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Cu Nanoparticles Have Different Impacts in *E. coli* and *L. brevis* than Their Micron-Sized and Ionic Analogs

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SUPPORTING INFORMATION AVAILABLE

Additional details relate to: Supplemental Materials and Methods, growth inhibition effects of Cu species in *E. coli* and *L. brevis* (Figure S1), individual growth inhibition curve including error bars for each of the Cu species in *E. coli* and *L. brevis* (Figure S2), area under growth-inhibition curve for Cu species in *E. coli* and *L. brevis* (Figure S3), comparison of regression lines for area under the growth inhibition curve as a function of % Cu dissolved in media for *E. coli* and *L. brevis* (Figure S4) schematic of sucrose gradient centrifugation procedure (Figure S5), standard curve of OD₆₀₀ and total number of cells (Figure S6), Cu bioavailability determined using bacterial biosensor strain (Figure S7), and confocal images of cells treated with n-FITC-CuO (Figure S8). Tables of data include: sources of Cu particles (Table S1), correlation coefficients for area under the growth inhibition curve and amounts of dissolved copper in media, cell-associated Cu, and Cu bioavailable in each of the bacterial species (Table S2), correlation between results from growth inhibition assays and results from the suite of sub-lethal assays (Table S3), and References for Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Abstract

Copper formulations have been used for decades for antimicrobial and antifouling applications. With the development of nano-formulations of copper that are more effective than their ionic and micron-sized analogs, a key regulatory question is whether these materials should be treated as new or existing materials. To address this issue, here we compare the magnitude and mechanisms of toxicity of a series of Cu species (at concentration ranging from 2–250 $\mu\text{g/mL}$), including nano Cu, nano CuO, nano Cu(OH)₂ (CuPro and Kocide), micro Cu, micro CuO, ionic Cu²⁺ (CuCl₂ and CuSO₄) in two species of bacteria (*Escherichia coli* and *Lactobacillus brevis*). The primary size of the particles studied ranged from 10 nm to 10 μm . Our results reveal that Cu and CuO nanoparticles (NPs) are more toxic than their micron-sized counterparts at the same Cu concentration, with toxicities approaching that of the ionic Cu species. Strikingly, these NPs showed distinct differences in their mode of toxicity when compared to the ionic and micron-sized Cu, highlighting the unique toxicity properties of materials at the nanoscale. *In vitro* DNA damage assays reveal that both nano Cu and micron-sized Cu are capable of causing complete degradation of plasmid DNA but electron tomography results show that only nanoformulations of Cu are internalized as intact intracellular particles. These studies suggest that nano Cu at the concentration of 50 $\mu\text{g/mL}$ may have unique genotoxicity in bacteria compared to ionic and micron-sized Cu.

Graphical abstract

Keywords

copper; nanoparticle; nanotoxicology; ecotoxicity; antimicrobial; antifouling; genotoxicity

Nanomaterials have contributed to rapid advances in diverse fields including engineering, materials development, medicine, energy conservation as well as space technology.¹ For nano-enabled products to be brought to market and have a sustainable future, we need to understand their toxicity and ecotoxicity. Here, we have chosen to focus on nanoformulations of Cu, which are used as lubricants, conducting polymers, surfactants and catalysts in chemical reactions^{2, 3} as well as drugs, deodorants, food additives and as antifouling agents in paint.^{4, 5} Nanoformulations of Cu that are used commercially in environmental settings include nano Cu(OH)₂ (e.g., CuPro and Kocide), nano CuO (n-CuO) and nano Cu (n-Cu). Many of the current and proposed applications for these materials take advantage of the potent antibacterial properties of Cu species. However, because of their antibacterial properties, Cu-based nanoparticles (Cu NPs) may also have unwanted effects in ecosystems. A central question is whether the magnitude and/or mechanisms of toxicity of Cu-based nanoparticles are the same as, or different from, those of other traditional Cu formulations that are already on the market.

Cu-based nanoparticles have been shown to exhibit toxicity against a wide-range of environmentally-relevant organisms. For example, CuO nanoparticles have been shown to induce growth inhibition and lead to cellular oxidative stress in green alga⁶, compromise the health of daphnia⁷ and lead to neurotoxic effects in mussels⁸ at the no observed effect level (NOEL) less than 100 mg/L, 0.8 mg/L and 0.01 mg/L respectively. Moreover, Cu nanoparticles have also been shown to cause gill injury and acute lethality in zebrafish with an LD₅₀ at 48 hours of 1.5 mg/L.⁹ In the bacterium *E. coli*, CuO nanoparticles have been shown to induce cytotoxicity and oxidative stress with an LD₅₀ at 30 minutes of 0.16 mg/L.¹⁰ There is also evidence to suggest that Cu-based nanoparticles may pose a threat to human health: CuO NPs were found to cause acute pulmonary inflammation at 20 µg when oropharyngeally administered in mice and lethality in human mesenchymal stem cells with an IC₅₀ value of 2.5 µg/mL.^{11, 12}

To address the question of whether the toxicity of nanoparticles is unique, it is critical to conduct direct comparisons between nanoparticles and their ionic and micron-sized counterparts under comparable conditions. For metallic nanoparticles, some researchers have suggested that the toxicity of the nanoparticles is due to dissolved metal ions, and hence metal nanoparticles simply constitute a novel delivery mechanism for an existing agent.¹³ For instance, Alvarez and co-workers reported that the toxicity of silver nanoparticles corresponds to the rate of the release of silver ions into solution, and that no particle-specific effects were observed when conducting the experiments in a completely anaerobic condition in bacteria.¹³ By contrast, other studies have shown that some (but not all) Ag nanoparticles exhibit mechanisms of toxicity that are different from their ionic analogs. For instance, studies comparing the toxicity of various Ag NPs with ionic Ag⁺ using microarray analysis¹⁴ and genome-wide single gene deletion mutants in *E. coli*¹⁵ revealed that the pathways involved in the response of this bacterium are distinct for different Ag NPs and vary from those seen for Ag⁺. In the gene deletion strain studies, the toxicity of Ag-BPEI, which is a cationic Ag NP, was shown to involve pathways similar to that of PS-NH₂, a cationic NP that contains no Ag⁺.¹⁵ Moreover, in erythroid (mammalian) cells, silver nanoparticles have been found to disrupt transcription by inhibiting RNA polymerase, a process that was distinctly different from cytotoxic pathways induced by silver ions.¹⁶

