all known ecosystems, and an understanding of the antimicrobial mechanisms exerted by nanomaterials may enable prevention, regulation, or manipulation of associated environmental impacts. Bacteria may also serve as models to identify cytotoxicity mechanisms and as sensitive indicators of potential toxicity to higher order organisms. There are few studies on the antibacterial nature of fullerenes, and each offers different explanations for the antibacterial mechanism. C(60)-bis(*N*,*N*-dimethylpyrrolidinium iodide) reportedly inhibited *E. coli* growth by interfering with glucose respiration (*35*). Some studies have shown fullerene derivatives incorporating into artificial lipid membranes (*36*), with carboxyfullerenes being able to puncture Gram-positive bacterial membranes (*37*). Given that ROS production was recently ruled out as the antibacterial mechanism (*31*), we explore here two alternative toxicity mechanisms: (1) nC_{60} could physically disrupt the cell membrane, leading to a leaky cell and cell death, and/or (2) nC_{60} could behave as a strong oxidant upon contact with the cell, uncoupling electron transport and energy transduction during respiration. We find that while nC_{60} does not physically puncture bacteria cells, it exerts an ROS-independent oxidative effect at the membrane interface which hinders respiration.

Materials and Methods

Bacterial Growth, Preparation of THF/nC_{60}, and Antibacterial Activity Measurements. *Escherichia coli* K12 (ATCC #25404) and *Bacillus subtilis* 168 (ATCC #31578) were maintained on Luria−Bertani (LB) plates and cultured in LB broth or minimal Davis medium (per 1 L, 0.7 g of K_2HPO_4, 0.2 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.5 g of sodium citrate, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 1 g of glucose) (*4*). The nC_{60} was

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prepared using the method for THF/nC_{60} described previously (*4*). Briefly, a 25 mg/L solution of C_{60} (99.5% pure, MER Corporation, Tucson, AZ) in THF that had been passed through a 0.22 μm nylon filter was vigorously stirred while an equivalent amount of DI water was added at a rate of 750 mL/min. THF was removed by evaporation (Buchi Rotavapor; Buchi Labortechnik AG, Flawil, Switzerland), and the final nC_{60} suspension was stored overnight before being filtered through a 0.22 μm nylon filter.

Flow Cytometry to Assess Cell Health and Function. Flow cytometry was performed using a BD FACScalibur System (BD Biosciences, San Jose, CA) and kits or dyes obtained from Molecular Probes (Invitrogen, Carlsbad, CA).

Assessing Membrane Integrity. Membrane integrity was assessed using propidium iodide (PI, Molecular Probes, Invitrogen), a dye which enters permeablized cells, binds DNA, and fluoresces at 617 nm when stimulated by a laser at 535 nm during flow cytometry. In intact cells, PI remains in the medium and does not fluoresce; in compromised cells, PI enters the cell and binds DNA which makes it fluoresce. The dye was used according to the manufacturer's protocol. Briefly, exponentially growing cells were diluted to an OD_{600} of 0.05 in MD with 5 mg/L nC_{60} and incubated at 37 °C for 1 h, a sufficient amount of time to observe toxicity (*20*). The cells were harvested by centrifugation in a microcentrifuge at 13,000 rpm and resuspended in 1 mL of phosphate buffered saline (PBS). The samples were diluted to an appropriate OD in 1 mL of PBS, and stained with 2 μL of a 1 mg/mL propidium iodide stock. Twenty μL of toluene, known to puncture membranes, acted as a positive control. The samples were incubated for 10 min prior to running on FCM. All experiments were performed in triplicate.

Assessing Membrane Potential. The BacLight Bacterial Membrane Potential Kit (Molecular Probes, Invitrogen) was used according to the manufacturer's instructions. The key component is $DiOC_2$ (3,3′-diethyloxacarbocyanine iodide), a dye that changes from green to red fluorescence with increasing membrane potential. In the protocol, exponentially growing cultures were added to 1 mL of PBS to an OD_{600} 0.005. The cells were either exposed to the ionophore CCCP as a positive control or to 1 mg/L nC_{60}. They were stained with 10 μL of 3 mM $DiOC_2$ at room temperature for 15 min prior to analysis by FCM.

