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Charge-Associated Effects of Fullerene Derivatives on Microbial Structural Integrity and Central Metabolism

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ABSTRACT

The effects of four types of fullerene compounds (C_{60}, C_{60}–OH, C_{60}–COOH, C_{60}–NH_2) were examined on two model microorganisms (*Escherichia coli* W3110 and *Shewanella oneidensis* MR-1). Positively charged C_{60}–NH_2 at concentrations as low as 10 mg/L inhibited growth and reduced substrate uptake for both microorganisms. Scanning electron microscopy (SEM) revealed damage to cellular structures. Neutrally charged C_{60} and C_{60}–OH had mild negative effects on *S. oneidensis* MR-1, whereas the negatively charged C_{60}–COOH did not affect either microorganism's growth. The effect of fullerene compounds on global metabolism was further investigated using [3-^{13}C]L-lactate isotopic labeling, which tracks perturbations to metabolic reaction rates in bacteria by examining the change in the isotopic labeling pattern in the resulting metabolites (often amino acids).[1−3] The ^{13}C isotopomer analysis from all fullerene-exposed cultures revealed no significant differences in isotopomer distributions from unstressed cells. This result indicates that microbial central metabolism is robust to environmental stress inflicted by fullerene nanoparticles. In addition, although C_{60}–NH_2 compounds caused mechanical stress on the cell wall or membrane, both *S. oneidensis* MR-1 and *E. coli* W3110 can efficiently alleviate such stress by cell aggregation and precipitation of the toxic nanoparticles. The results presented here favor the hypothesis that fullerenes cause more membrane stress[4−6] than perturbation to energy metabolism.[7]

Introduction. Nanotechnology is being applied to a diverse array of products, including cosmetics, printer toners, clothing, electronics, and even drug delivery vehicles. With this explosion in applications, it is important to address concerns, either legitimate or exaggerated, about the potential toxic effects of nanoparticles both in the environment and for medical applications (for review see Colvin[8] and Nel[9]). Carbon nanomaterials,[10−12] such as fullerenes and nanotubes, have been the most extensively used nanoparticles due to their unique and superior physical and chemical properties, including large surface areas, high electrical conductivity, and excellent mechanical strength. Fullerenes, also called C_{60} or buckyballs, and other fullerene derivatives, are the most well studied and most commonly used carbon nanomaterials. Recently, fullerenes were investigated as potential microbicides; other potential in vivo applications were explored as well. However, the toxicological definition for fullerene is still quite controversial. Early studies have indicated that a repeating subchronic topical dose of fullerenes on mouse skin for up to 24 weeks is noncarcinogenic.[13] The Ames assay also indicates that the fullerene is not mutagenic and of no toxicological significance.[14] Yet, recently, fullerenes have been suggested to be carcinotoxic[15,16] and genotoxic, although only upon photosensitization.[17,18] In addition, C_{60} derivatives have demonstrated superoxide dismutase mimetic properties; they can also generate free radicals[19,20] and can be photosensitized and mutagenic.[17,18] In contrast, others indicate that fullerenes have avid reactivity with free oxidative radicals, acting as radical scavengers and antioxidants instead.[14,15,21−24]

In general, water-soluble fullerenes are cytotoxic,[25,26] which can be attenuated by surface derivatization.[15] However, a recent report demonstrated the opposite results.[27] Isakovic et al.[28] suggested that the trace amount of THF in the fullerene toxicity studies was responsible for the cytotoxicity. It has also been shown that cationic fullerenes are moderately toxic,[29] and it has been suggested that these fullerenes affect the energy metabolism.[7,30,31] In contrast, anionic fullerenes have been shown to be relatively less toxic by some reports, yet other reports indicate that anionic fullerenes can inhibit

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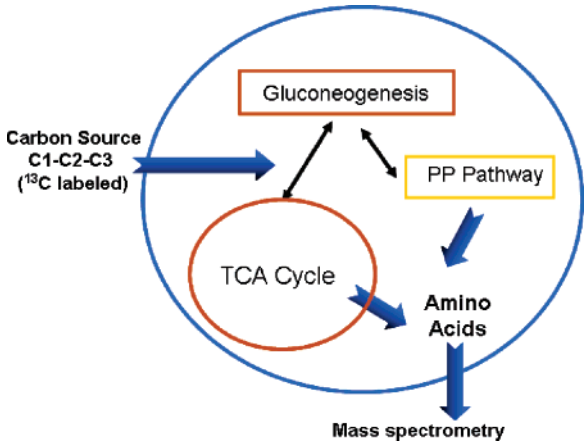

Figure 1. Schematics for the isotopic approach of investigating cellular global metabolism via ^{13}C labeling pattern in amino acids. Labeling patterns of metabolites were used to evaluate the perturbation of central carbon metabolism in this study.

bacterial growth,[4,32] more specifically that anionic fullerene derivatives (carboxyfullerene) affect Gram positive bacteria (such as *Streptococcus pyogenes*) but have no affect on Gram negative bacteria (such as *Escherichia coli*) at concentrations up to 500 mg/L.[4] The strong cytotoxicity of cationic fullerene compounds (e.g., ammonium or other amino acid-derivatized fullerene) has been shown in many microorganisms.[7,33−35] It has also been shown that the presence of light-induced reactive oxygen species (ROS) enhance fullerene antimicrobial activity.[18] The current hypothesized nanotoxicity mechanisms include suppression of energy metabolism (e.g., TCA cycle),[7] oxidative damage to crucial proteins and enzymes, and increased membrane permeability, causing its rupture.[4−6] Since mechanisms of cytotoxicity obtained from animal/human cell models may not be compatible with microbial models, the exact molecular mechanisms for the inhibition of bacterial growth are still not fully understood. Furthermore, the majority of nanoparticle antibacterial experiments were not performed at the molecular and metabolic levels, which are central for bacterial survival and proliferation.