Similarly, reports as to whether Cu nanoparticles are toxic simply because they release Cu ions or whether they can exhibit nano-specific toxicity has been a subject of debate in the literature. Bondarenko and coworkers have reported toxicity studies comparing nano CuO to CuSO₄ and micron-sized CuO at the exposure concentration of 0.001 – 10000 mg/L in *E. coli*.¹⁷ Based on their results, they suggested that nano CuO was toxic from copper ion release into solution because all three copper species resulted in biotic production of reactive oxygen species (ROS) and single-stranded DNA damage; additionally, these effects could be inhibited by addition of a Cu-chelating agent (EDTA).¹⁷ By contrast, in a separate study of *Pseudomonas chlororaphis*, only CuO nanoparticles, but not their bulk or ionic counterparts, were found to modify bacterial metabolism and cellular reprogramming at the exposure concentration of 200 mg/L.¹⁸ In addition, a study in the microcrustacean *Daphnia magna* and in the bacterium *Vibrio fischeri* revealed that CuO particles at the nano-size are about 10 times more toxic than their bulk analogs at the exposure concentration of 3.8 – 250 mg/L for *D. magna* and 0–4000 mg/L for *V. fischeri*.¹⁹

These somewhat conflicting results point to the need for a systematic and thorough analysis of the difference in toxicity mechanisms for multiple species of bacteria from related Cu species at the nano-size and the micron-size in comparison to ionic Cu. Here, we demonstrate the use of a suite of sub-lethal assays consisting of membrane damage, cellular ROS generation, electron transport activity and membrane potential to unveil the molecular mechanisms of toxicity of a panel of nanoparticles (n-Cu, n-CuO and Cu(OH)₂) compared to their micron-sized analogs (m-Cu and m-CuO) as well as ionic Cu (CuCl₂ and CuSO₄). In addition, we evaluated the toxicity of the Cu species in two very different taxa of bacteria: *Escherichia coli*, a gram negative bacterium, and *Lactobacillus brevis*, a gram positive bacterium. We intentionally selected two different species of enteric bacteria so that we could explore possible implications of Cu-based nanoparticles entering into liquid waste treatment systems that rely on microorganisms. These results have important implications for both the regulation of Cu-based nanoparticles and for the design of safer products containing Cu species.

RESULTS AND DISCUSSION

Cu species varied in size from nanoscale to microscale

The toxic effects of the selected particles (Table S1) were determined in gram negative bacterium and gram positive enteric bacteria. Our material selection included Cu particles that varied in size (nanoscale to microscale) as well as chemical composition (elemental Cu and CuO and Cu(OH)₂). Six different Cu particulates were studied: nano Cu (n-Cu), nano CuO (n-CuO), nano Cu(OH)₂ (2 forms, n-Cu(OH)₂-a and n-Cu(OH)₂-b), micro Cu (m-Cu), and micro CuO (m-CuO). Because n-Cu(OH)₂ constitutes the most widely-used class of nano-sized Cu fungicide/bactericides, we tested two different commercial sources of CuPro; n-Cu(OH)₂-a and Kocide; n-Cu(OH)₂-b to determine whether there are any manufacturer-dependent differences in environmental effects for this class of Cu NPs. In addition, two different forms of ionic Cu (CuCl₂ and CuSO₄) were tested as controls. The toxic effects of these particles were determined in *Escherichia coli* and *Lactobacillus brevis*. *E. coli* is a gram negative bacterium and *L. brevis* is a gram positive bacterium.

The primary sizes (diameter) of the particles (as shown in Table 1) were less than 1,000 nm for all of the nano-scale particles (n-Cu, n-CuO, n-Cu(OH)₂-a and n-Cu(OH)₂-b) and ranged from 200 nm to >10 µm for micro-scaled particles (m-Cu and m-CuO). It is important to note that the “nano-Cu” species was reported by the manufacturer (US Research Nanomaterials) to be “40 nm”.²⁰ However, when we looked at this sample using TEM, we found that the primary size to range between 200–1000 nm. Nonetheless, we have continued to refer to this sample as “nano-Cu” to be consistent with the designation given to this material in prior publications from our center, UC-CEIN.^{20, 21} The hydrodynamic diameter of these particles in purified water was less than 1,400 nm for all of the nano-scaled particles and ranged in size between ~1,300 nm and > 3 µm for the micro-scaled particles. When dispersed in *E. coli* minimal (MMD) media, the average hydrodynamic diameter of the particles was approximately the same as that observed in purified water (see Table 1). However, when the particles were dispersed in Lactobacilli MRS broth, the average hydrodynamic diameter was slightly larger (280–1,600 nm for nano-scaled particles and > 3 µm for micro-sized particles) than that observed in purified water. This slight increase of hydrodynamic diameter found in nutrient-rich Lactobacilli MRS broth might be the result of adsorption of proteins from the media onto the surface of the particles.²² All of the particles remained stable in both *E. coli* and *L. brevis* media after 24 hours as shown in Table 1, when dispersed using humic acid as described in the **Materials and Methods**. The zeta potential of the particles ranged from –16.5 to –53.8 mV in purified water (Table 1). The percentage of Cu ion dissolution from each particle in purified water, *E. coli* MMD media and Lactobacilli media are provided in the Table 2. n-Cu and the two n-Cu(OH)₂ nanoparticles (CuPro and Kocide) dissolved more than the rest of the particles in both bacterial media studied, but still not completely (10%, 10% and 11% in MMD media and 21%, 22% and 18% in *Lactobacillus* media after 24 hours for n-Cu, n-Cu(OH)₂-a and n-Cu(OH)₂-b, respectively). Interestingly, for all of the particles studied, the particles dissolved most in the Lactobacilli media, an intermediate amount in the MMD media, and least in purified water. This dissolution trend presumably reflects the greater ionic strength and organic materials present in Lactobacilli media > MMD media > water; increasing ionic strength and the presence of some organic species have both been shown previously to facilitate the dissolution of Cu ions from the particles.²⁰