Assessing Redox Activity. The BacLight RedoxSensor Green Vitality Kit (Molecular Probes, Invitrogen) was used to determine reductase activity. Briefly, exponentially growing cells were diluted to an OD_{600} of 0.005 in PBS. The cells were incubated with electron-transport disruptors, sodium azide or CCCP, as positive controls, or with 1 mg/L nC_{60} for 15 min prior to staining with RedoxSensor Green. The cells were then fixed with 1.6% formaldehyde or placed on ice. To assess the effect of an antioxidant, glutathione was added to 5 μM.

Submitochondrial Particle Analysis (SMP) to Verify Electron Transport Chain Function. The Mitoscan test focusing on reverse electron transfer (RET) using submitochondrial particle (SMP) analysis (*38*) was performed by AquaTOX Research Inc. (Syracuse, NY).

Monitoring Oxidative Damage to Cellular Components. *Thiol Oxidation.* Thiols, also known as sulfhydryl groups or mercaptans, are susceptible to oxidation in proteins exposed to ROS or other oxidants. The level of thiols in proteins was assessed using the Thiol and Sulfide Quantitation Kit (Molecular Probes, Invitrogen) following the manufacturer's protocol. This assay relies on thiol groups to release the active form of the enzyme papain, which then acts on a chromogenic substrate. Two different assays were performed to determine the ability of nC_{60} to oxidize proteins, both in and out of cells. First, *E. coli* cells, at an OD_{600} of 2.00, are exposed to 10 mg/L of nC_{60} in MD for 1 h at 37 °C. Second, cell-free assays were performed with the protein bovine serum albumin (BSA), which has 1 thiol group per protein. BSA, at 1 μg/mL, is incubated with nC_{60} in water. In both sets of experiments, negative controls are incubated with water and positive controls are incubated with 5 mM tert-butylhydroperoxide. The cells are then harvested by centrifugation, rinsed in the buffer used in the assay, and resuspended in that buffer. In a Coy anaerobic chamber, the cells are lysed by sonication 12 W for 1 min, centrifuged to pellet cell debris, and the supernatant was transferred to a fresh tube. Protein concentrations were quantified using a Pierce BCA protein assay kit, and enough cell extract was used to have at least 20 μg of protein per reaction. The results are shown as nmol of thiol in the sample.

Lipid Oxidation. Lipid oxidation was measured using the Lipid Hydroperoxide Kit (Calbiochem, EMD Biosciences) which is based on the ability of lipid hydroperoxides to interact with ferrous ions to produce ferric ions, which then react with the chromogen thiocyanate. The chromogen is quantified using a visible spectrophotometer, with higher absorbance indicating higher levels of lipid hydroperoxides.

Statistical Analysis. All experiments were run at least in triplicate. Error bars representing the standard error of the mean are included in the figures. Where appropriate, samples were analyzed for statistical difference using Student's *t* test at the 95% confidence level.

RESULTS

nC_{60} Does Not Compromise the Physical Integrity of Bacterial Cells. The bacterial cell membrane performs many essential functions, such as regulating transport of materials and establishing a membrane potential for ATP-generation; cells unable to repair damaged membranes quickly perish. Membrane integrity was assessed using PI. In Figure 1, the positive control containing toluene, a solvent known to perforate bacterial cells (*39*), showed increased fluorescence compared to the negative controls for both *B. subtilis* and *E. coli*. The cells treated with nC_{60} for 1 h did not exhibit increased fluorescence, indicating that the cell membranes were not perforated within the same time span needed to observe toxicity (*20*). These results agree with a study examining the interaction of nC_{60} with supported lipid bilayers, which shows that nC_{60} particles associated with the zwitterionic or cationic lipid head groups but did not penetrate or disrupt the membrane (*19*), and a second study demonstrating that if fullerene aggregates entered cells, they would not induce membrane disruption (*40*). This differs from a previous report using the same PI assay on (larger) eukaryotic cell lines, which showed membrane damage after 6 h incubation with nC_{60} (*14*). However, it was not determined whether this extended incubation period allowed killed cells to begin degrading and thus exhibit loss of membrane integrity as a symptom rather than cause of lost viability.

