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Antibacterial Activity of Fullerene Water Suspensions: Effects of Preparation Method and Particle Size†

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Fullerene research in biological systems has been hindered by the compound's relative insolubility in water. However, C_{60} molecules can be made to aggregate, forming stable fullerene water suspensions (FWS) whose properties differ from those of bulk solid C_{60}. There are many different protocols for making FWS. This paper explores four of these methods and establishes the antibacterial activity of each resulting suspension, including a suspension made without intermediary solvents. The aggregates in each polydisperse suspension were separated by size using differential centrifugation and tested for antibacterial activity using *Bacillus subtilis* as a test organism. All suspensions exhibited relatively strong antibacterial activity. Fractions containing smaller aggregates had greater antibacterial activity, although the increase in toxicity was disproportionately higher than the associated increase in putative surface area. This suggests the need for improved understanding of the behavior of FWS towards organisms and in the environment to determine how C_{60} can be safely used and disposed.

Introduction

Since the discovery of fullerenes in 1985 this third allotrope of carbon has been a promising compound for a number of applications, such as catalysts and sensors (*1*−*3*). The cagelike structure of fullerenes allows them to encapsulate other molecules (*4*). These doped fullerenes can be used as medical therapeutic agents, tissue-specific fullerene-based drugs, and other medical tools (*5*, *6*). Fullerenes can also be derivatized or polymerized to tailor them to a purpose (*7*). For example, nanocomposites incorporating fullerenes are used in nonlinear optics and photoelectrochemistry applications (*8*).

Fullerene production has steadily increased since 1990, when a method was first developed for its mass production (*9*). Frontier Carbon built a plant capable of annually producing 10 tons of C_{60} by 2007 (*10*). The economy of fullerene production indicates that fullerene-containing products will soon be widely available. The manufacture, use, and disposal of these items are not currently regulated, although the U.S. House Scientific Committee has prioritized legislation of nanomaterial handling and disposal (*11*−*13*). In the interest of making informed decisions, it is imperative to determine the health and environmental implications of fullerene use and disposal.

In an attempt to contribute to proactive risk management, this and previous papers have examined some of the potential impacts of fullerene use. Numerous papers on fullerene derivatives show both positive and negative health effects, depending upon the derivative, application, and organism/system of study (*14*−*22*). It must be stressed that the effects depend on the specific fullerene derivative; there is no consensus in the literature regarding the toxicity of fullerenes in general. Fewer studies have been performed on the effects of fullerene derivatives on bacteria. Most of these studies found that different derivatives had antibacterial properties, but the level of toxicity again depended on the test organism and the specific derivative (*23*−*31*).

With a solubility of less than 10^{-9} mg/L, powdered C_{60} is virtually insoluble in water, and studies examining C_{60} powder alone did not find antibacterial activity (*32*−*34*). Several papers have proposed different methods of making fullerene water suspensions (FWS) (*35*−*39*). These methods commonly involve dissolving C_{60} in a solvent followed by addition of water and subsequent removal of the solvent. A FWS contains nanoscale C_{60} aggregates in a suspension whose properties differ from those of C_{60} dissolved in the relevant solvent (*34*, *40*). FWS are typically yellow or brown suspensions that do not settle out over time. It has been hypothesized that water molecules stabilize the C_{60} molecules in the aggregates (*41*). In the interest of concise and consistent nomenclature in this paper, we call these stable FWS "nC_{60}"and use an appropriate prefix to designate the production method. One solubilization method used, adapted from Yamakoshi et al., does not actually produce nC_{60} but uses a stabilizer common in the pharmaceutical industry, namely poly(vinylpyrrolidone), to encapsulate C_{60} molecules (*38*).

It has been proposed that nC_{60} may be the most environmentally relevant form of C_{60} (*34*). In the event of a spill of C_{60} powder or C_{60} dissolved in a solvent, it is conceivable that nC_{60} could be formed (*42*). nC_{60} has therefore been the subject of several toxicological studies that have received widespread attention (*32*, *34*, *43*−*47*). Studies have shown nC_{60} to be toxic to bacteria, largemouth bass, and human cell lines (*32*, *43*, *44*). Each of these studies has used nC_{60} prepared using tetrahydrofuran (THF) as the intermediate solvent, raising concern that the toxicity is attributable to residual solvent rather than the C_{60} aggregates themselves (*42*, *48*).

