Published in final edited form as:

ACS Appl Mater Interfaces. 2020 November 11; 12(45): 50203–50211. doi:10.1021/acsami.0c11052.

Comparative Determination of Cytotoxicity of Sub-10-nm Copper Nanoparticles to Prokaryotic and Eukaryotic Systems

Savanna S. Skeeters¹, Ana C Rosu¹, Divyanshi², Jing Yang³, Kai Zhang^{1,*}

¹Department of Biochemistry, School of Molecular and Cellular Biology, 600 S Matthews Avenue, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

²Department of Cell and Developmental Biology, School of Molecular and Cellular Biology, 600 S Matthews Avenue, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

³Department of Comparative Biosciences, 2001 South Lincoln Avenue, University of Illinois at Urbana-Champaign, Urbana, IL 61802.

Abstract

Copper nanoparticles demonstrate antibacterial activity but their toxicity to eukaryotic systems is less understood. Here, we carried out a comparative study to determine the biocompatibility and cytotoxicity of sub-10 nm copper nanoparticles to a variety of biological systems, including prokaryotic cells (*Escherichia coli*), yeast, mammalian cell lines (HEK293T, PC12), and zebrafish embryos. We determined the bearing threshold for the cell-death-inducing concentration of copper nanoparticles by probing cell growth, viability, as well as embryological features. To exclude the partial toxicity effect from the remnant reactants, we developed a purification approach using agarose gel electrophoresis. Purified CuONP solution inhibits bacterial growth and causes eukaryotic cell death at 170 and 122.5 ppm (w/w) during the 18 h of treatment, respectively. CuONP significantly reduces the pigmentation of retina pigmented epithelium of zebrafish embryos at 85 ppm. The cytotoxicity to eukaryotic cells could arise from the oxidative stress induced by CuONP. This result suggests that small copper nanoparticles exert cytotoxicity in both prokaryotic and eukaryotic systems, and therefore, caution should be used to avoid direct contact of copper nanoparticles to human tissues considering the potential use of copper nanoparticles in the clinical setting.

Graphical Abstract

*Corresponding Author To whom correspondence should be addressed: Kai Zhang, kaizkaiz@illinois.edu.

Author Contributions

A.C.R. synthesized the CuONPs and developed methods for isolating them. S.S.S. and K.Z. conceived the experimental design for measuring toxicity in bacteria, yeast, and HEK293T, and prepared the figures. D., K.Z., and J.Y. conceived the experimental design for fish embryological assay. S.S.S., A.C.R. and D. conducted the experiments. S.S.S., A.C.R. and K.Z. prepared the manuscript. All authors have given approval to the final version of the manuscript.

Supporting Information

Calibration of the concentration of the remnant CuCl₂ reactant. CuONP induces oxidative stress in HEK293T cells. The hydrodynamic radius of purified CuONP. Zeta potential and conductivity of purified CuONP. (PDF)

The authors declare no competing interests.

Keywords

Copper Nanoparticles; Green Synthesis; Cytotoxicity; Prokaryotic; Eukaryotic; Zebrafish Embryos

INTRODUCTION

Metallic nanoparticles have attracted investigators from a number of research fields, including physics, chemistry, material sciences, and biological sciences, owing to their advantages in catalysis¹, bio-sensing²⁻⁷, and pharmaceuticals⁸⁻¹⁰. As the size of the nanoparticle decreases, its increased surface-area-to-volume ratio changes the physical, optical, and biological properties of the particle. Of particular interests are the inert metallic nanoparticles (silver, gold, and copper). Because surface plasmon resonance in silver and gold results in enhanced light absorption and scattering in the visible light spectrum, both silver and gold nanoparticles have been widely used in biological imaging and sensing. In addition, silver nanoparticles have been widely studied for their cytotoxicity towards bacterial and mammalian cells¹¹⁻¹². However, the suboptimal stability limits the application of silver nanoparticles in biological systems.

Being more stable than silver, copper has thus gained considerable attention for its antimicrobial properties. Multiple studies have shown that copper nanoparticles exert cytotoxicity like silver nanoparticles¹³⁻¹⁶. Indeed, solid copper and copper films have been used to create sterile surfaces on countertops, catheters, and cotton¹⁷⁻¹⁸. Copper nanoparticles of 20 to 500 nm exhibit toxicity towards multidrug-resistant bacteria and biofilms¹⁹⁻²¹. Because the reactivity of metals can increase as the size of the nanoparticle decreases¹¹⁻¹², it is expected that smaller copper nanoparticles could be more effective in their biological applications. Unfortunately, currently developed synthetic routes for copper nanoparticles mostly generate particles larger than 10 nm¹. Production of sub-10 nm copper particles has been challenging until recently. For example, the Wu group reported an assay of synthesizing small copper nanoparticles (sub-2 nm in diameter) without the use of extreme temperatures or pressures²². These sub-10 nm copper nanoparticles have shown great potential in biomedical and engineering, particularly in bioimaging²³, biosensing²⁴,

production of conductive nanoink²⁵, electrode design²⁶, catalysis²⁷, and photovoltaics²⁸. The convenient synthetic approach and the resulting stable particle products inspire us to further characterize the physicochemical and biological properties of sub-10 nm copper nanoparticles. Indeed, the antibacterial effects of embedded small copper nanoparticles (2.5 nm)²⁹, as well as the cytotoxicity of larger (25-100 nm) copper nanoparticles to mammalian cell lines³⁰, have been reported. However, comparative studies for the cytotoxicity of the sub-10-nm copper nanoparticles in prokaryotic and eukaryotic systems remains limited.