Changes in Membrane Potential. The membrane potential, essential for energy transduction during respiration, was measured in both *E. coli* and *B. subtilis* after exposure to nC_{60} using the BacLight Membrane Potential kit (Molecular Probes, Invitrogen). The kit contains $DiOC_2$ which maintains green fluorescence in cells, but this fluorescence shifts toward red emission at higher membrane potentials due to intracellular accumulation of the dye. Results are expressed as the ratio of red fluorescence to green fluorescence, with a larger ratio indicating a higher membrane potential. The positive control provided in the kit, CCCP, eliminates membrane potentials by behaving as a proton ionophore. The *B. subtilis* control had a red/green fluorescence ratio of 5.85 $\pm$ 0.44 while the nC_{60}-exposed *B. subtilis* had a significantly lower ($p = 0.0025$) ratio of 0.94 $\pm$ 0.20. On the other hand, *E. coli* showed no significant difference in red/green ratio after nC_{60} exposure, with ratios of 3.24 $\pm$ 0.007

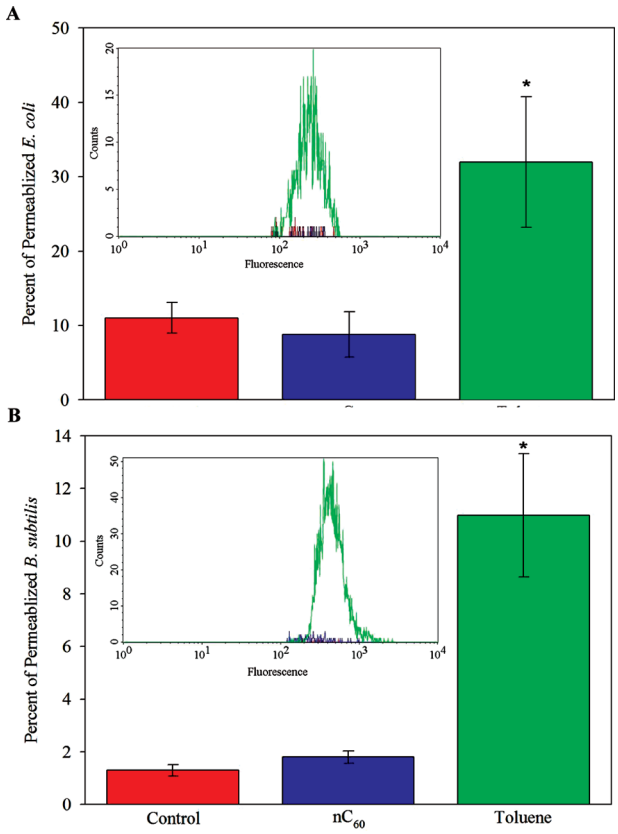

FIGURE 1. (A) *E. coli* and (B) *B. subtilis* are not permeablized by nC_{60}, as determined by flow cytometry with propidium iodide (PI), as shown by the calculated percentage of cells permeablized. The insets show the histogram data. Statistically significant differences relative to the control are indicated with an asterisk (*). Using a one-tailed *t* test at the 95% confidence interval, there was no difference between the control and nC_{60} ($p = 0.125$ for *E. coli* and $p = 0.13$ for *B. subtilis*), but there was a signficant difference between the control and the toluene samples ($p = 0.013$ for *E. coli* and $p = 0.022$ for *B. subtilis*).