Bacteria exhibit differential sensitivities to different Cu species, with gram positive bacteria more sensitive than gram negative bacteria

To study the antibacterial effects of Cu particles, growth inhibition assays were performed on both *E. coli* and *L. brevis*. Dose-dependent declines in bacterial growth were observed for all of the Cu species tested in both species of bacteria (Figure S1 A, *E. coli*; B, *L. brevis* and Figure S2 showing individual growth-inhibition curves with error bars for each of the Cu species in *E. coli*, A and *L. brevis*, B) but the magnitude of the toxicity (as measured by IC₅₀) differed significantly between the Cu species studied and between the two species of bacteria (Table 2). Markedly, a severe interruption of growth was observed when *E. coli* was treated with CuCl₂, n-Cu, CuSO₄, and n-CuO (the IC₅₀ was determined to be 38, 120, 140 and 160 mg/L for these species, respectively, Table 2). The observed difference in toxicity between CuCl₂ and CuSO₄ agrees with the previously published data showing the distinct behaviors in bioreduction and biosorption between the two Cu compounds²³ and the

influence of chloride and sulfate anions on the bioavailability of Cu and subsequently the uptake of Cu into starch granules in potato and wheat starch.²⁴ A relatively mild response (IC_{50} = 250 mg/L) was observed when *E. coli* was treated with n-Cu(OH)₂-a and n-Cu(OH)₂-b as well as micron-sized particles (m-Cu and m-CuO). Notably, *L. brevis* was more sensitive (as indicated by a smaller IC_{50} value) to each of the Cu species tested than was *E. coli*. n-CuO, n-Cu(OH)₂-a, n-Cu, n-Cu(OH)₂-b and CuCl₂ exhibited IC_{50} values of 4, 4, 6, 6 and 8 respectively in *L. brevis*, which meets the criteria for “toxic to aquatic life” according to the globally harmonized system of classification and labelling of chemicals (GHS) part 4: Environmental hazard).²⁵ A relatively modest response (IC_{50} = 120 mg/L) was observed in *L. brevis* for m-Cu and m-CuO (Figure S1, S2 and Table 2). To accurately distinguish between toxic and non-toxic Cu species, the area under the growth inhibition curves (as shown in Figure S1) was calculated for all of the Cu species (Figure S3). The calculation provided a continuous measurement for non-toxic particles whose exact IC_{50} could not be determined. (Note that the IC_{50} of these non-toxic particles was listed as = 250 mg/mL in Table 2). In agreement with the IC_{50} values, nano-sized particles and ionic Cu exhibit a significantly smaller area under their respective growth inhibition curves when compared to their micron-sized particles in both species of bacteria confirming that the nano-sized Cu particles are more toxic than their micron-sized counterparts. In both species of bacteria, the toxicity of the n-Cu was equivalent to that of toxic ionic Cu species (CuCl₂, CuSO₄, Table 2, Figure S3). Interestingly, the gram-positive *L. brevis* exhibited an elevated toxicity to all of the particles relative to gram-negative *E. coli*, suggesting a possible role for the double membrane structure found specifically in gram negative bacteria in protecting against toxicity of Cu particles. However, it is important to note that toxicity assays in the two bacterial species were conducted in two different types of media; MMD for *E. coli* and MRS media for *L. brevis*. Metal ion dissolution was found to be significantly higher in the MRS media, suggesting that the increased sensitivity of *L. brevis* towards Cu species could be partially due to elevated dissolved Cu ions in *L. brevis* media compared to *E. coli* media. To address this issue, we performed a regression analysis of the area under the growth inhibition curve as a function of the concentration of Cu dissolved in the bacterial media where data for both *E. coli* and *L. brevis* were considered both separately and together (Figure S4). This analysis reveals that the regression lines for the two species are quite similar but not identical, suggesting that the difference in toxicity observed in the two species may be largely (but not completely) due to differences in the extent of the dissolution of the copper species in the media. However, the observation that *L. brevis* is more sensitive than *E. coli* is also consistent with the previous finding that gram positive bacteria are more sensitive to nanoparticles compared to gram negative counterpart in the case of Cu nanoparticles and nano-sized metal oxide halogen adducts^{26, 27} and that gram positive bacteria tend to be more sensitive than gram negative bacteria to ionic copper and other metals ions.^{28, 29}

Magnitude of toxicity of Cu species correlates with amount of cell-associated copper

One possible explanation for why bacteria are more sensitive to nano-sized copper species than to micron-sized copper species was proposed previously by Rossetto *et al.*, *i.e.* that the percent dissolution of the nano copper species is greater than for micron-sized Cu species due to the larger surface to volume ratio for nano-sized particles.¹⁹ To test this possibility,

we looked at whether the area under the growth inhibition curve for the different Cu species correlates with the amount of dissolved Cu in the bacterial media. The area under the dose-response curve was determined to provide a continuous measure of toxicity, which is necessary because the IC_{50} could not be determined exactly for the non-toxic species (*i.e.*, those Cu species where $IC_{50} > 250$ mg/L). The correlation between area under the growth inhibition curve and the amount of Cu dissolved in the bacterial media was found to be moderate for *E. coli* ($r = 0.65$) and strong for *L. brevis* ($r = 0.83$). (See Table S2)³⁰

Because we had recently observed that the toxicity of silver nanoparticles is dependent upon their ability to bind to the outside of cells and perturb the outer membrane,¹⁵ we also tested whether the nano Cu species were more toxic because they were better able to bind to and deliver Cu to the bacterial cells. To assess this, we used sucrose gradient centrifugation coupled with ICP-MS to determine the concentration of Cu associated with bacteria. A detailed schematic of this experiment is shown in Figure S5. The total number of bacterial cells collected from the sucrose gradient was determined using a linear equation derived from a standard curve relating OD_{600} and total number of cells (colony forming units) as shown in Figure S6. Generally, there was more Cu associated with cells exposed to the more toxic Cu species than for the cells exposed to the less toxic Cu species (Table 2). In particular, n-Cu, with the smallest IC_{50} value, exhibited the greatest amount of cell-associated Cu, both in *L. brevis* and *E. coli*. The correlation between the area under the growth inhibition curves and the Cu associated with the cells were found to be 0.73 and 0.74 for *E. coli* and *L. brevis*, respectively (Table S2). This correlation is roughly equivalent to that observed between area under the growth inhibition curves and the amount of Cu dissolved in the bacterial media (0.65 and 0.83 for *E. coli* and *L. brevis*, respectively). One caveat to the sucrose gradient separation is that ionic Cu cannot be effectively separated from the cellular components and hence this method cannot be used to ascertain cell-associated Cu for bacteria treated with ionic Cu. However, these results suggest that the ability of the Cu species to associate with cells may play a crucial role in determining their toxicity and suggests the need for more detailed studies on the different mechanisms of toxicity for the Cu species.