This research has two aims: first to determine whether nC_{60} has antibacterial properties, regardless of solvents or solubilizers used during preparation, and second to examine how the morphology of the nC_{60} aggregate affects its antibacterial activity. We prepared FWS using four different methods, using THF as a solvent (THF/nC_{60}), sonicating C_{60} dissolved in toluene with water (son/nC_{60}), stirring C_{60} powder in water (aq/nC_{60}), and using a solubilizing agent (PVP/C_{60}). We tested each preparation for antibacterial activity toward the Gram-positive bacterium *Bacillus subtilis*. We then examined fractions of the nC_{60} aggregates, paying particular attention to how the size and morphology of nC_{60} affect antibacterial activity.

Experimental Section

Producing C_{60} FWS by Four Different Methods. The four different types of C_{60} FWS were produced as described in the literature with minor alterations.

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THF/nC$_{60}$. Tetrahydrofuran/nC$_{60}$, or THF/nC$_{60}$, was prepared by following the method of Deguchi et al. and incorporating the modifications described by Fortner et al. (*34*, *39*). Briefly, 100 mg of 99.5% pure C$_{60}$ (SES Research, Houston, TX, or MER Corp., Tucson, AZ) was dissolved in 4 L of spectra-analyzed THF (Fisher Scientific, Houston, TX). The THF was flushed with nitrogen to prevent oxidation prior to the mixture being stirred overnight at room temperature. The solution was then filtered through a 0.22 μm Osmonics nylon membrane (Fisher Scientific) to remove any undissolved particles. Five hundred milliliters of the C$_{60}$−THF solution was stirred vigorously while an equal volume of Milli-Q (Millipore, Billerica, MA) water was added at a rate of 1 L/min. The THF was removed by evaporation in a heated vacuum system, specifically using a Büchi Rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) complete with hot water bath, refrigerated condenser, and vacuum pump. The C$_{60}$−THF−water mixture was heated in a round-bottom flask to 65 °C, allowing the THF to evaporate. The vapor was cooled in the condenser, which was flushed with water at 2−10 °C, and the liquid THF was collected and disposed of appropriately. One liter of the C$_{60}$−THF−water mixture would yield approximately 400 mL of THF/nC$_{60}$ prior to concentration and filtration, which is discussed below.

Son/nC$_{60}$. Sonicated nC$_{60}$, or son/nC$_{60}$, is produced using the method of Andrievsky et al. (*37*). This FWS was originally named C$_{60}$FWS and the aggregates called HyFn, but for the purposes of clarity in this study, we term the suspension son/nC$_{60}$. A solution of 1 g/L C$_{60}$ in toluene was prepared, and 50 mL of this solution was added to 500 mL of Milli-Q (Millipore) water. This layered mixture was then sonicated using a Sonifier Cell Disruptor (Heat Systems-Ultrasonic Inc., Plainview, NY) at 80−100 W for 15 min intervals (allowing the machine to cool for 5 min between) until all of the toluene had evaporated, or for approximately 10 15-min intervals. The brown suspension was then filtered sequentially through a 1 Whatman filter (Fisher Scientific), a 0.45 μm Osmonics nylon membrane (Fisher Scientific), and a 0.22 μm nylon membrane to remove aggregates larger than 200 nm. This suspension was concentrated and filtered further as discussed below.

Aq/nC$_{60}$. Aqueous nC$_{60}$, or aq/nC$_{60}$, was prepared by stirring 1 g of C$_{60}$ powder in 1 L of Milli-Q water over low heat (40 °C) for 2−4 weeks (*36*). The brown suspension was filtered sequentially through a 1 Whatman filter, a 0.45 μm nylon membrane, and a 0.22 μm nylon membrane to remove aggregates larger than 200 nm. This suspension was concentrated and filtered further as discussed below.

PVP/C$_{60}$. PVP/C$_{60}$ was prepared according to the method of Yamakoshi et al., in which poly(vinylpyrrolidone) (PVP) was used to encapsulate C$_{60}$ molecules (*38*). Since PVP/C$_{60}$ is not composed solely of C$_{60}$, the term nC$_{60}$ is not applied. One hundred milliliters of a 0.31% PVP (*k* value 13−19, Sigma-Aldrich, St. Louis, MO) in chloroform solution was added to 25 mL of a 1 g/L solution of C$_{60}$ in toluene. The mixture was stirred and heated overnight at 45 °C until the solvents evaporated. The residue was resuspended in 500 mL of Milli-Q water. The yellow suspension was filtered through a 0.45 μm nylon membrane and a 0.22 μm nylon membrane to remove aggregates larger than 200 nm. This suspension was concentrated and filtered further as discussed below.