In an attempt to mechanistically study the cellular and biomolecular machineries affected by fullerenes and to determine the effect of nanoparticles on central carbon and energy metabolism, we examined the perturbation of metabolic flux distributions of cells treated with fullerene compounds (Figure 1). A metabolic flux distribution is a map of the rates of metabolic reactions in the cell. When cells are exposed to environmental stresses, changes in gene regulation and protein function may lead to altered activities of cellular enzymes and thus metabolic flux distribution. Using tracer experiments, such global metabolic responses (comprising hundreds of enzymatic reactions) can be determined by changes in the labeling pattern of cellular metabolites.[1,2] In this study, we fed bacteria [^{13}C]lactate and measured the isotopomer distribution in amino acids from fullerene-exposed cell populations to give insight into whether the toxicity of the fullerrene affects an organism's entire metabolome (change of the metabolic pathway or fluxes). Two Gram negative bacteria were used as model organisms to study the nanotoxicity of the fullerene compounds: *E. coli* W3110, a human related enterobacterium and *Shewanella oneidensis* MR-1, an environmentally important bacterium with versatile metabolism.[36]

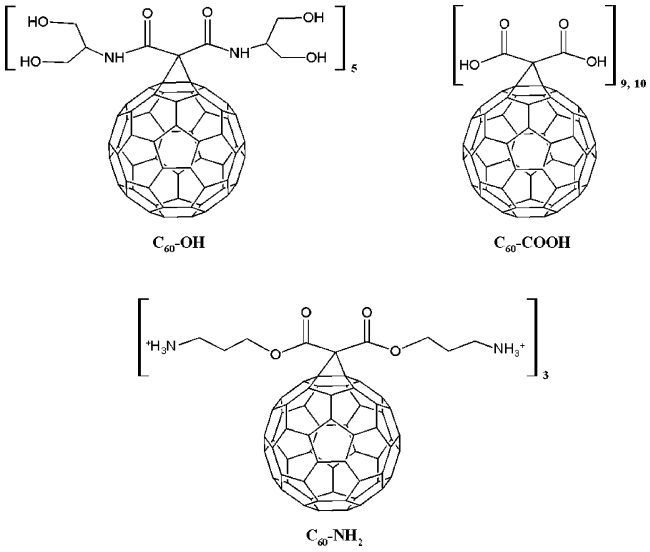

Figure 2. C_{60} fullerene derivatives used in this study. The fullerene−COOH derivative carries negative charges in solution, the fullerene−NH_3^+ derivative is positively charged. The C_{60}-serinol (−OH) is neutral.

Materials and Methods. (1) Nanoparticle Preparation. Sublimed C60 fullerene (purity 99.95%+) was purchased from MER. The carbon multiwall carbon nano-onions (MWCNOs) used in this study were produced using a modified direct-current electric-arc discharge method.[37,38] The multiadduct C_{60} serinol (C_{60}−OH),[39,40] carboxylic acid (C_{60}−COOH),[41] and amine (C_{60}−NH_2)[42] derivatives were synthesized using Bingel chemistry.[43] In brief, C_{60} derivatives were prepared by dissolving 50 mg (0.069 mmol) of C_{60} in 250 mL of anhydrous toluene. To this solution, the malonate derivative, CBr_4, and DBU were added sequentially. The solution was stirred at room temperature overnight to help ensure the desired degree of functionalization. Toluene was removed under reduced pressure to give the crude products, which were purified by column chromatography. After solvent removal, the purified solids were dried overnight. This gave the desired C_{60} derivatives. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to verify the products. The protecting groups of the C_{60} derivatives were then removed using established deprotection procedures to give the water-soluble fullerene compounds (Figure 2).

(2) Tracer Experiments. *Shewanella oneidensis* MR-1 and *Escherichia coli* W3110 were cultured in minimal medium.[44] The medium was supplied with 30 mM (98% [3-^{13}C]L-lactate) (Cambridge Isotope, USA) as the sole carbon source to support cell growth. The inoculum was prepared in LB medium, which was grown overnight. This culture was inoculated into the minimal medium with a 0.09% inoculation volume. Both *S. oneidensis* and *E. coli* were cultured in shake flasks at 30 °C (at 200 rpm in dark). The fullerene compounds (C_{60}, C_{60}−OH, C_{60}−COOH, and C60-NH_2) were added to the medium to different final

concentrations (from 1 to 80 mg/L) before the exponential phase (OD_{600} = 0.09−0.10). The culture's optical density at a wavelength of 600 nm (OD_{600}) was measured in a spectrophotometer (DU640, Beckman Instruments, Palo Alto, CA) every 2−3 h. The carbon source (lactate) concentrations at each time point were determined using an enzyme test kit (r-Biopharm Inc., Darmstadt, Germany).