In contrast to the antibacterial effect of copper nanoparticles in isolation, the examination of the antibacterial effect within eukaryotic hosts requires a comparative, parallel study of the toxicity in both bacterial and eukaryotic systems, which remains unavailable in the literature. Thus, in this work, we set out to determine the biocompatibility and selectivity of the antimicrobial effects of sub-10 nm copper nanoparticles between multiple eukaryotic and prokaryotic systems. Here, we define biocompatibility as the copper concentration that the eukaryotic system (host) can withstand (safe bearing level). We define selectivity as the differential toxicity effect of copper nanoparticles to bacteria and eukaryotic cells (effective bacterial killing). An excellent antibacterial reagent would ideally have high biocompatibility and high selectivity. In this work, we used HEK293T and PC12 cells as representative eukaryotic model systems and *Escherichia coli* as representative bacteria. We also determined the toxicity of CuONP in yeast, a eukaryotic system with the cell wall. We further determined the effects of sub-10 nm CuONP in developing zebrafish embryos, particularly on the pigmentation of retina pigmented epithelium, a crucial tissue for eye development. The goal of this work is to provide a comparative study of the biological effects of sub-10 nm copper particles on multiple systems from bacteria, yeast, mammalian cells, and live zebrafish embryos.

RESULTS AND DISCUSSION

Synthesis and characterization of copper/copper oxide nanoparticles

Copper nanoparticles were synthesized by following the previous studies^{22, 29}. Briefly, CuCl₂ was reduced by L-ascorbic acid, which also acts as the surfactant, followed by incubation in the convection oven at 80 °C for 22 h. UV-vis spectroscopy of the resulting solution (Figure 1A) shows the appearance of an absorption peak around 350 nm, as well as the disappearance of the 640-840 nm absorption from the CuCl₂ solutions, indicating the formation of copper/copper oxide nanoparticles (referred to as CuONP). It was postulated that the reduction of Cu²⁺ to Cu⁰ by L-ascorbic acid generates a semi dehydroascorbic acid intermediate²². We further outline a possible mechanism for this reaction: deprotonated L-ascorbic acid coordinates with Cu²⁺ and allows for a two-electron transfer process to produce dehydroascorbic acid and Cu⁰ (Figure 1B). Consumption of ascorbic acid was confirmed by the disappearance of 950-1960 cm⁻¹ peaks when we overlaid the Fourier-transform infrared (FT-IR) spectra of particle solution and pure ascorbic acid (Figure 1B).

The formation of the CuONP nanoparticle was further confirmed by electron microscopy (Figure 1C). Larger aggregates in the solution were removed by centrifugation, and the remaining supernatant was dried on the TEM grid before imaging. Representative copper/copper oxide particles are indicated by dashed circles in Figure 1C. The inset shows the

crystalline lattice of the nanoparticles. Thus, we confirmed the successful production of sub-10 nm CuONP. The estimated stock concentration of CuONP is 300 ppm (w/w).

Copper nanoparticles reduce the growth rate of bacteria and yeast and cause dose-dependent toxicity

Copper nanoparticles are well established as antimicrobial agents²¹, but the biotoxicity of sub-10 nm copper/copper oxide nanoparticles has yet been characterized. To determine the biotoxicity of these small CuONP, we measured the growth curve of bacteria with dose-dependent particle solutions. We first produced a starter culture by growing an ampicillin-resistant *E. Coli* strain to the log phase in Lennox broth containing ampicillin. From this starter, solutions containing freshly synthesized CuONP (at 3, 6, 12, and 15 ppm, w/w) were inoculated and grown overnight in a plate reader at 37 °C with constant shaking and continuous measurement of optical density at 600 nm (Figure 2A). Cells with 3 ppm CuONP solution showed typical growth curves, whereas those in 6 ppm CuONP solution grew at a slower rate and did not reach the same endpoint stationary optical density. Cells in 12 ppm CuONP solution grew significantly slower and reached lower endpoint optical density than those in 6 ppm CuONP solution. Cells with 15 ppm solution did not grow at all.

To determine if the CuONP solution inhibits cell growth or caused cell death, we recover the endpoint cells on marked LB plates supplemented with ampicillin. Ten microliters of cells were dropped on the plate, incubated overnight, and checked for colonies the following day. Cells containing 3-9 ppm CuONP solution consistently grew colonies. Cells with 12 ppm solution grew colonies, but the size of the spread is far smaller than the 3-9 ppm group (Figure 2B). Cells with 15-21 ppm CuONP solution never grew colonies. These results indicated that the copper oxide nanoparticles exert a bactericidal effect at concentrations greater than 12 ppm.