Evaporation and Filtration. All FWS were concentrated by evaporating excess water with a Büchi rotary evaporator to a final concentration of between 2 and 15 mg/L C$_{60}$. The FWS were heated in the water bath to 75 °C under a vacuum, allowing the water to evaporate. The water cooled in the 2−10 °C condenser and was collected and disposed of. The concentrated suspensions were filtered−sterilized through a 0.22 μm cellulose syringe filter or a 0.22 μm MCE membrane vacuum filter (Fisher Scientific).

Bacterial Growth. The Gram-positive, facultative anaerobe *B. subtilis* was chosen as a test organism. *B. subtilis* CB310 (courtesy of Dr. Charles Stewart, Rice University, Houston, TX) was grown as described previously (*32*). The culture was maintained on Luria−Bertani plates but grown in minimal Davis medium (MD) for experimentation. MD is a variation of Davis medium in which the potassium phosphate concentration was reduced by 90% (*49*). The medium consists of 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 (added after autoclaving) in 1 L of Milli-Q water. MD medium was chosen as the antibacterial test medium as previous research shows nC$_{60}$ aggregates precipitate out of suspension in media containing high phosphate concentrations (*32*).

Assessing Antibacterial Activity. The minimal inhibitory concentration (MIC) of each preparation was determined using Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) methodology as described by Tsao et al. (*31*) with a few modifications. Briefly, to calculate the MIC, MD media tubes containing nC$_{60}$ were inoculated with an overnight culture of *B. subtilis* (to a final OD$_{600}$ of 0.002). The concentration of nC$_{60}$ that resulted in no growth was the MIC. Growth was visually assessed by changes in turbidity and quantified by measuring the optical density at 600 nm (OD$_{600}$) using a Turner SP-830 spectrophotometer 135 (Barnstead, Dubuque, IA).

Size Separation of nC$_{60}$ Aggregates. The nC$_{60}$ aggregates in suspension were separated into large and small particle size fractions by differential centrifugation. The suspensions were centrifuged for 1 h at 25 000*g* in a Beckman J2-MC centrifuge (Beckman Coulter, Fullerton, CA). The supernatant was removed with a pipet from the bottles while they were still in the centrifuge to maintain the pellet's integrity. The supernatant was termed the "smaller" fraction. The pellet was resuspended in Milli-Q water to equal the volume of the supernatant, and it was termed the "larger" fraction.

Determining the Size and Concentration of the nC$_{60}$ Suspensions. Particle size range was estimated using a dynamic light scattering (DLS) device (Brookhaven Instrument Corp., Holtsville, NY) and corroborated by transmission electron microscopy (TEM). For the DLS calculation, the mean diameters were weighted according to the number of particles of each size, not by the intensity of the signal. Bright field TEM images were taken on a JEOL-2010 transmission electron microscope (JEOL-USA Inc., Peabody, MA) operating at 100 kV. TEM grids were prepared by evaporating approximately 20 μL of nC$_{60}$ solution onto a Ted Pella 300 mesh grid (Ted Pella Inc., Redding, CA) with removable Formvar and a 5−10 nm thick amorphous carbon film.

The concentration of nC$_{60}$ was determined by extracting the C$_{60}$ from suspension and analyzing it in an Ultrospec 2100*pro* spectrophotometer (Amersham Biosciences, Piscataway, NJ) at 336 nm. The extraction was performed by adding 1 mL of 100 mM magnesium perchlorate and 2 mL of toluene to 2 mL of nC$_{60}$ suspension. The vial was sealed, and the mixture was stirred for 2 h. The vial was then placed in a −20 °C freezer, the aqueous phase allowed to freeze, and the toluene removed for analysis. The absorbance at 336 nm was compared to a standard curve prepared by dissolving varying amounts of C$_{60}$ in toluene.