(3) Isotopomer Analysis. Isotopomer analysis was performed as described previously.[45,46] Briefly, 10 mL of culture in the exponential phase (OD_{600} = 0.6−0.8) was harvested and centrifuged at 10000*g*. The cell pellets were suspend in 1 mL of sterile nanopure water and sonicated for 3 min with a 3 s on/1 s off cycle. The protein from the resulting lysate was precipitated using trichloroacetic acid. The protein pellet was washed with cold acetone two times and then hydrolyzed in 6 M HCl at 100 °C for 24 h. Gas chromatography−mass spectrometry (GC−MS) samples were prepared in 100 μL of tetrahydrofuran (THF) and 100 μL of *N*-(*tert*-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide (Sigma-Aldrich, USA). All samples were derivatized in a water bath at 65−80 °C for 1 h, producing *tert*-butyldimethlysilyl (TBDMS) derivatives. One microliter of the derivatized sample was injected into GC−MS: a gas chromatograph (Agilent, model HP6890) equipped with a DB5-MS column (J&W Scientific, Falsom CA) and analyzed using a mass spectrometer (Agilent, model 5973, Wilmington, DE). The GC operation conditions are as follows: the GC column was held at 150 °C for 2 min, heated at 3 °C/min to 280 °C, heated at 20 °C/min to 300 °C, and held for 5 min at that temperature.[47] Unfragmented molecules, $[M - 57]^+$ for all amino acids were clearly observed by MS in this study. M is the total molecular mass of the derivatized hydrolysate component, and 57 indicates the loss of 57 mass units, e.g., a *tert*-butyl group. The natural abundance of isotopes, including ^{13}C (1.13%), ^{18}O (0.20%), ^{29}Si (4.70%), and ^{30}Si (3.09%) (Si occurs in amino acids derivatized for gas chromatography separation), change the mass isotopomer spectrum and were corrected using a published algorithm to get final GC−MS data.[48] Fourteen amino acids were used in this study (tryptophan, proline, isoleucine, arginine, glutamine, and asparagine mass peaks were not used due to their degradation during hydrolysis or due to the overlay of their main MS peaks with other MS peaks).

(4) Light Microscopy and Scanning Electron Microscopy (SEM) Pictures. *S. oneidensis* and *E. coli* cultures in the late exponential growth phase (OD = 0.7−0.9) exposed to nanoparticles were collected and directly observed under light microscopy (LEICA DM4000B). Meanwhile, SEM imaging was carried out as follows:[49] Samples were fixed with 2% glutaradehyde in 0.1 M sodium cacodylate buffer at pH 7.2 for an hour. Fixed samples were rinsed in buffer and then immersed in secondary fixative (1% aqueous osmium tetroxide in buffer) for 30 min. Dehydration of sample was carried out through increasing concentrations of ethanol, further dried in critical point drier, and coated with 1.2 nm of iradium in a MED-020 sputter coater. Images were collected with a Hitachi S-5000 field emission scanning electron microscope, operated at 10 kV.

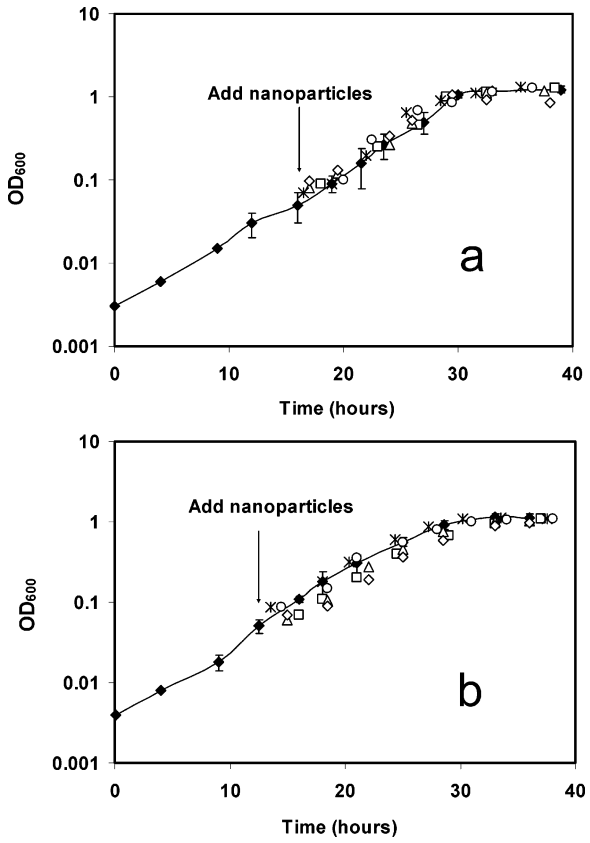

Figure 3. The effect of C_{60}, C_{60}−OH, and C_{60}−COOH fullerene compounds (neutral or anionic charge) on *E. coli* W3110 (a) and *S. oneidensis* MR-1 (b) growth: ◆, control, 0 mg/L; □, C_{60}, 20 mg/L; △, C_{60}−OH, 20 mg/L; ◇, C_{60}−OH, 80 mg/L; ∗, C_{60}−COOH, 20 mg/L; ○, C_{60}−COOH, 80 mg/L.

Results and Discussion. This study used two model microorganisms *E. coli* W3110 and *Shewanella oneidensis* MR-1. *E.coli* is a human enterobacterium, which is also an important industrial microorganism, whose phenotype is well-known. *E.coli* is the most widely used model bacterium in research with its metabolic pathways best understood. *Shewanella oneidensis* MR-1 is one of the most environmentally important bacteria with versatile metabolism mechanisms, allowing it to survive in a natural environment. *Shewanella oneidensis* is useful for bioremediation of many toxic compounds (such as chromium). Study of this strain will give us insights on how the nanoparticles pose the potential negative effect in the environment and microbial ecosystem and how the environmental microorganism responds to the nanoparticle stresses.