Next, we sought to understand if this cytotoxicity was limited to bacteria or also affected eukaryotes. First, we repeated the cell growth assay with *Saccharomyces cerevisiae* yeast cells with different doses of CuONP solution. Consistent with the bacteria result, we observed a strong inhibition of cell growth when more than 6 ppm CuONP was incubated (Figure 2C). We then proceeded to determine the cytotoxicity of CuONP in mammalian cells by selecting two cell lines as the model systems: human embryonic kidney cells (HEK293T cell line) and rat neuronal cells (PC12 NS1). These two cell lines were selected because 1) they are commonly used in biomedical research, and 2) their biological make-up represents distinct eukaryotic cell types (epithelial and neuronal). Cell viability was determined by dual staining with calcein AM (stains live cells) and propidium iodide (stains dead cells). In HEK293T cells, CuONP cytotoxicity began at as little as 6 ppm with nearly complete cell death by 21 ppm (Figure 2 D-E). In PC12 cells, toxicity began at 6 ppm with nearly complete cell death at 12 ppm (Figure 2 F-G). Therefore, this CuONP solution was not solely toxic to bacteria but to rat and human cell lines.

It is well documented that Cu(II) could induce oxidative stress in bacteria and exerts the toxicity effect³¹. Here, we want to determine if the cytotoxicity in mammalian cells involves oxidative stress as well. To do this, we compared HEK293T cell death in cultured treated with CuONP or CuONP and 50 mM beta-mercaptoethanol (BME), a reducing agent. We

expect that BME could partially rescue cell viability given an appropriate level of oxidative stress. Consistent with previous result, CuONP (6 ppm) caused significant cell death measured by propidium iodide staining. In the presence of 50 mM BME, however, we observed more than half of the cell population is stained as viable by Calcein AM (green) (Supplementary Figure S2). As the concentration of CuONP continues to increase, this rescuing effect is less significant. This result suggests that the cytotoxicity of CuONP in mammalian cells also involves its capacity to induce oxidative stress.

Unreacted reactants for the synthesis of copper nanoparticles cause partial toxicity in bacteria and mammalian cells

Although we could successfully reproduce the previous synthetic route to generate sub 10-nm copper nanoparticles, we note that no purification step was developed in previous work. Although the majority of physical and chemical properties of nanoparticles may not be affected by the remnant reactants, we cannot ignore their contribution to cell toxicity. We first determine the remaining concentration of CuCl_2 by UV/Vis spectroscopy. CuCl_2 has a broad absorption peak at 800 nm. This peak virtually disappeared in the final diluted CuONP solution. But in high concentrations of CuONP solution, light scattering significantly broadens the CuONP absorption peak at 800 nm, which may artificially inflate the estimation of particle concentration. With this in mind, if none the CuCl_2 was consumed in the reaction, the maximum possible concentration of CuCl_2 in a 21 ppm solution would be 0.7 mM (Supplemental Figure S1). Based on the UV-Vis absorption spectrum of the CuONP, we estimate the remnant CuCl_2 concentration should be less than 0.1 mM, which should not cause significant cytotoxicity based on the dose-dependent study of CuCl_2 alone (Figure 3A). We also expect that unreacted L-ascorbic acid could partially cause cytotoxicity. When prepared in isolation, 5 mM L-ascorbic acid was sufficient to cause significant cell death (Figure 3A), which is assayed by calcein AM/PI staining in HEK293T cells (Figure 3B). The partial contribution from the L-ascorbic acid should, therefore, be separated from that of CuONP alone.

Develop a purification strategy for copper nanoparticles with agarose gel electrophoresis

Next, we aim to quantify the cellular toxicity of purified sub-10-nm nanoparticles. However, we found that traditional purification assays, such as chromatography and ultracentrifugation, could not be used to purify the sub-10-nm copper particles due to the small particle size. Additionally, extractions using harsh solvents could leave behind toxic components convoluting the direct killing efficiency of the copper oxide nanoparticles. To address these challenges, inspired by previous work of gold nanoparticles purification³²⁻³³, we developed a purification strategy based on agarose gel electrophoresis. While the copper nanoparticles themselves are not charged, we speculated that the surfactant could provide enough negative charge to allow for particle movement in an electric field (Figure 4A).

To test this idea, we prepared 2% agarose gel in 40 mM Tris-acetate buffer (TA buffer) and set two wells at the initial position (IP) and final position (FP), which locate approximately 2 cm apart (Figure 4B). Crude CuONP solution containing unreacted CuCl_2 and L-ascorbic acid was loaded into the well in IP. The nanoparticles, which are tinted brown from Cu^0 , can be readily visualized through the opaque agarose gel. When an electric field is applied, as

expected, we observed the collective movement of nanoparticles toward the positive charge, indicating that surfactants provide negative charges on the particle surface. When traveling to the final position (FP), the dark band containing particles was collected by a long pipet tip. The collection should consist of CuONP primarily because the L-ascorbic acid and CuCl_2 should not travel in the same band. The solution was then dried in a rotary evaporator, massed, and resuspended in DEPC water at a concentration of 2000 ppm (w/w). UV/Vis spectroscopy confirmed the presence of the nanoparticles in the purified solution, as evidenced from the peak around 380 nm (Figure 4C).