Both the aq/nC$_{60}$ and the PVP/C$_{60}$ extracted poorly. Their concentrations were determined by measuring their absorbance at 336 nm without extraction. These readings were compared to a standard curve made by correlating the concentrations determined by the extraction method above to the absorbance of the nC$_{60}$ suspension itself.

Statistical Analyses. All experiments were performed at least in triplicate. Error bars for the MIC values reflect the actual range of values observed. Student *t*-test was used to

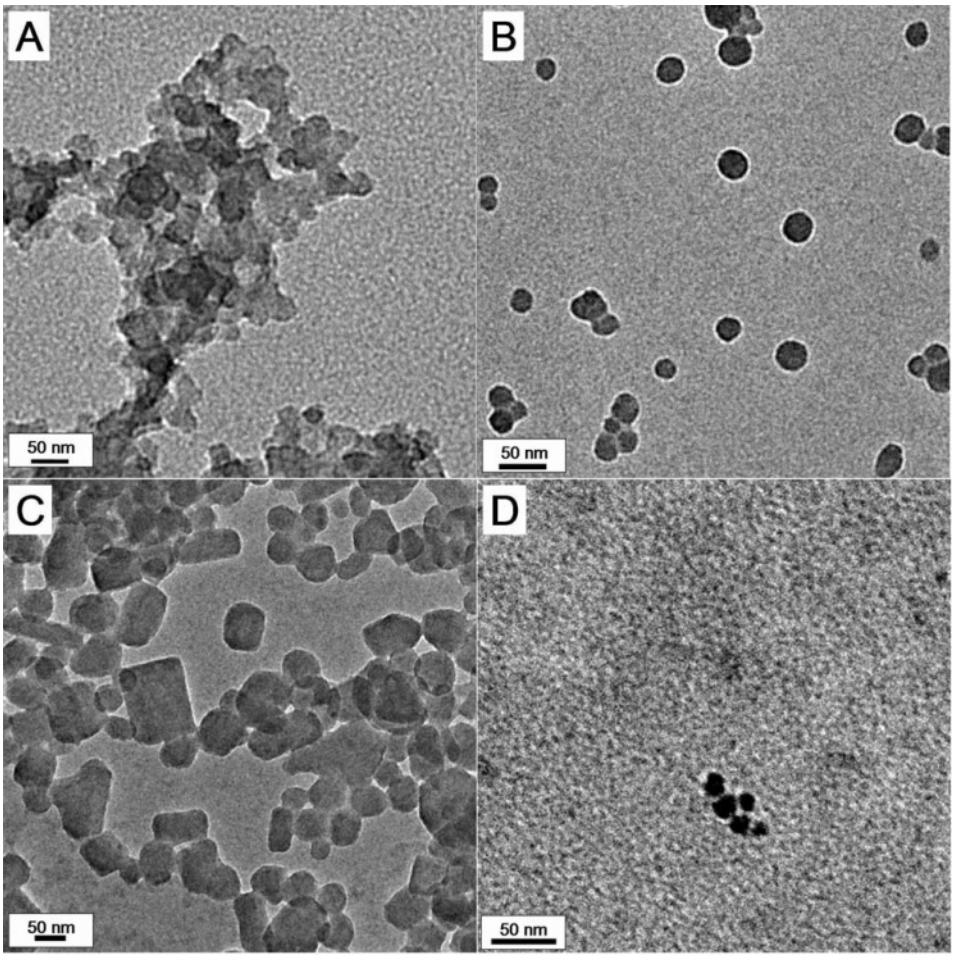

FIGURE 1. TEM micrographs of (A) aq/nC_{60}, (B) son/nC_{60}, (C) THF/nC_{60}, and (D) PVP/nC_{60}.

analyze whether there were any statistical differences in the means of the data at a 95% confidence interval.