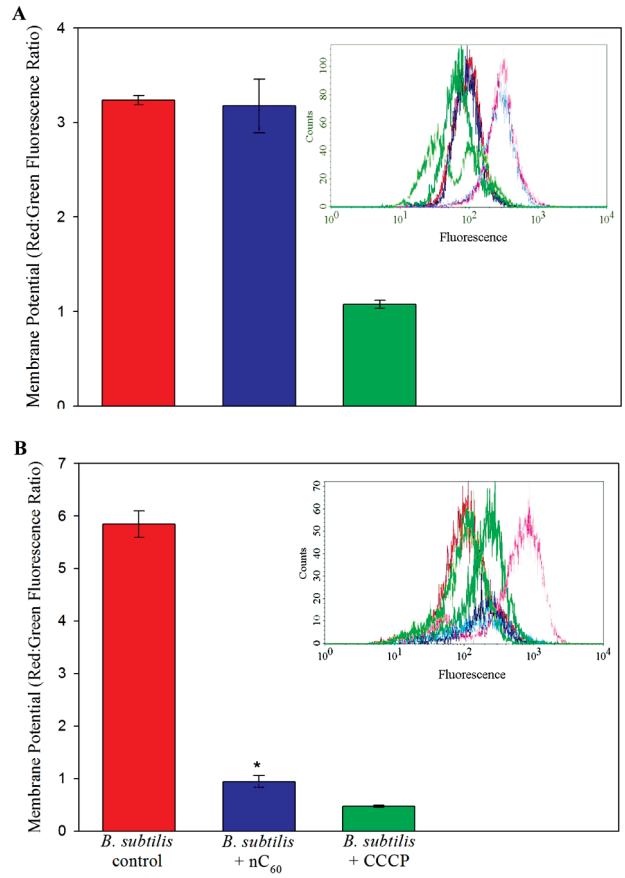

FIGURE 2. Membrane potential of (A) *E. coli* and (B) *B. subtilis* exposed to nC_{60} expressed as red/green ratios, with higher ratios indicating larger membrane potentials. CCCP, an ionophore, is the positive control. There was no difference between the control and nC_{60}-exposed *E. coli* ($p = 0.426$), but there was a significant difference, indicated with an asterisk, between the control and nC_{60}-exposed *B. subtilis* ($p = 0.0025$). The insets are the raw histogram data from the flow cytometer.

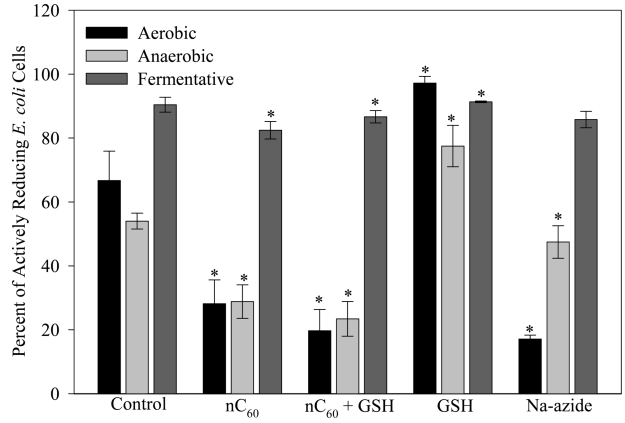

FIGURE 3. nC_{60} Exposure reduces the percentage of *E. coli* cells with active reductases under aerobic ($p = 0.047$) and anaerobic nitrate-reducing conditions ($p = 0.023$), but not under fermentative conditions. In both figures, "Na-azide" stands for sodium azide and "GSH" stands for reduced glutathione. Statistically significant differences ($p = 0.05$) relative to the negative control are indicated with an asterisk (*).

before exposure versus 3.18 ± 0.24 after nC_{60} exposure. Thus, *B. subtilis*, but not *E. coli*, experienced a reduction in membrane potential after exposure to nC_{60} (Figure 2), although both bacteria are equally susceptible to nC_{60} (*20*). The difference between the responses of the Gram-positive versus Gram-negative bacteria is most likely due to physiological differences in cell envelope structure, although no specific hypothesis has been investigated.