TEM images of purified CuONP show that most particles are spherical (Figure 4D), with an average diameter of approximately 5 nm (Figure 4E). To determine if particle aggregation could occur, we measured the hydrodynamic radius of these particles using dynamic light scattering. Purified CuONP at 2000 ppm showed a hydrodynamic radius of 648.2 nm, significantly larger than the size of single particles. Intriguingly, the diluted sample showed a reduction of the average hydrodynamic radius. At 2.5 ppm, the hydrodynamic radius was reduced to 142.5 nm (Table S1). This result indicates that although particle agglomeration occurs, they do not form a large, hard agglomerate but could dissociate into stable, smaller clusters. Indeed, the average zeta potential of purified CuONP at 0.3 mg/mL is -29.7 mV, indicating a stable structure with negative surface charges, consistent with the migration direction in the gel electrophoresis experiment (Table S2).

Determine the cytotoxicity of purified nanoparticles

The purified nanoparticles were then tested for their toxicity in bacteria and human kidney cells. Ampicillin-resistant *E. Coli* cultures were treated with 8.5, 42.5, 85, 122.5, and 170 ppm of purified CuONP solutions. As a control, cells were also treated with TA buffer at the equivalent volume of corresponding CuONP solutions (1%, 5%, 10%, 15%, and 20% v/v). Cells experienced toxicity at 170 ppm CuONP treatment but in none of the buffer-treated conditions, indicating that the toxicity seen was the direct result of the isolated nanoparticles (Figure 5A).

Isolated CuONP toxicity was then investigated on human cells. HEK293T cells were also treated with 8.5, 42.5, 85, 122.5, and 170 ppm purified CuONP solutions, or TA buffer. Calcein AM and propidium iodide staining showed that HEK293T cells experienced about 20% death at 122.5 ppm, and the death percentage increased to 40% at 170 ppm purified CuONP (Figure 5B). In order to better understand the effect of CuONPs on the metabolism of mammalian cells, we used MTS assay (MTS tetrazolium based protocol) to measure the population of metabolically active cells based on the activity of NAD(P)H-dependent dehydrogenase. Consistent with the live/dead cell staining results, HEK293T cells show decreased metabolic activity as the concentration of CuONP increases from 8.5 to 170 ppm in a dose-dependent manner. TA buffer alone had no significant effects on the metabolic activity of HEK293T cells (Figure 5C).

CuONP reduces pigmentation of retina pigmented epithelium in zebrafish embryos

To determine if sub-10 nm CuONP exerts toxicity in living organisms, we set out to probe the effect of CuONP on early embryonic development in zebrafish (Figure 6A). At 24 hours

post fertilization (hpf), we manually removed the chorion from the embryos and treated embryos with 85 ppm CuONP. At 48 hpf, the development of retina pigmented epithelium (RPE) became evident in the majority (83.6%) of the untreated control embryos, judged by pigmentation in the RPE (Figure 6B). In contrast, the development of RPE was clearly delayed in CuONP treated embryos, with 61.4% of embryos showing significantly reduced pigmentation accumulated in the RPE (Figure 6C). This result suggests that CuONP may selectively affect specific cell types during early vertebrate development, likely related to the oxidative stress that CuONP induced in developing embryos.

With growing production and applications of nanometer-sized materials, considerations should be taken so that the nanoparticle material is safe for use and, of arguably equal importance, can safely be disposed of. While many nanoparticles are tested for human contacts, such as in wound healing³⁴⁻³⁶, food³⁷⁻³⁹, sunscreens⁴⁰, disinfectants⁴¹, and biosensors for drug delivery³⁷, many are produced with little or no information on their biocompatibility⁴². Furthermore, it is also not always required to disclose nanoparticles as ingredients⁴³.

Here, we focus on copper/copper oxide nanoparticles, which are used as “catalysts, magnetic storage media, solar energy transformer, solar cells and lithium batteries, semiconductors and field emission, gas sensors, biosensor in drug delivery, electronic chips, and heat transfer nanofluids”³⁷. Increasing research is being done to understand and manage the risks to the food chain, aquatic ecosystem, and microorganisms^{40, 44-45}. But a comparative study for the cytotoxicity of copper nanoparticles in prokaryotic and eukaryotic cells has not been available.

We used a protocol based on an environmentally friendly synthetic route for copper (oxide) nanoparticles to produce sub-10-nm copper nanoparticles²². This synthetic route utilizes L-ascorbic acid (Vitamin C) and copper chloride with no extreme pressures or temperatures. This promising synthesis is one of the growing collection of green synthesis techniques⁴⁶. We sought to further characterize the biocompatibility of these nanoparticles to gain insight into their applications as well as assess their potential risks. The main difference in our synthesis was that the size of the nanoparticles was slightly larger than 2 nm but still sub-10 nm, which may result from different heating methods.

Our results indicate that the residual reactants, particularly the L-ascorbic acid, in the solution can contribute to some of the toxicity (Figure 3). To determine the contribution of death directly from the nanoparticles, we purified the sub-10-nm nanoparticles using gel electrophoresis. We found that the minimal inhibitory concentration for purified nanoparticles in bacteria was 170 and 122.5 ppm in human cells (Figure 5), respectively. To further determine the cytotoxicity of sub-10 nm copper particle for live organisms, we probed the effect of purified CuONP on the developmental process of zebrafish embryos. At 85 ppm, we found that CuONP significantly reduced the pigmentation of the embryos, particularly at the retina pigmented epithelium. Ongoing efforts aim to further determine the effect of surface bio-conjugation on the cytotoxicity of sub-10-nm copper nanoparticles.