To test whether the most important factor in determining toxicity was the amount of Cu delivered to the inside of the cells, we used a genetically-engineered bacterial biosensor, *E. coli* MC1061 (pSLcueR/pDNPcopAlux), in which bioluminescence is specifically induced by intracellular, bioavailable Cu.¹⁷ The copper species that were found to be most toxic to cells (*i.e.*, $CuCl_2$ and nano-sized Cu particles) resulted in more bioluminescence than the less toxic particles (*i.e.*, micron-sized particles). (See Figure S7). The level of bioavailable Cu for cells treated with n-Cu was about the same or higher than that of cells treated with $CuCl_2$, suggesting that the nanoparticle form of Cu has a unique advantage over Cu ions in entering the cells and that particles at nano-scale are more effective in Cu ion delivery than particles at micro-scale. The correlation coefficient between area under the growth inhibition curve and the intracellular amount of bioavailable Cu, was very strong ($r = 0.98$, Table S2) suggesting that the best predictor of bacterial toxicity is the amount of intracellular, bioavailable Cu.

Sub-lethal assays reveal that different Cu species exhibit different mechanisms of toxicity

To elucidate the mechanisms of toxicity for the Cu species, a suite of assays measuring membrane potential, membrane damage, cellular ROS generation and electron transport activity were employed in both *E. coli* and *L. brevis*. A detailed description of these methods is also provided in the Supplemental Materials and Methods section within the Supporting Information. The outcomes of these four assays are shown in the heat map in Figure 1. In the heat map, red indicates that the results are similar to the positive control (*i.e.*, NaN₃ for the membrane potential assay, ethanol for membrane damage, H₂O₂ for cellular ROS generation, and CCCP for electron transport activity), and green indicates that the results are identical to the negative controls (*i.e.*, PBS with no added Cu species). While both nano-sized Cu and Cu ions resulted in significant membrane damage and a decrease in electron transport activity in *E. coli*, the micro-sized Cu particles did not. This observation strongly agrees with the growth inhibition results, in which nano-sized Cu and Cu ions exhibit lower IC₅₀ values and smaller areas under the growth inhibition curve than the micro-sized Cu species in *E. coli* (Table 2, Figure S3). Strikingly, oxidative stress (measured by biotic ROS generation) was only observed in *E. coli* treated with Cu NPs, but not with *E. coli* treated with ionic copper (CuCl₂ or CuSO₄) or micron-sized Cu at the concentrations studied herein. Conversely, severe disruption of membrane potential was observed only in *E. coli* treated with Cu ions and not those treated with nano- or micron-sized Cu particles. In both species, the toxicity of the Cu species (as measured by area under the growth curve) was strongly correlated with the decrease in electron transport activity (correlation coefficient = 0.76 in *E. coli*, Table S3A and 0.64 in *L. brevis*, Table S3B). Similar trends were observed in *L. brevis* (Figure 1), with the following exceptions: (1) overall, a stronger response was observed in the sub-lethal assays in *L. brevis* for the more toxic particles; (2) significant membrane damage was observed for both Cu(OH)₂ (CuPro and Kocide)-and CuCl₂/CuSO₄ in *L. brevis*; and (3) biotic ROS generation was observed in *L. brevis* treated with n-Cu and n-CuO, as well as both of the Cu(OH)₂ species (CuPro and Kocide). Inter-species correlation (Table S3C) revealed that a strong agreement between the two species were observed in biotic ROS generation ($r = 0.90$) and membrane potential ($r = 0.86$). Consistent with the results from the growth inhibition assay, however, we typically observed stronger responses in *L. brevis* than in *E. coli* for a given Cu species for each of the assays. The observation that overall toxicity does not correlate strongly with biotic ROS generation for the series of copper species ($r = 0.02$ in *E. coli* and $r = 0.60$ in *L. brevis*) is important because it suggests that different toxicity mechanisms are relevant to different Cu species. Although ionic Cu is known to cause oxidative stress,³¹ this mechanism does not predominate for ionic Cu at the concentrations studied herein. By contrast, cells treated with particulate forms of Cu (both nano-sized and micron-sized) exhibited significant ROS production even at the low Cu concentrations studied herein. Our observation suggests that n-Cu provokes a particularly strong biotic ROS response — even at these low concentrations — is consistent with prior reports that Cu NPs lead to greater cellular oxidative stress in bacterial cells,³² yeast cells,³³ and mammalian cells³⁴ than does ionic Cu. Our finding that, out of the species studied, only ionic Cu severely disrupts membrane potential is consistent with a previous study showing that ionic Cu promotes proton leakage across the plasma membrane and hinders the respiratory chain downstream of coenzyme Q.³⁵ These results highlight important differences in the mechanistic pathways of toxicity for Cu NPs and Cu ions.