Evaluating Reductase Activity. The BacLight RedoxSensor Green Vitality Kit contains a dye, RedoxSensor Green reagent, which enters the cell in its oxidized form and then fluoresces when reduced by a reductase. Bacterial reductase activity is an indicator of normal electron transport chain function. Fluorescence decreases when cells are treated with uncouplers, such as sodium azide which interrupts electron transport in *E. coli*, or carbonyl cyanide 3-chlorophenylhydrazone (CCCP) which interrupts electron transport in *B. subtilis*. Results under aerobic conditions show that both *E. coli* (Figure 3) and *B. subtilis* (Supporting Information, Figure A) experience a decrease in reductase activity upon exposure to nC_{60}. Addition of glutathione (GSH), which protects thiol/disulfide bonds in *E. coli* and scavenges ROS (*41*), increased the reductase activity of healthy cells but could not rescue cells from nC_{60} toxicity, providing further evidence that ROS are not involved in the antibacterial mechanism. The same assay was performed with cells grown under anaerobic conditions (with nitrate as the electron acceptor) and fermentative (with glucose) conditions. The reductase-inhibiting properties of nC_{60} persist under anaerobic, nitrate-reducing conditions in *E. coli* (Figure 3). Under fermentative conditions, there is no significant decrease in reductase activity after exposure to nC_{60}. In *E. coli*, fermentation involves only intracellular electron transport, with no electrons

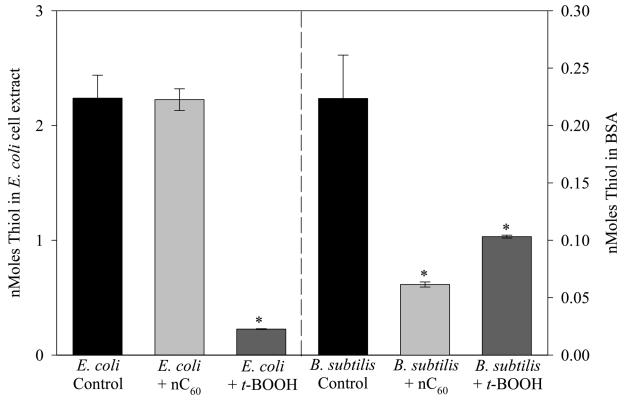

FIGURE 4. Protein oxidation in *E. coli* and in BSA as measured by loss of thiol groups. The left half of the graph uses the left axis and the right half uses the right axis. There was no difference between the *E. coli* cell extract control and the cell extract exposed to nC_{60} ($p = 0.473$), but there was a significant difference, indicated with an asterisk, between the negative control and nC_{60}-exposed BSA ($p = 0.023$).

TABLE 1. EC_{50} of Various Chemicals in an RET Assay

chemical	EC_{50} (mg/L)
acetylsalicylic acid	936
chloramphenicol	165
dichloromethane	33.7
nickel	2.2
sodium dodecyl sulfate	0.713
nC_{60}	**0.62**
silver	0.14
pentachlorophenol	0.088

crossing the membrane, explaining why there was no difference in reductase activity. Sodium azide inhibits the action of cytochromes involved in electron transport across the membrane (*42*), which explains the lack of effect observed under fermentative conditions.

Monitoring Electron Transport. The reverse electron transfer (RET) assay uses submitochondrial particles (SMPs) to examine whether respiration is interrupted by exposure to a substrate. Briefly, SMPs isolated from beef heart are inverted such that the electron transport and oxidative phosphorylation enzymes on the surface of the SMP are exposed to the substrate (*43*). In the RET test, the electron transport chain and oxidative phosphorylation are reversed, with ATP being hydrolyzed and NADH being produced (*43*). The test monitors the amount of NADH produced via spectrophotometry and expresses the results as an EC_{50} (i.e., the effective concentration of a compound that decreases NADH production to one-half of the baseline value). The RET assay of SMPs exposed to nC_{60} showed an EC_{50} of 0.62 mg/L, indicating that nC_{60} uncoupled electron transport. For comparison, the EC_{50} values for other compounds are listed in Table 1; nC_{60}'s low EC_{50} indicates its potency as an electron transport inhibitor.