CONCLUSION

In this work, we carried out a comparative study to determine the biological effects of sub-10 nm copper nanoparticles on biological systems ranging from bacteria, yeast, mammalian cell lines, and live zebrafish embryos. Using cell viability and embryological assays, we determined that the safe concentration threshold for purified nanoparticle is approximately 10 mg/mL. Above this threshold, sub-10 nm copper particles inhibit the growth of bacteria and yeast, induce mammalian cell death, and cause developmental defects in the pigmentation of retina pigmented epithelium in zebrafish embryos. The result of cytotoxicity of CuONP to the tested prokaryotic and eukaryotic systems suggests that caution should be used to avoid direct contact of copper nanoparticles to human tissues considering the potential use of copper nanoparticles in the clinical setting.

EXPERIMENTAL SECTION

Synthesis of copper oxide nanoparticles

Copper oxide nanoparticles (CuONPs) were synthesized using the Wu group's method with some modifications. First, 5 mL of 600 mM L-ascorbic acid was heated to 80 °C and then 5 mL of 10 mM CuCl₂ was added dropwise to magnetically stirred L-ascorbic solution. The mixture was then transferred to a capped bottle and put in an MTI gravity convection oven (serial number JST 2010) at 80 °C for 22 hours.

The resulting CuONP solution was centrifuged twice at 4900 G for 10 minutes in the Sorvall ST 16R Centrifuge (serial number 41717319). The supernatant was collected with a BD 10 mL syringe (serial number 300912) and filtered through a Millex Syringe-driven Filter Unit of 0.22 µm from Merck Millipore (serial number SLMP025SS). CuONPs were stored in a capped bottle at room temperature in the dark.

UV/Spectroscopy

Solutions of 600 mM L-ascorbic acid, 10 mM CuCl₂, and 600 mM L-ascorbic acid + 10 mM CuCl₂ were left in an MTI convection oven for a period of 22 hours. The resulting L-ascorbic acid, CuCl₂, and CuONP solutions were calibrated using a NANODROP 2000c Spectrophotometer (serial number P10R41191). L-ascorbic acid solution and CuCl₂ solution were measured at 600 mM, 300 mM, 150 mM, and 10 mM, 5 mM, 2.5 mM, respectively.

Infrared Spectroscopy

Synthesized nanoparticles were characterized by IR. Both a CuONP solution and an unreacted 600mM L-ascorbic acid spectrum were obtained using a Perkin Elmer Spectrum Two FT-IR.

Transmission electron microscopy

Electron Microscopy data collection was done with staff from the University of Illinois at Urbana Champaign on the JOEL2200FS TEM microscope using Graphene Oxide on Quantifoil R2/4 200 grid mesh Cu grids (Structure Probe Inc., 4510C-BA). Sample preparation followed a previous protocol using ultrasonic nebulization⁴⁷.

Isolating CuONPs using gel electrophoresis

An agarose gel was made by mixing 2 g of agarose with 100 mL of 40 mM Tris-acetate solution not containing EDTA (TA buffer). Two gel combs were placed about 2 cm apart in the gel until solidified. After covering gel with TA buffer, 75 μ L of CuONP solution was added to each of the wells in the set closest to the anode. The gel was then run at 140 mV until the CuONPs reached the set of wells closest to the cathode. Then 100 μ L of solution was collected from each well. To ensure that as many CuONPs were recovered as possible, the agarose gel was run for additional 30 seconds and 100 μ L was collected from every well in the set closest to the cathode. This process was repeated until all CuONPs were collected.

To concentrate the CuONPs isolated using gel electrophoresis, solution was transferred to a 1.5 mL and the liquid was removed by a rotary evaporator. The dry CuONPs were then weighed and dissolved in DEPC water. Considering the salt in the TA buffer will also contribute to the dry weight, we used the initial reactant CuCl₂ to estimate the final concentration of CuONP particles assuming a complete reaction. A 10 mL of crude CuONP was estimated to produce 1.6 mL of 2.0 mg/mL (2000 ppm, w/w) of purified particles. This stock was then diluted to produce the final concentration used in cell- and zebrafish-based assays.

Dynamic light scattering and zeta potential measurement

Purified CuONP was diluted to different concentration in the nanopure water from the stock solution. Dynamic light scattering and zeta potential measurements were done with a Malvern Zetasizer NanoZs using purified nanoparticles. Data analysis was performed using the Malvern Zetasizer software.

Bacterial and yeast growth

E. coli (Stellar Cells, CAT) were grown transformed with a pCS2 plasmid containing EGFP and ampicillin resistance. These cells were grown in Lennox Broth containing ampicillin to log phase to make a starter culture. Each biological replicate came from a new starter culture from that same bacterial stock. From this starter culture, cells were added to culture tubes with freshly autoclaved LB + ampicillin to a final optical density at 600 nm of 0.1. Dose-dependent of purified CuONPs in TA buffer were used for cell treatment, whereas equal volume of TA buffers alone were used as a control. Bacteria was incubated for 18 hours. The optical density at 600 nm was measured either continuously via Biotek Synergy™ H4 Hybrid Multi-Mode Microplate Reader or Nanodrop before and after incubation for endpoint experiments.