Different Cu species also result in different types of DNA damage *in vitro*

A previous study had demonstrated that ionic Cu, CuO nanoparticles, and micron-sized CuO particles all result in single-stranded DNA damage,¹⁷ but we wished to explore here if *other* types of DNA damage were caused by the different Cu species studied herein. To study the intrinsic potential of the different Cu species to damage double-stranded DNA, we used a plasmid-based *in vitro* DNA damage assay.³⁶ In this experiment, the plasmid pUC19 (where 90% of the plasmid is supercoiled and 10% is in single-strand nicked form as purchased)³⁷ was incubated with each of the Cu species for 24 hours; the resulting state of the plasmid was assayed by gel electrophoresis. The gel electrophoresis results for each Cu species are shown in Figure 2A. The positive controls used in this study were a UV-treated plasmid (lane 2 in Figure 2A, which completely degrades the DNA and appears as smeared band) and plasmid treated with the restriction enzyme *Pst*I, (lane 3 in Figure 2A), which results in double strand breaks and hence appears as a linearized plasmid. The DNA damage was classified into linearized DNA (plasmid DNA with a double-strand break), single-stranded, nicked DNA (plasmid DNA with single strand breaks) and fragmented DNA (plasmid DNA with multiple strand breaks). All of the Cu species tested resulted in some form of DNA damage, but the nature and the severity of the damage varied significantly from one Cu species to the next. The most severe DNA damage observed for n-Cu and m-Cu, both of which induced complete degradation of plasmid DNA, resulting in smeared bands at lower molecular weights (Figure 2A). By contrast, both forms of n-Cu(OH)₂ (CuPro and Kocide) resulted in complete conversion of the native form of the supercoiled plasmid to open circular (single-strand breaks) and linearized plasmid (double-strand breaks). The remaining Cu species tested (n-CuO, m-CuO and ionic Cu) resulted in partial conversion of the supercoiled DNA to open circular and linearized plasmid.

To test whether the ability of the Cu species to cause DNA damage *in vitro* is linked to the ability of the various species to generate ROS abiotically, (*i.e.* in the absence of cells), we studied each of the particles using an abiotic ROS generation assay using dichloro-dihydro-fluorescein diacetate (DCFH). In this assay, DCFH is generated *in situ* by cleaving the acetate functional group from H₂DCFDA using NaOH.³⁸ The solution containing DCFH was mixed with each of the Cu species and the fluorescent signal at 530 nm, which indicates the amounts of oxidized dye, was measured after 2 hours.³⁸ A detailed description of the method used for abiotic ROS generation is also provided in the Supplemental Materials and Methods section within the Supporting Information. Only n-Cu and m-Cu — the same two of the particles that resulted in complete DNA degradation — resulted in significant oxidation of DCFH *in vitro* (Figure 2B). This result strongly suggests that the Cu species studied herein that cause significant DNA damage do so by means of DNA oxidation. This result is consistent with previous findings in mammalian cells that nanoparticles induce single/double stranded DNA breaks as well as chromosomal damage using micronucleus, comet and γ -H₂AX assays.³⁹

Cellular imaging studies reveal that Cu nanoparticles enter the bacterial cells intact

The observation that both n-Cu and m-Cu generate significant ROS under abiotic condition and exhibit significant DNA-damage potential *in vitro* but n-Cu exhibits significantly higher toxicity towards bacterial cells begs the question of whether there is a difference in the

ability of the two particles to enter the bacterial cells, and hence have direct access to intracellular DNA. To address this question, we examined TEM images of *E. coli* exposed to each of the different Cu species. For these experiments, we chose to focus on *E. coli* because it is a well characterized bacterium and has been used previously to study uptake of nanoparticles.^{40, 41} To ensure that particles loosely associated with the bacteria were removed, the samples were washed with PBS 3 times prior to obtaining the images. As shown in Figure 3, *E. coli* treated with nano-sized particles (n-Cu, n-CuO, n-Cu(OH)₂-a and n-Cu(OH)₂-b) were observed to have particles-associated with them. By contrast, no Cu particles were observed in cells treated with micron-sized particles (m-Cu and m-CuO). Interestingly, n-Cu(OH)₂-a, n-Cu(OH)₂-b and CuCl₂-treated bacteria showed a marked distorted conformation of the cells, as well as membrane damage.

To ascertain whether the particles were actually inside the cells, we constructed 3D images of *E. coli* treated with n-Cu from a tomogram series. The resulting image (Figure 4) revealed n-Cu particles inside of the *E. coli*, suggesting that the nanoparticles were able to cross the cell membrane intact. Consistent with the results presented herein, Kumar *et al.* have previously reported the direct uptake of nanoparticles (ZnO and TiO₂) in the bacteria *E. coli*, which they assessed using flow cytometry.⁴⁰ Although *E. coli* does not have endocytotic machinery, it is possible that nanoparticles could enter the bacterial cells intact. Results from this study and Rastogi *et al.* demonstrate that Cu NPs lead to membrane damage and the cytoplasmic leakage in *E. coli*,⁴² which might permit the internalization of the nanoparticles into the bacterial cells. This result supports the hypothesis that n-Cu can penetrate into the cells, and mediate toxicity through effects of the nanoparticle surface (*e.g.*, DNA damage) in addition to toxicity due to release of Cu ions. In addition, we conducted confocal imaging of both *E. coli* and *L. brevis* that were stained with Hoechst fluorescent dye (which stains nucleic acids inside the cells blue) and treated with n-CuO labeled with FITC (green fluorescent)⁴³. In both cases, we observed co-localization of the n-FITC-CuO and Hoechst signals. (Figure S8) The results indicate that n-CuO is able to be internalized in both of the species of bacteria studied herein. Taken together, these findings help to explain why we observed both a greater magnitude of toxicity and different mechanisms of toxicity for nano Cu species compared with their micron and ionic analogs.

CONCLUSIONS

The studies reported herein provide strong evidence that nano-sized Cu particles cannot only be more toxic than their micron-sized analogs, but can also exhibit significantly different mechanisms of toxicity than both ionic Cu and micron-sized Cu. Critically, we found that only nano Cu species were either strongly bound to or internalized within *E. coli* and demonstrated that both n-Cu and n-CuO can be internalized into the bacteria intact. Because n-Cu was also found to generate significant ROS and cause extremely deleterious damage DNA *in vitro*, the potential that n-Cu may exhibit unique genotoxicity inside cells is particularly of concern. Taken together, these studies suggest that additional safety testing should be conducted on nano Cu species in other organisms.