Results and Discussion

Characterization of nC_{60}. The TEM images of THF/nC_{60}, aq/nC_{60}, and son/nC_{60} show faceted, high-contrast particles characteristic of crystalline aggregates (Figure 1). The THF/nC_{60} and son/nC_{60} aggregates were similar in size and shape to those published previously (*34*, *40*). The aq/nC_{60} aggregates have more rounded edges than the either THF/nC_{60} or son/nC_{60}, indicating overall less crystallinity. The PVP/C_{60} aggregates are not expected to be crystalline, due to the coating of PVP, but they are dense enough for visualization. In conjunction with the hydrodynamic diameters determined by DLS, the TEM micrographs indicate that these suspensions have a wide size range, with the THF/nC_{60} particles ranging from 50 to 150 nm in diameter and the aq/nC_{60} ranging from 30 to 100 nm. The sizes of the particles are all below 200 nm, since the suspensions were filtered through 0.22 μm filters. The son/nC_{60} and PVP/C_{60} suspensions are more uniformly dispersed than the THF/nC_{60} and aq/nC_{60}. The DLS reports their particle diameters at roughly 2 nm, but the DLS cannot accurately determine the size of particles smaller than 10 nm. The TEM images indicate that there are some particles around 10−25 nm (Figure 1B,D) but that the average particle size is below the DLS quantification limit.

Antibacterial Activity of Four Different Types of C_{60} FWS. The Gram-positive bacterium *B. subtilis* was chosen as a test organism because it is a well-studied soil organism that grows easily in the laboratory under both aerobic and anaerobic conditions. Previous studies indicate that the Gram-negative species *Escherichia coli* behaves similarly to *B. subtilis* after nC_{60} exposure, so it is assumed that *B. subtilis* behavior is a good indicator of both Gram-positive and -negative bacterial responses to nC_{60} (*32*). When grown at 37 °C in a shaking incubator, *B. subtilis* initially has the same growth rate in MD medium as in Davis medium, but it does not reach the same density, due to the reduced buffering capacity of the MD medium (data not shown).

All four FWS displayed antibacterial activity toward *B. subtilis*, as reflected by the MIC data (Table 1, Figure 2). THF/nC_{60} had an MIC of 0.09 ± 0.01 mg/L, son/nC_{60} had an MIC of 0.7 ± 0.3 mg/L, aq/nC_{60} had an MIC of 0.5 ± 0.13 mg/L, and PVP/C_{60} had an MIC of 0.95 ± 0.35 mg/L. Other researchers have shown PVP/C_{60} to suppress the growth of *B. subtilis* at concentrations of about 13 mg/L in nutrient broth, an undefined medium (*50*). These data are within the same range as our results. At a 95% confidence interval, there is no statistical difference between the antibacterial activity of the son/nC_{60}, aq/nC_{60}, and PVP/nC_{60} preparations (Figure 2). THF/nC_{60} appears to have a more potent antibacterial effect than the other preparations, having an MIC 1 order of magnitude smaller. This may be due to variability in the extraction procedure and thus the reported concentration of the suspension.

Some researchers have argued that the solvents involved in producing the various types of nC_{60} are responsible for the observed antibacterial activity (*42*, *48*). There are two pieces of evidence that obviate this concern. First, controls in which the various preparation methods were performed with solvents but without fullerene had either a lower toxicity, as in the case of the sonication preparation method, or they had no observed toxicity, as in the case of the THF and PVP preparation methods (data not shown). This does not mean

TABLE 1. MIC's of the Four Different Preparations and Their Constituent Small and Large Aggregate Fractions[a]

	MIC (mg/L)	mean diameter (nm)	surface area: volume ratio[b]
THF/nC_{60}	0.08−0.10	75.6	0.079
THF/nC_{60} small	0.008−0.010	39.1	0.153
THF/nC_{60} large	0.6−0.8	97.4	0.062
son/nC_{60}	0.4−0.6	~2[c]	3
son/nC_{60} small	0.15−0.20	~2[c]	3
son/nC_{60} large	0.6−0.8	~2[c]	3
aq/nC_{60}	0.4−0.6	74.9	0.080
aq/nC_{60} small	0.1−0.23	~2[c]	3
aq/nC_{60} large	0.75−1.5	142.3	0.042
PVP/C_{60}	0.6−1.0	~2[c]	3

[a] "Large" refers to particles that were pelleted by the differential centrifugation method, and "small" refers to the supernatant. [b] Surface area and volume were calculated by assuming the mean diameter given is a good reflection of the population and that the aggregates are spherical. [c] Theoretical detection limits of the DLS are from 10 nm to 1 μm.