We used minimal medium containing [3-^{13}C]L-lactate to grow both organisms and test the impact of fullerenes (concentrations range from 0 to 80 mg/L) on their growth. Since C_{60} is not soluble in water and cannot be uniformly suspended in the culture solution, we only applied concentrations less than 20 mg/L in the studies of nonderivatized C_{60}. Our results indicated that the addition of anionic fullerene (C_{60}−COOH) and neutrally charged fullerene (C_{60} and C_{60}−OH) did not significantly inhibit (concentrations up to 80 mg/L) *E. coli* and *S. oneidensis* growth (Figure 3). This evidence is consistent with previous conclusions that fullerene

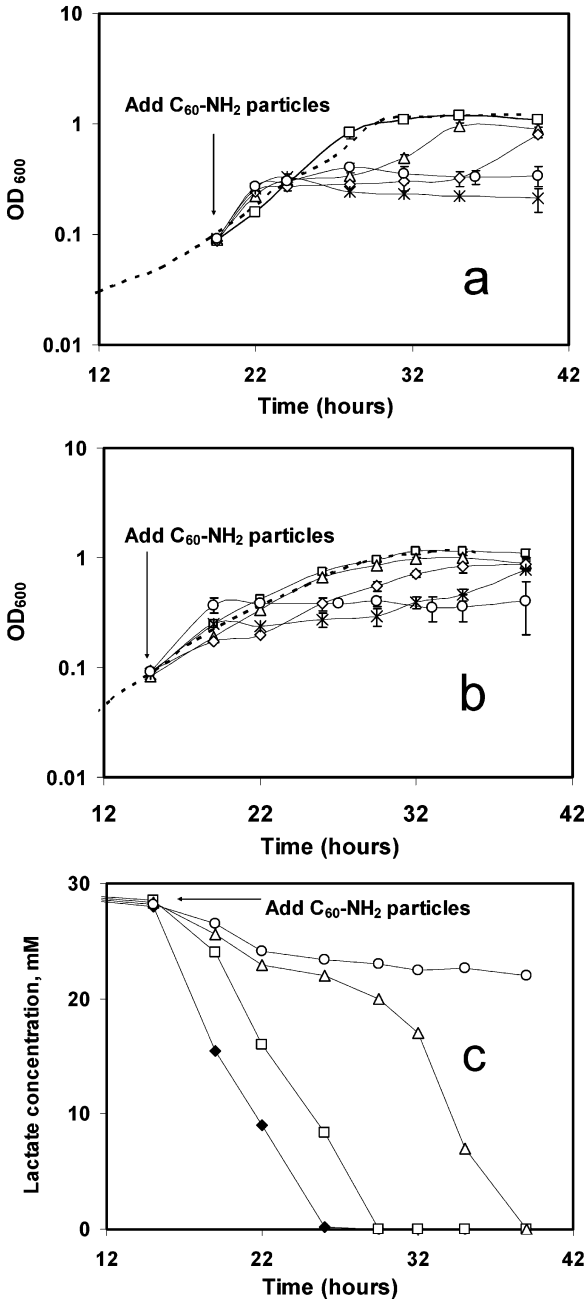

Figure 4. $C_{60}-NH_2$ fullerene (cationic charge) affected *E. coli* W3110 growth (a), *S. oneidensis* MR-1 growth (b), and *S. oneidensis* MR-1 lactate uptake (c). (a) and (b) —, 0 mg/L; □, 1 mg/L; Δ, 10 mg/L; ◇, 20 mg/L; ∗, 40 mg/L; ○, 80 mg/L. (c) ◆, 0 mg/L; □, 20 mg/L; △, 40 mg/L; ○, 80 mg/L.

and anionic fullerene compounds do not affect Gram-negative bacteria.[4,50] At high concentrations (>40 mg/L), $C_{60}-OH$ only mildly slowed *S. oneidensis* growth (not for *E. coli*) by reducing the growth rate 10−20%. On the other hand, the positively charged $C_{60}-NH_2$ strongly inhibited the growth of both microorganisms at concentrations as low as 10 mg/L. The cell growth was completely inhibited at high concentration (80 mg/L). It is interesting that at various C_{60} concentrations (20 and 40 mg/L), *E. coli* and *S. oneidensis* growth slowed after addition of nanoparticles and then resumed their normal growth rate after certain period of lag (several hours) (Figure 4a,b). For the slow growth under