MATERIALS AND METHODS

Bacterial strains

Two bacterial strains were used in this study: *Escherichia coli* strain ATCC 25922, a standard strain widely used for antimicrobial disk susceptibility tests, and *Lactobacillus brevis* strain (Orla-Jensen) ATCC 14869. Chemicals and media components used in these studies are detailed in the Supplemental Materials and Methods section of the Supporting Information.

Physiochemical characterization of copper species

The copper species tested herein were obtained from a variety of sources as described in Table S1. Filtered deionized water was used to make stock solution at 20 mg/mL. Dynamic light scattering (DLS, ZetaPALS, Brookhaven Instruments Ltd., UK) was utilized to analyze the average size and size distribution of copper species (50 µg/mL) in water, *E. coli* media (MMD) and *L. brevis* media (Lactobacilli MRS broth). The ζ-potential values of each Cu species in aqueous solution were determined using a ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments Ltd, UK). Transmission Electron Microscopy (TEM) was used to determine the primary size and morphology of the particles. To prepare samples for TEM, a drop of each Cu species in purified water was applied to carbon-coated TEM grids and evaporated at room temperature. Images were taken with a JEOL 1200 EX TEM microscope.

The percent dissolution of each of the Cu species in water and bacterial media was measured by quantifying dissolved Cu by ICP-OES (ICPE-9000 plasma atomic emission spectrometer, Shimadzu). For the analysis, 1 mg/mL of Cu species was suspended in water or bacterial growth media to yield a final volume of 1 mL for 24 hours and then was centrifuged at 15,000 rpm for 30 minutes to precipitate any remaining particles. The supernatant was collected and transferred to a clean tube for acid digestion. 10 mL of nitric acid (HNO₃, 65–70%, Trace Metal Grade) was added to the supernatant before incubating in a HotBlock (SC100, Environmental Express) at 80°C for 6 hours. The temperature was then raised to 95°C overnight to evaporate all liquid present in sample. The dried sample was allowed to cool down at room temperature before being dissolved in 2% (v/v) nitric acid at 80°C for 3 hours. The extract was transferred to a 15 mL ICP-OES analysis tube to measure Cu ion concentration.

Growth inhibition effects and IC₅₀ calculation

To assess the half-maximal inhibitory concentrations (IC₅₀), a growth inhibition curve was constructed for each Cu species. A 20 mg/mL stock of Cu species was diluted to 10 mg/mL with 2X media (MMD for *E. coli* and Lactobacilli MRS broth for *L. brevis*). Humic acid was added to MMD media as a dispersing agent and was chosen because of its relevance for environmental systems. In this experiment, humic Acid (HA) was added to a final concentration of 0.01 mg/mL and then the resulting mixture were sonicated in water bath (Branson 2510, CT, USA) for 15 minutes at room temperature. 10 mg/mL of each Cu species was then diluted with 1X media supplemented with 0.01 mg/mL HA to a step-wise concentration gradient at 2, 3.9, 7.8, 15.6, 31.3, 62.5, 125 and 250 mg/mL. 50 µL of NP at

each concentration were pipetted into 384-well polystyrene microplates. Nine replicates were performed for each concentration. In a separate plate, 50 μ L of a log-phase bacterial culture (OD₆₀₀ between 0.5 – 0.7) was pipetted into the 384-well plate and then a plastic 384 pin replicator (Genetix Molecular Devices) was used to inoculate bacteria from this plate to the plate containing the serial dilution of Cu species. Sterility and blank controls (bacterial media with no inoculation) were also included for each concentration. A Biotek Synergy plate reader (BioTek, VT) was used to monitor OD₆₀₀ every 30 minute at 37°C for 24 hours. A growth curve was constructed using equation:

$$\text{Growth}(\%) = \frac{A_{\text{NP,B}} - A_{\text{NP,A}}}{A_{\text{BI,B}} - A_{\text{BI,A}}} \times 100$$

In the above equation, $A_{\text{NP,B}}$ is the absorbance of the bacterial culture in the presence of each concentration of Cu NPs (average of 9 replicates); $A_{\text{NP,A}}$ is the absorbance of the Cu NPs at the respective concentrations which contain no bacteria (average of 3 replicates); $A_{\text{BI,B}}$ is the absorbance of the bacterial culture in blank (no Cu species) media (average of 9 replicates), and $A_{\text{BI,A}}$ is the absorbance of media with no bacteria (average of 3 replicates). The growth inhibition curve was plotted using the program Origin version 9 (OriginLab Corporation) using the category Growth/Sigmoidal, function Logistic. The IC₅₀ and standard error were calculated for each data set using the same program.

***In vitro* assays**

***In vitro* DNA damage assay**—Purified plasmid pUC19 (Thermo Scientific, catalog #SD0061) was incubated in the presence of 100 mg/L Cu species for 24 hours in purified water. Centrifugation at 15000 rpm for 30 minutes was used to separate plasmid DNA from residual Cu NPs. The supernatant containing the plasmid DNA was collected and loaded into a 1.2% Trisacetate EDTA buffer, TAE agarose gel. Electrophoresis was performed at 5 V/cm for 1 hour and the resulting gel was stained with ethidium bromide for 30 minutes. A Bio-Rad FX™ imaging system was used to image the gel; the band intensities were quantified using QuantityOne™ software. To linearize the pUC19 (first positive control), the restriction enzyme, *Pst*I was incubated with the plasmid at 37° C for 1 hour. To induce random nicking and DNA fragmentation (second positive control), the plasmid was treated with a xenon arc UV-B lamp (Asahi Spectra, LAX-Cute) for 10 minutes at 1000 mJ/cm². For the negative control, pUC19 was loaded into the electrophoresis gel whereas the majority of the plasmid as purchased was in the supercoiled-formed of DNA.