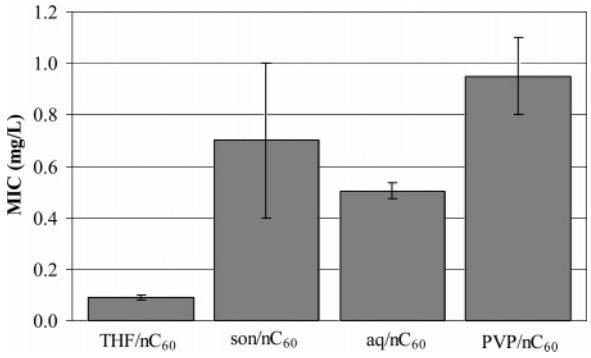

FIGURE 2. Minimal inhibitory concentrations (MIC's) of the various FWS preparations. Error bars represent the range of the measured MIC values ($n = 5$).

that those solvents are not toxic to bacteria, but rather that they were not toxic at the concentrations found in these C_{60} FWS preparations. The second piece of evidence is that the aq/nC_{60} displayed antibacterial activity at 0.5 mg/L, which is similar to the MIC's of the other FWS prepared with solvents (Table 1, Figure 2). The aq/nC_{60} preparation involved no solvent or solubilizing agent and would best mimic a spill or disposal scenario in the environment.

nC_{60} Aggregate Size and Morphology Affects Antibacterial Activity. Differential centrifugation was used to separate larger and smaller aggregates in each suspension, and the two fractions were tested for antibacterial activity as reflected by their MIC (Table 1). Only the polydisperse suspensions, e.g., THF/nC_{60} and the aq/nC_{60}, were successfully separated by size. Their mean diameters were determined by DLS and corroborated by TEM (Table 1, Figure 3). Unlike the THF/nC_{60} and aq/nC_{60}, the son/nC_{60} particles were below the DLS size detection capabilities and therefore particle diameters could not be determined by this method. TEM images confirm that the two fractions of son/nC_{60} were not noticeably different (Figure 3C,D). The PVP/C_{60} suspension was monodisperse and was therefore excluded from these experiments. In certain images, there appears to be a matrix that encapsulates the particles (Figure 3C). It is unknown whether the matrix is an artifact of drying or if it is part of the FWS.

In a survey of different THF/nC_{60} batches, suspensions with smaller aggregates tended to have a higher level of antibacterial activity than those with larger aggregates (Figure 4). The MIC increases as the mean diameter increases. In one batch, the "small" THF/nC_{60} MIC was about 80 times lower than the "large" THF/nC_{60} MIC (Table 1). This increase in antibacterial activity was disproportionate to the increase in putative surface area, which was only about 2.5 times higher. The increase in MIC for the "small" versus "large" aq/nC_{60} was 6.8-fold, while the increase in surface area: volume ratio was approximately 70-fold. Even though the DLS was unable to detect a change in particle size between the "small" and "large" fractions of the son/nC_{60}, there was a 4-fold increase in antibacterial activity for the "small" fraction. There is a lack of a linear relationship between toxicity and particle size (Figure 5), indicating that other factors besides an increase in surface area are likely responsible for the higher antibacterial activity of the smaller aggregates. Furthermore, differently prepared suspensions containing aggregates of the same size did not necessarily have the same MIC (Table 1), possibly due to differences in the production methods (e.g., reagents, temperature, rate of addition of water, rate of stirring) or discrepancies in the determination of the concentration of the suspension.

While all the suspensions possess antibacterial activity, their morphologies are different, suggesting that there are influential differences in surface chemistry and/or morphology. The separated nC_{60} suspensions contain both crystalline and amorphous aggregates (Figure 3). Following centrifugation, the aggregates in the "smaller" size fraction had a higher level of amorphous aggregates than the "larger" size fraction, as evident in the TEM images. The small aggregates inherently appear amorphous, due to a lack of repeating structure that is needed to establish a defined crystal. Aggregates with diameters of 2−3 nm have been calculated to contain only 4−13 molecules of C_{60} (*41*). It is unclear whether the greater antibacterial activity of the "smaller" size fraction is attributed directly to the smaller size itself or with the presence of an amorphous structure.