The *Saccharomyces cerevisiae* (BJ3505 strain) cells⁴⁸ were grown at 30 °C overnight in YPD media with ampicillin. From this starter culture, cells were diluted into fresh YPD media containing copper nanoparticles in 96 well plates. Cells were grown at 30 °C in a plate reader with constant shaking for 18 hours before OD at 600 nm was measured. YPD media contains 10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose.

Determining bacterial colony-forming units (CFUs)

After incubation for 18 hours and growth measurement at OD of 600 nm, cells from each condition were diluted 1:10⁶ in the LB broth. Then, 10 μ L of diluted cells were pipetted on a marked agar plate and incubated 20 hours at 37 °C in VWR incubator (serial number P10R41732).

CuONP treatment of HEK293T cells

In a 24 well plate HEK293T were treated with media (DMEM + 10% FBS (Dulbecco's Modification Eagle's Medium with 4.5 g/L glucose, L-glutamine & sodium pyruvate, serial number 07517002)) containing CuONP at 8.5, 42.5, 85, 122.5, and 170 ppm or, as a control, equal volume of TA buffer. To determine toxicity, copper nanoparticle or TA-containing media was removed and replaced with DMEM + 10% FBS containing calcein AM (life technologies, serial number C3100MP) and propidium iodide (PI) and incubated as recommended in manufacturer instructions. The calcein AM and PI stain-treated cells were imaged using a Lecia DM6i microscope with LAS X software. Analysis and quantification were performed using FIJI software.

MTS Assay

One hundred (100) μ L CellTiter 96 AQueous One Solution Cell Proliferation Assay (serial number G3580) was added to each well of a 24-well plate. The plate was then placed in an incubator at 37 °C, 5% CO₂, for one hour as according to the manufacturer's protocol. The OD of the wells at 495 nm was measured using Biotek Synergy™ H4 Hybrid Multi-Mode Microplate Reader.

CuONP treatment of zebrafish embryos

The cleavage-stage zebrafish (*Danio rerio*) embryos were collected from breeding set up between wild type AB line Zebrafish parents 0.5-1 hour post-fertilization (hpf). At 24 hpf, embryos were dechorionated and were grown in 1 $\times$ E3 media with methylene blue (untreated control) or were treated with 10 mg/mL CuONP (diluted in the same E3 medium). At 24-30 hours post-treatment, the embryos were fixed with 4% paraformaldehyde in 1 $\times$ phosphate buffer saline (PBS) for one hour, followed by thorough rinsing with 1 $\times$ PBS. 1 $\times$ E3 media was diluted from the 60 $\times$ stock that is made by dissolving 17.52 g NaCl, 0.76 g KCl, 2.92 g CaCl₂·H₂O, and 4.88 g MgSO₄·7H₂O in water. To every 1 L of 1 $\times$ E3, 1 mL of 0.01% methylene blue was added to prevent fungal growth. Embryos were imaged using the Olympus XC30 camera and the cellSens application. All experimental procedure on zebrafish embryos was approved by the Illinois Institutional Animal Care and Use Committee (IACUC).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

A.C.R. would like to thank David Bergandine (University Laboratory High School, Urbana, IL) for his mentorship. S.S.S. thanks Prof. Catherine Murphy and the lab members (UIUC) for their insight and recommendations and the

staff at MRL (UIUC) for assistance with TEM. We also thank Prof. Tobias Meyer (Stanford University) for the gift of PC12 NS1 cells, Prof. Lin-Feng Chen (UIUC) for the gift of HEK293T cells, and Prof. Rutilio Fratti (UIUC) for the gift of yeast strains.

Funding Sources

This work is supported by Westcott Bioscience Fellowship from the Department of Biochemistry at UIUC (S.S.S.) and NIH grants R35GM131810 (J.Y.) and R01GM132438 (K.Z.).

ABBREVIATIONS

CuONP	copper/copper oxide nanoparticles
calcein-AM	calcein acetoxymethyl
FT-IR	Fourier-Transfer Infrared
HEK293T	human embryonic kidney 293T
LOEC	lowest observed effects concentration
PC12	adrenal phaeochromocytoma
PI	propidium iodide
TEM	transmission electron microscopy