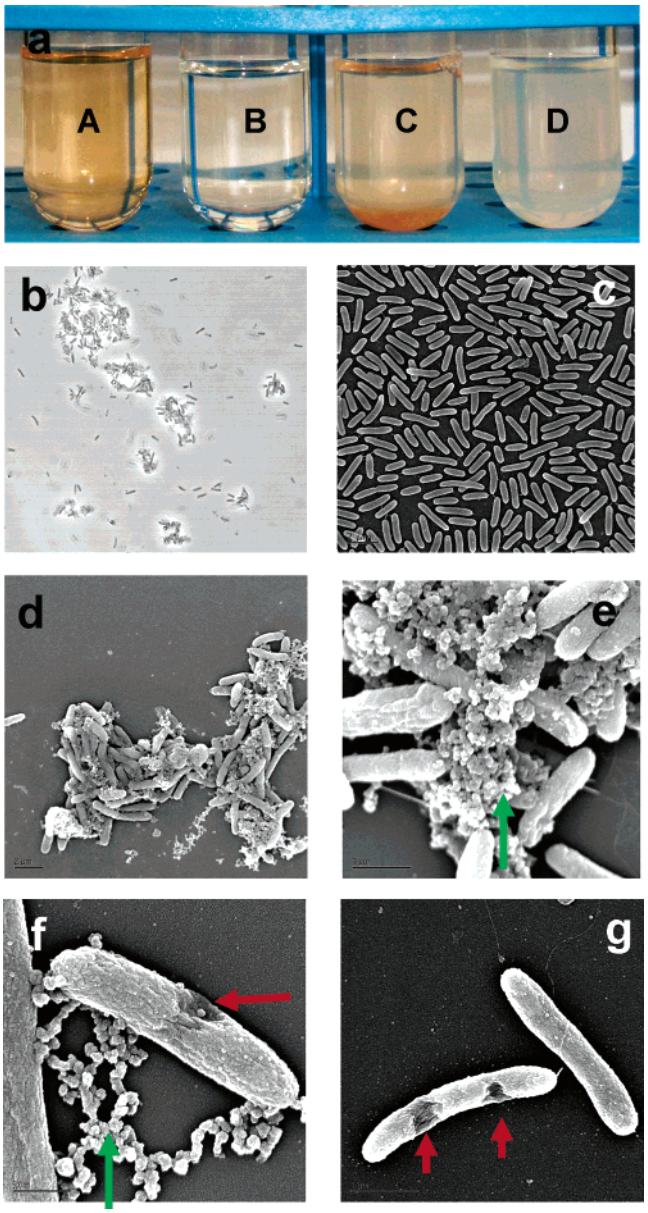

Figure 5. Images of *S. oneidensis* MR-1 exposed to $C_{60}-NH_2$. Micrographs were taken 40 min after addition of 10 mg/L $C_{60}-NH_2$ to log phase cell cultures. Cell samples were fixed for SEM images approximately 1 h after exposed to 20 mg/L $C_{60}-NH_2$. (a) Precipitation of *S. oneidensis* MR-1 with nanoparticles (A, no cells, $C_{60}-NH_2$; B, no cells, no nanoparticles; C, *S. oneidensis* MR-1 cells at OD_{600} 0.26, $C_{60}-NH_2$ added; D, *S. oneidensis* MR-1 at OD_{600} 0.26, no nanoparticles). (b) Light microscopy showed cell aggregation. (c) SEM of *S. oneidensis* MR-1 (no effect from NPs). (d) SEM of *S. oneidensis* MR-1 aggregation in the presence of $C_{60}-NH_2$. (e) SEM of *S. oneidensis* MR-1 in the presence of $C_{60}-NH_2$ (green arrow points to nanoparticles). (f) SEM of *S. oneidensis* MR-1 in the presence of $C_{60}-NH_2$ aggregation (red arrow points to the damaged part of the cell). (g) SEM of individual *S. oneidensis* MR-1 cells (red arrow points to the damaged part of the cell).

$C_{60}-NH_2$ stress, slower lactate consumption rates were also observed (Figure 4c).

In order to further understand the cells' response to nanoparticle stress, changes in bacterial morphology were examined with light microscopy and SEM. Both *E. coli* and *S. oneidensis* aggregated after addition of $C_{60}-NH_2$; no

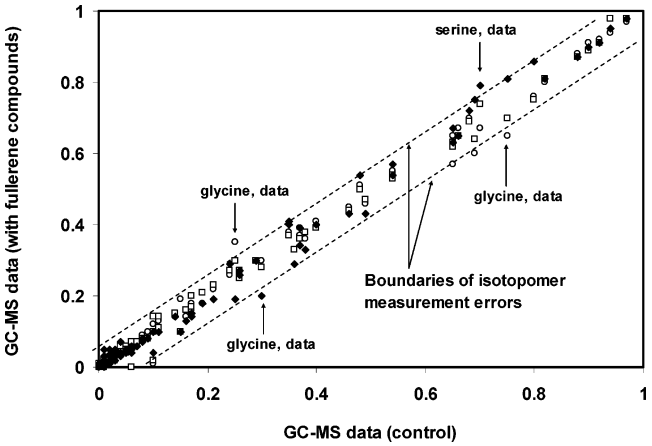

Figure 6. Isotopomer distribution in proteogenic amino acids of *S. oneidensis* MR-1 cultured in [3-^{13}C]L-lactate medium. The GC−MS data include 14 amino acids ($(M-57)^+$ and $(M-159)^+$ mass values) for all three nanoparticle-stressed experiments (○, 80 mg/L C_{60}−OH; □, 80 mg/L C_{60}−COOH; ◆, 20 mg/L C_{60}−NH_2), which indicates the central metabolism pathways for producing small molecules are not altered by the presence of nanoparticles. The dotted lines represent absolute variances (±0.05) of measured GC−MS data based on batch cultures.