Monitoring Oxidative Damage to Cellular Components. *E. coli* exposed to nC_{60} was examined for oxidative damage to proteins and lipids. Cell extracts, consisting primarily of cytoplasmic proteins, and the protein bovine serum albumin (BSA) were examined for loss of thiol groups as evidence of protein oxidation using a thiol oxidation kit (CalBioChem). In the cell extracts, the control sample had 2.24 ± 0.35 nmoles of thiol and the nC_{60}-exposed sample had 2.23 ± 0.16 nmoles (Figure 4). Thus, no thiol oxidation occurred after exposure to nC_{60} under anoxic conditions. These results agree with previously published results using a different assay, which showed no protein oxidation in nC_{60}-exposed cell extracts (*31*). However, there was significant oxidation ($p = 0.023$) and loss of thiol groups in the BSA samples under anoxic conditions (Figure 4). Each BSA molecule has 1 thiol group, and the theoretical amount of thiol in the 10 μg of BSA used in this assay is 0.151 nmoles. The control samples had 0.224 ± 0.065 nmoles thiol, which is slightly higher than the theoretical amount, versus $0.0614 \pm 3.8 \times 10^{-3}$ nmoles for nC_{60}-exposed samples. The oxidation of the BSA (per thiol loss) reveals that nC_{60} can oxidize proteins; the lack of oxidation in the cell extract, which consists primarily of cytoplasmic proteins, reveals that nC_{60} remains primarily outside the cell and any oxidation would have occurred at the membrane interface. Previous research has shown that nC_{60} sorbs to bacterial membranes (*4*).

Lipids in the membranes can be damaged by ROS, leading to lipid peroxidation and areas of leakage in the membrane (*29*). In a previous publication, no lipid peroxidation was observed, although the method was interfered with by the nC_{60} itself (*31*). In this research, Lipid Hydroperoxide Kit (Calbiochem, EMD Biosciences), which was not susceptible to such interference, was used to assess lipid oxidation after exposure to nC_{60} under aerobic conditions. The negative control sample had 1.08 ± 0.24 nmoles of hydroperoxide while the nC_{60}-exposed sample had 0.77 ± 0.21 nmoles, which is statistically indiscernible at the 95% confidence level ($p = 0.155$). Thus, there was no lipid oxidation in nC_{60}-exposed samples. The *t*-BOOH positive control showed lipid oxidation with 34.8 ± 3.17 nmoles hydroperoxide, thus validating the assay.