REFERENCES

- (1). Gawande MB; Goswami A; Felpin FX; Asefa T; Huang X; Silva R; Zou X; Zboril R; Varma RS Cu and Cu-Based Nanoparticles: Synthesis and Applications in Catalysis. *Chem Rev* 2016, 116 (6), 3722–3811. [PubMed: 26935812]
- (2). Han Y; Zhang F; Gong H; Cai C Double G-quadruplexes in a copper nanoparticle based fluorescent probe for determination of HIV genes. *Mikrochim Acta* 2018, 186 (1), 30. [PubMed: 30564958]
- (3). Can Chen FG, Wang Yongxiang, Yu Haidong, Li Lin, Yaang Sheng, Liu Jinhua, Huang Wei. Design of a nanoswitch for sequentially multi-species assay based on competitive interaction between DNA-templated fluorescent copper nanoparticles, Cr³⁺ and pyrophosphate and ALP. *Talanta* 2019, 205.
- (4). Chawla S; Rawal R; Pundir CS Fabrication of polyphenol biosensor based on laccase immobilized on copper nanoparticles/chitosan/multiwalled carbon nanotubes/polyaniline-modified gold electrode. *J Biotechnol* 2011, 156 (1), 39–45. [PubMed: 21864588]
- (5). Dhanasekaran Solairaj PR, Muthukumaran Palanisamy, jWilson Jeyara. Studies on electrochemical glucose sensing, antimicrobial activity and cytotoxicity of fabricated copper nanoparticle immobilized chitin nanostructure. *International Journal of Biological Macromolecules* 2017, 101, 668–679. [PubMed: 28363648]
- (6). Ibupoto ZH; Khun K; Beni V; Liu X; Willander M Synthesis of novel CuO nanosheets and their non-enzymatic glucose sensing applications. *Sensors (Basel)* 2013, 13 (6), 7926–7938. [PubMed: 23787727]
- (7). Wang Z; Wang Z; Lu WW; Zhen W; Yang D; Peng S Novel biomaterial strategies for controlled growth factor delivery for biomedical applications. *NPG Asia Materials* 2017, 9 (10), e435.
- (8). Sharma Arun K., A. K., Sahu Megha, Sharma Gunjan, Datusalia Ashok K., Rajput Satyendra K.. Exercise Preconditioning and Low Dose Copper Nanoparticles Exhibits Cardioprotection Through Targeting GSK-3 β Phosphorylation in Ischemia: Reperfusion Induced Myocardial Infarction. *Microvascular Research* 2018, 120, 56–66.

- (9). Cholewinska E; Ognik K; Fotschki B; Zdunczyk Z; Juskiewicz J Comparison of the effect of dietary copper nanoparticles and one copper (II) salt on the copper biodistribution and gastrointestinal and hepatic morphology and function in a rat model. *PLoS One* 2018, 13 (5), e0197083. [PubMed: 29758074]
- (10). Arancibia Sergio, Andrea; Barrientos JT; Alejandro Escobar CJ; Beltrán. Copper oxide nanoparticles recruit macrophages and modulate nitric oxide, proinflammatory cytokines and PGE2 production through arginase activation. *Nanomedicine* 2016, 11, 1237–1251. [PubMed: 27079258]
- (11). Perde-Schrepler M; Florea A; Brie I; Virag P; Fischer-Fodor E; Vâlcău A; Gurzu E; Lisencu C; Maniu A Size-Dependent Cytotoxicity and Genotoxicity of Silver Nanoparticles in Cochlear Cells In Vitro. *Journal of Nanomaterials* 2019, 2019, 1–12.
- (12). Korshed P; Li L; Liu Z; Mironov A; Wang T Size-dependent antibacterial activity for laser-generated silver nanoparticles. *Journal of Interdisciplinary Nanomedicine* 2019, 4 (1), 24–33.
- (13). Yoon KY; Hoon Byeon J; Park JH; Hwang J Susceptibility constants of *Escherichia coli* and *Bacillus subtilis* to silver and copper nanoparticles. *Sci Total Environ* 2007, 373 (2-3), 572–575. [PubMed: 17173953]
- (14). Azam A; Ahmed AS; Oves M; Khan MS; Habib SS; Memic A Antimicrobial activity of metal oxide nanoparticles against Gram-positive and Gram-negative bacteria: a comparative study. *Int J Nanomedicine* 2012, 7, 6003–6009. [PubMed: 23233805]
- (15). Ashajyothi C; Harish KH; Dubey N; Chandrakanth RK Antibiofilm activity of biogenic copper and zinc oxide nanoparticles-antimicrobials collocate against multiple drug resistant bacteria: a nanoscale approach. *Journal of Nanostructure in Chemistry* 2016, 6 (4), 329–341.
- (16). Rodhe Y; Skoglund S; Odnevall Wallinder I; Potacova Z; Moller L Copper-based nanoparticles induce high toxicity in leukemic HL60 cells. *Toxicol In Vitro* 2015, 29 (7), 1711–1719. [PubMed: 26028147]
- (17). McLean RJC; Hussain AA; Sayer M; Vincent PJ; Hughes DJ; Smith TJN Antibacterial activity of multilayer silver–copper surface films on catheter material. *Canadian Journal of Microbiology* 1993, 39 (9), 895–899. [PubMed: 8242490]
- (18). Schmidt MG; Attaway HH; Sharpe PA; John J Jr.; Sepkowitz KA; Morgan A; Fairey SE; Singh S; Steed LL; Cantey JR; Freeman KD; Michels HT; Salgado CD Sustained reduction of microbial burden on common hospital surfaces through introduction of copper. *J Clin Microbiol* 2012, 50 (7), 2217–2223. [PubMed: 22553242]
- (19). Chatterjee AK; Chakraborty R; Basu T Mechanism of antibacterial activity of copper nanoparticles. *Nanotechnology* 2014, 25 (13), 135101. [PubMed: 24584282]
- (20). Vincent M; Duval RE; Hartemann P; Engels-Deutsch M Contact killing and antimicrobial properties of copper. *J Appl Microbiol* 2018, 124 (5), 1032–1046. [PubMed: 29280540]
- (21). Shiv Shankar XT, Rhim Jong-Whan. Properties and characterization of agar:CuNP bionanocomposite films prepared with different copper salts and reducing agents. *Carbohydrate Polymers* 2014, 114, 484–492. [PubMed: 25263917]
- (22). Xiong J; Wang Y; Xue Q; Wu X Synthesis of highly stable dispersions of nanosized copper particles using l-ascorbic acid. *Green Chemistry* 2011, 13 (4), 900–904.
- (23). Wang C; Wang C; Xu L; Cheng H; Lin Q; Zhang C Protein-directed synthesis of pH-responsive red fluorescent copper nanoclusters and their applications in cellular imaging and catalysis. *Nanoscale* 2014, 6 (3), 1775–81. [PubMed: 24352741]
- (24). Wang C; Cheng H; Huang Y; Xu Z; Lin H; Zhang C Facile sonochemical synthesis of pH-responsive copper nanoclusters for selective and sensitive detection of Pb²⁺ in living cells. *Analyst* 2015, 140 (16), 5634–5639. [PubMed: 26133700]
- (25). Hokita Y; Kanzaki M; Sugiyama T; Arakawa R; Kawasaki H High-Concentration Synthesis of Sub-10-nm Copper Nanoparticles for Application to Conductive Nanoinks. *ACS Appl Mater Interfaces* 2015, 7 (34), 19382–19389. [PubMed: 26287811]
- (26). Wu S Preparation of fine copper powder using ascorbic acid as reducing agent and its application in MLCC. *Materials Letters* 2007, 61 (4), 1125–1129.