aggregation was observed in the presence of other fullerene compounds. For example, uniformly distributed *S. oneidensis* MR-1 started to aggregate right after addition of C_{60}−NH_2 at concentrations as low as 10 mg/L (Figure 5a,b). It has been reported previously that aggregation of *S. oneidensis* MR-1 was associated with oxidative stress and aggregation could be a protection mechanism.[44] Moreover, for the cells that aggregated upon addition of nanoparticles, some cells showed substantial damage (Figure 5c−g). The formation of cell debris and the large amount of cell/nanoparticle aggregates resulted in a sudden increase in the turbidity of the culture (measured by OD_{600}), right after addition of a high concentration of C_{60}−NH_2 (Figure 4a,b). This evidence supports previous observation that fullerene compounds (carboxyl-fullerene) intercalate into the cell wall and cell membrane in some Gram-positive bacteria.[4,50] The fullerene compounds might have caused the destruction of membrane integrity in bacteria. On the other hand, bacteria may produce certain membrane proteins to strengthen its membrane structure in response to nanoparticle stress to reduce its membrane's permeability.[7,51] We also observed that bacteria (especially *S. oneidensis* MR-1) can efficiently remove soluble C_{60}−NH_2 at high concentration by adsorbing and precipitating them from the supernatant of the culture (Figure 5a,e). After that, a normal growth rate and substrate uptake rate were resumed (Figure 4b,c). The fact that *S. oneidensis* MR-1 efficiently precipitates and absorbs the nanoparticles makes this microorganism a potential bioremediation candidate for soluble cationic nanoparticle pollution.

In previous studies with human cell lines, nanoparticles were found to inhibit enzyme activity by interacting with the hydrophobic cavity of certain enzymes and inducing oxidative stress and thus have been investigated as therapeutic agents against enteric pathogens.[5,33] These redox-active nanoparticles may interact with a variety of essential enzymes related to energy and biosynthesis pathways, such as cytochrome P450s,[34] cysteine and serine proteases, etc.[5,33] However, the influence of nanoparticles on microbial central metabolism is poorly understood. To better understand the metabolic fluxes under nanoparticle influence, we administered [3-^{13}C]lactate as the sole carbon source to *S. oneidensis* in order to monitor its growth under the effect of the nanoparticles. The fraction of ^{13}C label in the resulting 14 amino acids was analyzed. These amino acids were synthesized from the precursors in the central metabolic pathways

Table 1. Nanoparticle Effect on the Microbes

references	nanoparticles	bacteria types	conclusions
this paper	C_{60}, C_{60}−OH, C_{60}−COOH, and C_{60}−NH_2 (0−80 mg/L)	*E. coli* K12 and *Shewanella oneidensis* MR-1	only C_{60}−NH_2 had an acute effect
our unpublished data	gold (30 nm) (0−80 mg/L), carbon onion, 10 mg/L	*E. coli* K12 and *S. oneidensis* MR-1	no effect on both bacteria
Chiron et al.[50]	fullerene (~43 mg/L)	22 collection strains including *E. coli, B. subtilis,* etc.	no effect
Tegos et al.[18]	six functionalized C(60) with hydrophilic or cationic groups (10−100 μM)	Gram-positive bacteria, Gram-negative bacteria, and fungi	in combination with white light, cationic fullerenes were highly active in killing all tested microbes
Tsao et al.[4]	the trimalonic acid derivative of fullerene (50 mg/L)	20 strains, Gram negative or positive, including *E. coli* and *Streptococcus pyogenes*	damage to the cell membrane in Gram-positive, but not Gram-negative, bacteria was observed
Fontner et al.[25]	underivatized C_{60} and C_{60}−OH (up to 5 mg/L)	*E. coli* and *B. subtilis*	underivatized C_{60} at relatively low concentrations is inhibitory to prokaryotic microorganisms, C_{60}−OH did not affect the growth
Mashino et al.[30]	alkylated C(60)−bis(*N*,*N*-dimethylpyrrolidinium iodide) derivatives (up to 100 mg/L)	Gram-positive bacteria (*S. aureus*, *E. hirae*, *E. faecalis*)	the fullerene derivatives inhibited tested bacteria growth effectively

including the TCA cycle, pentose phosphate pathway, and glycolysis. The variance of ^{13}C-labeled isotopomers for batch cultures was expected to have <10% measurement error, and data for most amino acids in bacterial biomass (both *E. coli* and *S. oneidensis*) cultured in presence of different nanoparticles showed that there were no significant differences in the isotopomer distribution over the measurement noise compared to the control experiments, even for the cultures treated with high concentrations of $C_{60}-NH_2$ (Figure 6). Only isotopomer data for glycine and serine (related to C1 metabolism)[52] were slightly affected (if at all) by $C_{60}-NH_2$ and $C_{60}-OH$ nanoparticles. The fact that nanoparticle-stressed cells showed no significant differences in the isotopomer distributions of key metabolites indicates that most enzymes and proteins involved in central carbon metabolism and the amino acid biosynthesis pathway were not seriously perturbed. These observations are not surprising given the robustness of bacterial central metabolism.[3,54] Such stability of central metabolism against environmental stress is an important characteristic for bacterial survival under hazardous environmental conditions.

In addition, we also tried other nanoparticles including gold particles (30 nm) and carbon onions (onion-like fullerenes) (data not shown) for their effect on both *E. coli* and *S. oneidensis* growth; there was no effect on the growth of these bacteria (Table 1). The reported assessments of fullerene toxicity conflict with those in the literature. In general, nonderivatized, anionic and neutral fullerenes have the least impact on bacteria (especially Gram-negative bacteria), whereas cationic fullerene compounds have high antimicrobial activity. This can be explained by the negative charges on the bacterial surface that are responsible for the strong adsorption of positively charged nanoparticles and the enhanced interaction of the nanoparticles with the cell surface. Unlike animal cells, many bacteria are generally more resistant to nanoparticles. This may be due to the robust bacterial cell wall, which might provide extra protection against nanoparticles, and robust central metabolism.