Discussion

In this paper, two different hypotheses were tested to explain the antibacterial mechanism of nC_{60}: (1) perforation of bacterial cell envelopes or (2) exertion of ROS-independent oxidative stress. A series of tests showed that the cell structural integrity remained intact, but nC_{60} exerted oxidative stress and hindered respiration. Whereas there have been several assertions that ROS are responsible for nC_{60}'s toxicity (*3, 14, 17, 24, 30*), the lipid peroxidation assay and previous research from our laboratory indicated that ROS are not responsible for the antibacterial activity of nC_{60} (*31*). ROS are not the only mechanism by which oxidants exert toxicity; some oxidants can also uncouple oxidative phosphorylation (*44*). There are several lines of evidence pointing to nC_{60}'s oxidative effects. (1) The loss of membrane potential in nC_{60}-exposed *B. subtilis* (Figure 2), stopping the cells from establishing the proton gradient necessary for oxidative phosphorylation via electron transport. Bacteria lack subcellular organelles like mitochondria specializing in respiration, and instead bacterial respiration occurs at the cellular membrane. Often, a lowered membrane potential is accompanied by membrane damage. However, the propidium iodide tests showed that neither Gram-negative *E. coli* nor Gram-positive *B. subtilis* suffered from perturbation of membrane integrity (Figure 1). Thus, the change in membrane potential is probably due to perturbation of the proteins involved in establishing the membrane potential. (2) The RET assay indicated that nC_{60} interrupts electron transport (Table 1). This could either be due to nC_{60} compromising the membrane integrity (which was already negated), or due to nC_{60} interfering with the electron transport proteins. (3) Protein oxidation was observed in BSA exposed to nC_{60} (Figure 4), suggesting that membrane proteins may be oxidized after exposure to nC_{60}. The exact location of the nC_{60} particles interacting with bacteria has not been established. While it is theoretically possible that the particles are able to enter the cells without damaging the membrane (*40*), the lack of cytoplasmatic protein oxidation even while BSA was easily oxidized by nC_{60} indicates that nC_{60} remained outside the bacterial cell. Oxidation of membrane proteins was not

analyzed due to assay interference by the protein extraction reagents; attempts to dialyze out the reagents were hindered by the short-lived and oxygen-sensitive nature of the thiol oxidation. (4) The measured oxidation−reduction potential (ORP) of nC_{60} (411 mV at 5 mg/L (*31*)) and the potential ability of nC_{60} to oxidize ROS-detecting dyes (*31*) both indicate that nC_{60} is an oxidant.

The lack of membrane potential loss in *E. coli* raises questions about the target of the oxidation and how well a generic mechanism can be stated. Still, converging lines of evidence point to ROS-independent oxidation of respiratory proteins, like reductases. Under anaerobic or fermentative conditions, the primary target might change, but it is likely linked to the oxidative capacities of nC_{60}. While nC_{60} likely behaves as an oxidant upon contact with cells, disrupting energy transduction, the specific target(s) for nC_{60}'s antibacterial activity has not been established, which is also the case for more conventional antibacterial agents like silver and chlorine. To clarify the mechanism, future research might identify the impacted proteins using a modified protein pull-down assay, or use microarray analysis to elucidate the initial molecular response to nC_{60}.

There have been other studies proposing how oxidants act as antibacterial agents. Another nanomaterial, cerium oxide, was postulated to exert a similar antibacterial mechanism, with the nanoparticles being reduced upon exposure to cells (*45*). Electrolyzed water has high disinfection efficiency due to the combination of the high ORP and the presence of hypochlorous acid (HOCl) (*46*). The symptoms associated with HOCl exposure, such as loss of culturability, membrane potential, and respiratory activity, are very similar to those exhibited by cells exposed to nC_{60} (*4, 44, 47*). At the low HOCl levels needed for cell death, the cell exhibits little to no breach of the membrane and no oxidation of the cytosolic components. This mimics the lack of cytoplasmic protein oxidation observed after exposure to nC_{60}.

Previous reports of antibacterial nanomaterials typically indicate three different toxicity mechanisms. Metal oxides, such as TiO_2 and MgO (*48, 49*), produce ROS which mediate toxicity. The toxicity of other metal-containing nanomaterials, such as nanosilver or quantum dots (*50, 51*), is augmented by the release of toxic ions. Some nanomaterials physically puncture cells, leading to their death (*37*). nC_{60} is unique in that it does not puncture the cell nor release ROS or toxic products, but instead exerts toxicity as a particle via a chemical interaction upon direct contact. The behavior of nC_{60} as both an oxidizing chemical and as a nanoparticle is intrinsic to its reactivity.

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Supporting Information Available

A bar graph showing loss of reductase activity ($p = 0.008$) in *B. subtilis* as a result of nC_{60} exposure under aerobic conditions, as reflected by the BacLight RedoxSensor Green Vitality Kit. This material is available free of charge via the Internet at http://pubs.acs.org.

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