Alternative Factors Affecting Antibacterial Activity. Previous studies have shown that THF/nC_{60} attaches to bacterial and human cells (*32*, *45*), but it is not clear whether contact is necessary for antibacterial activity. It has been proposed that nC_{60} aggregates have an outer layer of hydrated or hydroxylated C_{60} molecules (*34*, *41*). It has also been previously demonstrated that hydroxylated C_{60} ($C_{60}(OH)_{22-24}$) does not display antibacterial activity at solubility limits (*32*). If the nC_{60} clusters are composed of an outer shell of nonbactericidal particles, this is an indication that direct interaction of the nC_{60} surface with the bacterial cell is not involved in the antibacterial mechanism. Indeed, PVP/C_{60} has an outer coating of PVP molecules (which themselves did not exhibit antibacterial properties, data not shown) that shield the cell from the C_{60} molecule. Yet, PVP/C_{60} has antibacterial properties, which highlights that direct contact may not be necessary for the antibacterial mechanism.

While the mechanisms behind the antibacterial activity have not been determined, reactive oxygen species (ROS) are believed to be responsible for eukaryotic cell membrane disruption and eukaryotic lipid peroxidation (*43*−*45*). It is unclear whether ROS are produced by nanomaterials themselves or by the eukaryotic cells immune response to nanomaterials. ROS have been implicated in the antibacterial mechanism of PVP/C_{60} (*50*), but they have not been confirmed as the main factors responsible for the bactericidal activity of any of the FWS. In a previous study of nC_{60} with bacteria, toxicity was not affected by the presence or absence of light that is needed to stimulate ROS production by nC_{60} (*32*). nC_{60} was also able to kill bacteria under anaerobic and fermentative conditions (results not shown) where O_2, a critical ROS precursor, was absent. All these lines of evidence indicate that photocatalyzed ROS production is probably not the sole antibacterial mechanism associated with nC_{60}. Thus, further research on the relationship between nC_{60} surface chemistry, morphology, and reactivity is warranted to