Determining cell-associated Cu using sucrose gradient centrifugation and ICP MS

E. coli and *L. brevis* were treated with 0.5 and 1 mg/L of a series of the Cu species. After 24 hours, the cells were washed 2 times with PBS and then sucrose gradient centrifugation was used to separate the cells from residual particulate Cu. To make the sucrose gradient, 0.3, 0.4 and 0.6 g/mL of sucrose (Sigma-Aldrich) were completely dissolved in water and filtered with a 0.22 μ m Millipore filter.⁴⁴ 1.2 mL of each sucrose concentration was carefully layered into a 15 mL Falcon tube and then 1 mL of cell suspension was placed on top of the gradient. The mixture was centrifuged (Eppendorf 5810 R) at 2916×g for 5 minutes at room

temperature. After centrifugation, a brown band of cells was clearly visible at the upper part of the gradient and Cu species were visibly precipitated at the bottom of the tube. (See Figure S4.) 1.2 mL of cells suspension were collected. To determine the total number of bacterial cells, a standard curve between OD₆₀₀ and the number of cells was constructed (Figure S5, A for *E. coli* and B for *L. brevis*). The total amount of cell-associated Cu was normalized as the Cu content per 10⁹ cells for each bacterial species. The number of cells was determined by measuring the OD₆₀₀ of 200 µL of this suspension. To determine the amount of cell-associated copper, 1 mL of the suspension was digested with 5 mL of pure HNO₃ for overnight and then was evaporated at 95°C until no liquid remained. 5% HNO₃ was used to resuspend the sample. Cu content was analyzed using ICP-OES (ICPE-9000, Shimadzu).

Determining bioavailable, intracellular Cu with biosensor strain of *E. coli*

The amount of bioavailable, intracellular Cu was determined using a genetically engineered *E. coli* biosensor strain in which bioluminescence specifically responds to Cu cellular bioavailability (MC1061 pSLcueR/pDNPcopAlux, “Cu-inducible strain”). The luminescent strain was constructed as previously described in Bondarenko *et al.*¹⁷ A colony of the bacteria was inoculated into 3 mL of fresh LB media supplemented with 100 mg/L ampicillin and allowed to grow overnight before being diluted 1:50 using fresh LB media with antibiotic. The culture was allowed to grow to reach log phase (OD₆₀₀ of 0.6) before the cells were harvested for the experiment. Growth was conducted at 30 °C, shaking at 200 rpm. 25 µL of the Cu species in MMD supplemented with 0.01 mg/mL humic acid were mixed with twenty-five µL of the biosensor bacteria in a 384-well plate to yield a final bacterial OD₆₀₀ of 0.1. The plate was kept at 30 °C for 2 hours to allow for luminescence induction, at which point the luminescence was quantified using a micro-plate reader (SpectraMax MS, Molecular Devices, CA).

Microscopy of bacterial cells exposed to Cu species

TEM sample preparation and microscopy—*E. coli* cells that had been treated with 0.1 mg/mL Cu species for 24 hours were washed 3 times with phosphate-buffered saline (PBS) before being fixed with 2% glutaraldehyde in 0.1 M PBS. Cells were washed with PBS 3 times before treating with osmium tetroxide (OsO₄) in PBS for 1 hr. After rinsing 3 times with PBS, the cell pellet was dehydrated in a graded series of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol for two hours each). Propylene oxide was used to remove any residual ethanol before the pellet was embedded in Epon resin (Sigma Aldrich). A Reichert-Jung Ultracut E ultramicrotome was used to cut 100 nm thick sections which were placed onto 1GG200 gold grids (Ted Pell Inc.). Sections were stained with uranyl acetate and Reynolds lead citrate, and examined on an FEI T12 transmission electron microscope operated at 80 kV in the Electron Imaging Center for Nanomachines (EICN) at UCLA.

Electron tomography and 3D reconstruction—*E. coli* cells that had been treated with Cu NPs for 24 hours were washed, dehydrated and embedded in Epon resin as described above. A Reichert-Jung Ultracut E ultramicrotome was used to make 250 nm thick sections from the block, which were placed onto Maxtaform 75/300 Rectangular Mesh copper grids

(Ted Pell Inc.). An FEI Tecnai F20 transmission electron microscope was used to capture the tomography tilt series. A Gatan 626 cryo specimen holder was used to collect images in tilt angles ranging from -70° to $+70^{\circ}$, with a 1° increment. The tilt series of 141 images were used to reconstruct 3D volumes using the Etomo tomography processing software in the Imod package (Boulder Laboratory for 3-D Electron Microscopy of Cells).⁴⁵

Confocal microscopy—*E. coli* and *L. brevis* were treated with 50 mg/L of n-FITC-CuO for 24 hours before being washed 3 times with PBS. The bacteria were fixed using 4% paraformaldehyde for 20 minutes at room temperature. After another 3 washes with PBS, the cells were stained with Hoechst 33342, which specifically stains bacterial DNA, yielding blue fluorescence. After 1 hour of incubation with Hoechst dye, the cells were washed 3 times and placed into an 8-well chamber slide (Lab Tek) before being visualized under a confocal microscope (Leica Confocal SP2 1P/FCS). For Hoechst dye, the excitation wavelength used was 358 nm, and $E_{\max}$ was monitored at 420–500 nm. For FITC, the excitation wavelength used was 488 nm, and $E_{\max}$ was monitored at 520–580 nm.

Safe Handling of Nanomaterials

Nanoparticles as dry powders were handled in a chemical fume hood or powder enclosure, and manipulated while the researcher was wearing a N95 filter mask. After suspension in aqueous solutions, standard good chemical hygiene practices were employed. Sonication can result in aerolization and thus was only performed on solutions that were in closed containers. More detailed recommendations are available in the *Nanotoolkit* developed by the *California Nanosafety Consortium of Higher Education* which is available online at: <http://www.cein.ucla.edu/new/p155.php>

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. A suite of sub-lethal assays was used to elucidate the mechanisms of toxicity of the Cu species

Cells were treated with Cu particles ranging from 2–250 mg/L for 24 hours before being treated with DiBAC, PI/SYTO, H₂DCFDA or XTT to assess membrane potential, membrane damage, biotic ROS generation or electron transport chain activity, respectively. Red indicates similarity for each treatment to the positive controls (NaN₃, Ethanol, H₂O₂ and CCCP for membrane potential, membrane damage, cellular ROS generation and electron transport activity assays, respectively), whereas green indicates the degree of similarity for each treatment to the negative controls (PBS alone). At least three biological replicates were performed at each concentration for each assay.