This study is one of the first to explore the microbial metabolic perturbation effected by nanoparticles. Although the central metabolism in the bacteria studied here does not seem to be strongly influenced by fullerene nanoparticles, the growth inhibition was obvious for the positively charged fullerenes. On the other hand, the SEM morphological results here favor the membrane stress hypothesis.[4−6] The association of positively charged fullerenes with the negatively charged membranes is more efficient than that of neutral and negatively charged fullerenes. These data support the charge-associated effects. Plausible explanations for the membrane damage are that the fullerenes induce redox damage on the membrane, or the fullerenes mechanically damage the lipid bilayers in the cell membrane. It seems less likely that the energy metabolism perturbation[7] is the major cause for the microbial growth inhibition observed here.

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References

(1) Stephanopoulos, G.; Aristidou, A.; Nielsen, J. *Metabolic Engineering Principles and Methodologies*; Academic Press: San Diego, 1998; pp 75, 120−130.
(2) Szyperski, T. *Q. Rev. Biophys.* **1998**, *31* (1), 41−88.
(3) Tang, Y. J.; Hwang, J. S.; Wemmer, D.; Keasling, J. D. *Appl. Environ. Microbiol.* **2007**, *73* (3), 718−729.
(4) Tsao, N.; Luh, T. Y.; Chou, C. K.; Chang, T. Y.; Wu, J. J.; Liu, C. C.; Lei, H. Y. *J. Antimicrob. Chemother.* **2002**, *49* (4), 641−649.
(5) Jensen, A.; Wilson, S.; Schuster, D. *Bioorg. Med. Chem. Lett.* **1996**, *9* (20), 2959−2962.
(6) Sayes, C.; Gobin, A.; Ausman, K.; Mendez, J.; West, J.; Colvin, V. *Biomaterials* **2005**, *26* (36), 7587−7595.
(7) Mashino, T.; Okuda, K.; Hirota, T.; Hirobe, M.; Nagano, T.; Mochizuki, M. *Bioorg. Med. Chem. Lett.* **1999**, *9* (20), 2959−2962.
(8) Colvin, V. L. *Nat. Biotechnol.* **2003**, *21* (10), 1166−1170.
(9) Nel, A.; Xia, T.; Madler, L.; Li, N. *Science* **2006**, *311* (5761), 622−627.
(10) Curl, R. F.; R.E., S.; Kroto, H. W.; O'Brien, S.; Heath, J. R. *J. Mol. Graphics Modell.* **2001**, *19* (2), 185−186.
(11) Iijima, S. *Nature* **1991**, *354*, 56−58.
(12) Iijima, S.; Ichihashi, T. *Nature* **1993**, *363*, 603−605.
(13) Nelson, M. A.; Domann, F. E.; Bowden, G. T.; Hooser, S. B.; Fernando, Q.; Carter, D. E. *Toxicol. Ind. Health* **1993**, *9* (4), 623−630.
(14) Mori, T.; Takada, H.; Ito, S.; Matsubayashi, K.; Miwa, N.; Sawaguchi, T. *Toxicology* **2006**, *225* (1), 48−54.
(15) Sayes, C. M.; Fortner, J. D.; Guo, W.; Lyon, D.; Boyd, A. M.; Ausman, K. D.; Tao, Y. J.; Sitharaman, B.; Wilson, L. J.; Hughes, J. B.; West, J. L.; Colvin, V. L. *Nano Lett.* **2004**, *4* (10), 1881−1887.
(16) Burlaka, A. P.; Sidorik, Y. P.; Prylutska, S. V.; Matyshevska, O. P.; Golub, O. A.; Prylutskyy, Y. I.; Scharff, P. *Exp. Oncol.* **2004**, *26* (4), 326−327.
(17) Sera, N.; Tokiwa, H.; Miyata, N. *Carcinogenesis* **1996**, *17* (10), 2163−2169.
(18) Tegos, G. P.; Demidova, T. N.; Arcila-Lopez, D.; Lee, H.; Wharton, T.; Gali, H.; Hamblin, M. R. *Chem. Biol.* **2005**, *12* (10), 1127−1135.
(19) Ali, S. S.; Hardt, J. I.; Quick, K. L.; Kim-Han, J. S.; Erlanger, B. F.; Huang, T. T.; Epstein, C. J.; Dugan, L. L. *Free Radical Biol. Med.* **2004**, *37* (8), 1191−1202.
(20) Rancan, F.; Rosan, S.; Boehm, F.; Cantrell, A.; Brellreich, M.; Schoenberger, H.; Hirsch, A.; Moussa, F. *J. Photochem. Photobiol., B* **2002**, *67* (3), 157−162.
(21) Oberdorster, E. *Environ. Health Perspect.* **2004**, *112* (10), 1058−1062.
(22) Frampton, M. W.; Utell, M. J.; Zareba, W.; Oberdorster, G.; Cox, C.; Huang, L. S.; Morrow, P. E.; Lee, F. E.; Chalupa, D.; Frasier, L. M.; Speers, D. M.; Stewart, J. *Res. Rep.−Health Eff. Inst.* **2004**, (126), 1−47; discussion 49−63.
(23) Block, M. L.; Wu, X.; Pei, Z.; Li, G.; Wang, T.; Qin, L.; Wilson, B.; Yang, J.; Hong, J. S.; Veronesi, B. *FASEB J.* **2004**, *18* (13), 1618−1620.
(24) Wolff, D. J.; Papoiu, A. D.; Mialkowski, K.; Richardson, C. F.; Schuster, D. I.; Wilson, S. R. *Arch. Biochem. Biophys.* **2000**, *378* (2), 216−223.
(25) 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* (11), 4307−4316.
(26) Lyon, D. Y.; Fortner, J. D.; Sayes, C. M.; Colvin, V. L.; Hughe, J. B. *Environ. Toxicol. Chem.* **2005**, *24* (11), 2757−2762.