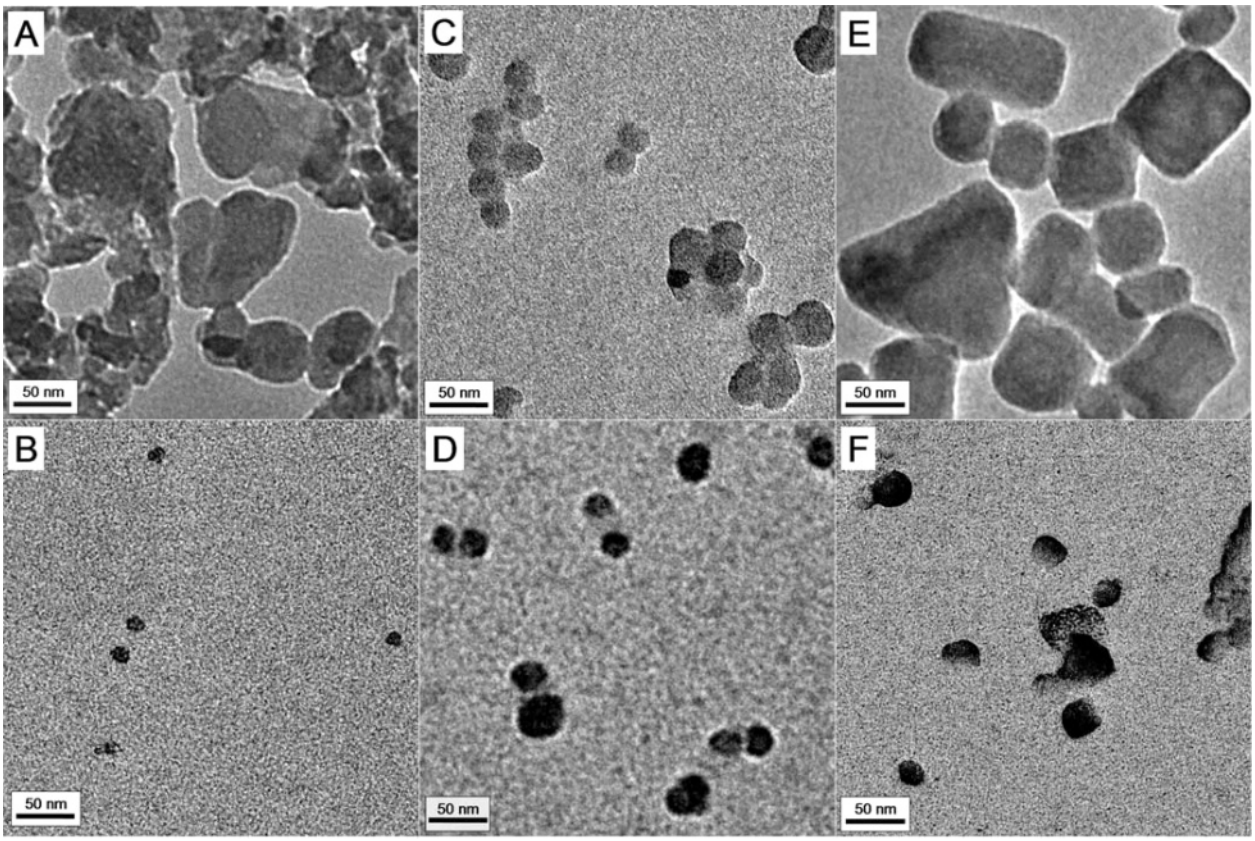

FIGURE 3. TEM micrographs of size-separated nC_{60}: (A) large aq/nC_{60}, (B) small aq/nC_{60}, (C) large son/nC_{60}, (D) small son/nC_{60}, (E) large THF/nC_{60}, and (F) small THF/nC_{60}

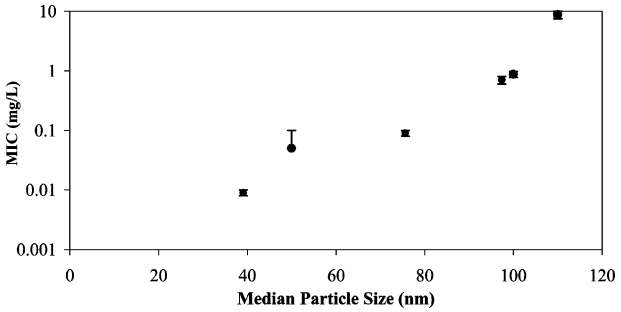

FIGURE 4. Size-separated THF/nC_{60} suspensions show that increasing aggregate size is associated with decreasing antibacterial activity. For each suspension of "small", "large", and noncentrifuged nC_{60}, the median particle size, as determined by DLS, is compared to the MIC of that suspension.

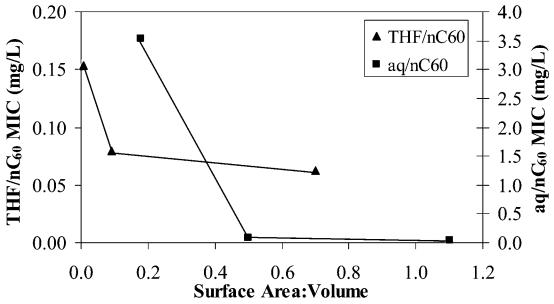

FIGURE 5. Relationship between MIC and aggregate surface area. There is no linear relationship between the mean MIC and the surface area to volume ratio calculated, indicating that the difference in surface area alone does not account for the difference in MIC between the small and large aggregates.

elucidate toxicity mechanisms and identify manufacturing and derivatization processes that decrease toxicity.

Environmental Relevance of nC_{60}. C_{60} powder has not been shown to have detrimental environmental or health effects (*32*−*34*). In a study using the same methods as this paper, C_{60} powder did not display antibacterial activity against either *B. subtilis* or *E. coli* (*32*). However, disposal or a spill of C_{60} as a powder or in a solvent could result in nC_{60} formation, and the full repercussions are as yet unclear. A comparison of the MIC's for *B. subtilis* presented in this paper with the MIC's for the antibiotic vancomycin shows that vancomycin has less antibacterial activity than nC_{60} (*51*). This indicates that nC_{60} is a potent antibacterial agent that warrants further investigation for both its implications in environmental health and for its application as an antibiotic or disinfectant.

This study addressed the antibacterial activity of four different FWS with a pure culture of bacteria in MD, a low salt medium. The ionic strength of MD is about 0.04 M, which is within the 0.01−0.1 M typical range for freshwater (*52*). Results for one laboratory strain in MD may not give an accurate reflection of the behavior of nC_{60} in aquatic environments with mixed cultures and higher ionic strength, where coagulation of the nC_{60} aggregates could occur (*40*, *42*, *53*). This coagulation results in the aggregates precipitating out of suspension and the loss of the antibacterial activity (*32*). Microbial activities are important to the health of all known ecosystems, and the observed bactericidal effects of nC_{60} suggest the need for caution against accidental releases and disposal of fullerenes.

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