Figure 2. Results from DNA damage assay and abiotic ROS generation

(A) The plasmid pUC19 was treated with the different Cu species for 24 hours and the resulting DNA species were separated and analyzed using gel electrophoresis. UV and restriction enzyme (*Pst*I) were used as positive controls for fragmented and linearized DNA, respectively. Electrophoresis was run at 5 V/cm for 1 hour. N-Cu and m-Cu generated the most severe DNA damage and resulted in complete degradation of the plasmid DNA. N-Cu(OH)₂-a and n-Cu(OH)₂-b induced complete conversion of supercoiled DNA to open circular and linear DNA, while exposure to the other Cu species resulted in only partial conversion of the plasmid DNA. (B) The capability of each particle to generate reactive

oxygen species (ROS) was tested *in vitro* using (2',7' – dichlorofluorescein) DCFH. The particles were treated with DCFH for 2 hours and the fluorescence intensity, which reflects the extent of oxidation, was measured using excitation/emission wavelengths of 530/630 nm. Three replicates were performed at each concentration of Cu species.

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Figure 3. TEM images of *E. coli* cells treated with Cu species

Cells were treated with 0.1 mg/mL of the Cu particles for 24 hours before being washed, embedded in resin and negatively stained before being imaged by TEM. Red arrows indicate Cu particles; green arrows indicate membrane damage. Particles-associated with cells were observed in cells treated with nano-sized particles (n-Cu, n-CuO, n-Cu(OH)₂-a and n-Cu(OH)₂-b).

Figure 4. Electron tomography 3D construction reveals the presence of intact Cu nanoparticles inside *E. coli* cells

Cells were treated with 0.1 mg/mL of n-Cu for 24 hours before being embedded in the resin, sectioned and stained (see Methods). A tilt series of 141 images was recorded by tilting the sample one degree at a time from -70° to $+70^\circ$ and used to construct a 3D tomogram of the sectioned cell. Example images in the tilt series (top panel) and two orthogonal slabs in the 3D tomogram are shown in the bottom panel, showing a single nano particle inside the cell (black dot).

Zeta potential, particle size and hydrodynamic diameter of Cu particles in purified water and bacterial media. Error bars indicate standard error of measurements.

Table 1

Particles	Zeta potential (mV)	Primary size (nm) ^a	Average Hydrodynamic Diameter (nm) ^c				
			Purified Water	MMD + HA ^d		Lactobacilli media	
				0 hr	24 hr	0 hr	24 hr
n-Cu	-46.3 ± 1.6	200–1000	1200 ± 200	1100 ± 200	1400 ± 300	1600 ± 500	1400 ± 500
n-CuO	-16.5 ± 0.8	20–100	420 ± 20	300 ± 2	460 ± 10	470 ± 4	1600 ± 300
n-Cu(OH) ₂ -a	-45.1 ± 0.8	10	900 ± 200	270 ± 1	300 ± 10	390 ± 10	400 ± 30
n-Cu(OH) ₂ -b	-53.8 ± 0.7	N/A ^b	1400 ± 100	240 ± 2	250 ± 10	280 ± 1	290 ± 2
m-CuO	-28.5 ± 0.9	200–2000	1300 ± 200	1300 ± 500	1500 ± 600	N/A ^e	N/A ^e
m-Cu	-32.5 ± 2.9	>10,000	N/A ^e	N/A ^e	N/A ^e	N/A ^e	N/A ^e

^a primary sizes were measured by TEM, n = 100

^b primary size could not be obtained because particles are of undefined morphology

^c Standard error was measured from three experiments

^d HA = humic acid, which was added as a stabilizing agent

^e N/A indicates that particles size exceeds instrumentation range (0.3 nm – 3 μM) according to the manufacturer of the Dynamic Light Scattering machine.

IC₅₀ values, % Cu ion dissolution, amount of cell-associated Cu by sucrose gradient centrifugation, and % Cu bioavailable by biosensor stain for different Cu species and different bacteria studied herein. Error bars represent standard deviation of measurements.

Table 2

NPs	<i>E. coli</i> IC ₅₀ (μg/mL)	<i>L. brevis</i> IC ₅₀ (μg/mL)	%Cu ion dissolution <i>in vitro</i>				Cu associated with cells by sucrose gradient centrifugation (ppm/10 ⁹ cells)		%Cu bioavailable (<i>E. coli</i> biosensor)
			water	<i>E. coli</i> (MMD) media	<i>L. brevis</i> MRS media		<i>E. coli</i> (1 mg/mL)	<i>L. brevis</i> (1 mg/mL)	
CuCl ₂	38 ± 8	7.8 ± 0.5	100	100	100		N/A ^a	N/A ^a	100
CuSO ₄	140 ± 23	24 ± 3	100	100	100		N/A ^a	N/A ^a	--
n-Cu	120 ± 14	5.7 ± 0.2	0.1 ± 0.0	9.9 ± 0.4	20.7 ± 0.4		10.4 ± 0.1	10.5 ± 0.3	100
n-CuO	160 ± 17	3.6 ± 0.1	0.0 ± 0.0	7.5 ± 0.1	10.9 ± 0.1		4.3 ± 0.1	3.9 ± 0.1	70
n-Cu(OH) ₂ -a	>250	4.0 ± 0.1	2.7 ± 0.1	9.9 ± 0.6	21.8 ± 0.6		8.3 ± 0.3	5.4 ± 0.1	17.5
n-Cu(OH) ₂ -b	>250	6.2 ± 0.9	5.4 ± 0.2	10.8 ± 0.6	17.9 ± 0.6		8.0 ± 0.4	5.6 ± 0.1	--
m-CuO	>250	>250	0.0 ± 0.0	2.9 ± 0.3	3.3 ± 0.3		1.2 ± 0.0	0.9 ± 0.0	4.4
m-Cu	>250	120 ± 24	0.1 ± 0.0	2.1 ± 0.8	5.6 ± 0.8		0.4 ± 0.0	1.5 ± 0.1	10.9

^aNA = not applicable, because it was not possible to separate ionic Cu from bacterial cells using sucrose gradient centrifugation.