(27) Chiron, J.; Lamande, J.; Moussa, F.; Trivin, F.; Ceolin, R. *Ann. Pharm. Fr.* **2000**, *58* (3), 170−175.
(28) Isakovic, A.; Markovic, Z.; Nikolic, N.; Todorovic-Markovic, B.; Vranjes-Djuric, S.; Harhaji, L.; Raicevic, N.; Romcevic, N.; Vasiljevic-Radovic, D.; Dramicanin, M.; Trajkovic, V. *Biomaterials* **2006**, *27* (29), 5049−5058.
(29) Bosi, S.; Da Ros, T.; Castellano, S.; Banfi, E.; Prato, M. *Bioorg. Med. Chem. Lett.* **2000**, *10* (10), 1043−1045.
(30) Mashino, T.; Nishikawa, D.; Takahashi, K.; Usui, N.; Yamori, T.; Seki, M.; Endo, T.; Mochizuki, M. *Bioorg. Med. Chem. Lett.* **2003**, *13* (24), 4395−4397.
(31) Mashino, T.; Usui, N.; Okuda, K.; Hirota, T.; Mochizuki, M. *Bioorg. Med. Chem.* **2003**, *11* (7), 1433−1438.
(32) Tsao, N.; Luh, T. Y.; Chou, C. K.; Wu, J. J.; Lin, Y. S.; Lei, H. Y. *Antimicrob. Agents Chemother.* **2001**, *45* (6), 1788−1793.
(33) Jensen, A.; Wilson, S.; Schuster, D. *Bioorg. Med. Chem.* **1996**, *4* (6), 767−779.
(34) Oberdorster, G.; Oberdorster, O.; Oberdorster, J. *Environ. Health Perspect.* **2005**, *113* (7), 823−839.
(35) Bosi, S.; Da Ros, T.; Spalluto, G.; Balzarini, J.; Prato, M. *Bioorg. Med. Chem. Lett.* **2003**, *13* (24), 4437−4440.
(36) Tiedje, J. M. *Nat. Biotechnol.* **2002**, *20* (11), 1093−1094.
(37) Ding, L.; Stilwell, J.; Zhang, T.; Elboudwarej, O.; Jiang, H.; Selegue, J. P.; Cooke, P. A.; Gray, J. W.; Chen, F. F. *Nano Lett.* **2005**, *5* (12), 2448−2464.
(38) Sano, N.; Wang, H.; Chhowalla, M.; Alexandrou, I.; Amaratunga, G. A. J. *Nature (London)* **2001**, *414* (29 Nov 2001), 506−507.
(39) Ashcroft, J. M.; Tsyboulski, D. A.; Hartman, K. B.; Zakharian, T. Y.; Marks, J. W.; Weisman, R. B.; Rosenblum, M. G.; Wilson, L. J. *Chem. Commun.* **2006**, 3004−3006.
(40) Wharton, T.; Kini, V. U.; Mortis, R. A.; Wilson, L. J. *Tetrahedron Lett.* **2001**, *42*, 5159−5162.
(41) Lamparth, I.; Hirsch, A. *J. Chem. Soc., Chem. Commun.* **1994** (14), 1727−1728.
(42) Richardson, C. F.; Schuster, D. I.; Wilson, S. R. *Org. Lett.* **2000**, *2* (8), 1011−1014.
(43) Bingel, C. *Chem. Ber./Recl.* **1993**, *126* (8), 1957−1959.
(44) Tang, Y. J. J.; Laidlaw, D.; Gani, K.; Keasling, J. D. *Biotechnol. Bioeng.* **2006**, *95* (1), 176−184.
(45) Tang, Y. J.; Meadows, A.; Kirby, J.; Keasling, J. D. *J. Bacteriol.* **2007**, *189* (3), 894−901.
(46) Tang, Y. J.; Pingitore, F.; Mukhopadhyay, A.; Phan, R.; Hazen, T. C.; Keasling, J. D. *J. Bacteriol.* **2007**, *189* (3), 940−947.
(47) Daunder, M.; Sauer, U. *Biotechnol. Prog.* **2000**, *16*, 642−649.
(48) Wahl, S. A.; Dauner, M.; Wiechert, W. *Biotechnol. Bioeng.* **2004**, *85* (3), 259−68.
(49) Tung, H. C.; Price, P. B.; Bramall, N. E.; Vrdoljak, G. *Astrobiology* **2006**, *6* (1), 69−86.
(50) Chiron, J. P.; Lamande, J.; Moussa, F.; Trivin, F.; Ceolin, R. *Ann. Pharm. Fr.* **2000**, *58* (3), 170−175.
(51) Lok, C. N.; Ho, C. M.; Chen, R.; He, Q. Y.; Yu, W. Y.; Sun, H.; Tam, P. K.; Chiu, J. F.; Che, C. M. *J. Proteome. Res.* **2006**, *5* (4), 916−924.
(52) Scott, J. H.; Nealson, K. H. *J. Bacteriol.* **1994**, *176* (11), 3408−3411.
(53) Fischer, E.; Sauer, U. *Nature Genetics* **2005**, *37* (6), 636−640.

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