- (27). Chahdoura F; Pradel C; Gómez M Copper(I) Oxide Nanoparticles in Glycerol: A Convenient Catalyst for Cross-Coupling and Azide–Alkyne Cycloaddition Processes. *ChemCatChem* 2014, 6 (10), 2929–2936.
- (28). Zhang J; Wang J; Fu Y; Zhang B; Xie Z Sonochemistry-synthesized CuO nanoparticles as an anode interfacial material for efficient and stable polymer solar cells. *RSC Advances* 2015, 5 (36), 28786–28793.
- (29). Sehmi SK; Noimark S; Weiner J; Allan E; MacRobert AJ; Parkin IP Potent Antibacterial Activity of Copper Embedded into Silicone and Polyurethane. *ACS Applied Materials & Interfaces* 2015, 7 (41), 22807–22813. [PubMed: 26418101]
- (30). Song L; Connolly M; Fernandez-Cruz ML; Vijver MG; Fernandez M; Conde E; de Snoo GR; Peijnenburg WJ; Navas JM Species-specific toxicity of copper nanoparticles among mammalian and piscine cell lines. *Nanotoxicology* 2014, 8 (4), 383–393. [PubMed: 23600739]
- (31). Lemire JA; Harrison JJ; Turner RJ Antimicrobial activity of metals: mechanisms, molecular targets and applications. *Nat Rev Microbiol* 2013, 11 (6), 371–384. [PubMed: 23669886]
- (32). Claridge SA; Liang HW; Basu SR; Frechet JM; Alivisatos AP Isolation of discrete nanoparticle-DNA conjugates for plasmonic applications. *Nano Lett* 2008, 8 (4), 1202–1206. [PubMed: 18331002]
- (33). Parak WJ; Pellegrino T; Micheel CM; Gerion D; Williams SC; Alivisatos AP Conformation of Oligonucleotides Attached to Gold Nanocrystals Probed by Gel Electrophoresis. *Nano Letters* 2003, 3 (1), 33–36.
- (34). Alizadeh S; Seyedalipour B; Shafieyan S; Kheime A; Mohammadi P; Aghdami N Copper nanoparticles promote rapid wound healing in acute full thickness defect via acceleration of skin cell migration, proliferation, and neovascularization. *Biochem Biophys Res Commun* 2019, 517 (4), 684–690. [PubMed: 31400855]
- (35). Rakhmetova AA; Alekseeva TP; Bogoslovskaya OA; Leipunskii IO; Ol'khovskaya IP; Zhigach AN; Glushchenko NN Wound-healing properties of copper nanoparticles as a function of physicochemical parameters. *Nanotechnologies in Russia* 2010, 5 (3-4), 271–276.
- (36). Tao B; Lin C; Deng Y; Yuan Z; Shen X; Chen M; He Y; Peng Z; Hu Y; Cai K Copper-nanoparticle-embedded hydrogel for killing bacteria and promoting wound healing with photothermal therapy. *J Mater Chem B* 2019, 7 (15), 2534–2548. [PubMed: 32255130]
- (37). Maharramov AM; Hasanova UA; Suleymanova IA; Osmanova GE; Hajiyeva NE The engineered nanoparticles in food chain: potential toxicity and effects. *SN Applied Sciences* 2019, 1 (11), 1362.
- (38). Carrillo-Inungaray ML; Trejo-Ramirez JA; Reyes-Munguia A; Carranza-Alvarez C Use of Nanoparticles in the Food Industry: Advances and Perspectives. In *Impact of Nanoscience in the Food Industry*; 2018; pp 419–444.
- (39). Singh T; Shukla S; Kumar P; Wahla V; Bajpai VK Application of Nanotechnology in Food Science: Perception and Overview. *Front Microbiol* 2017, 8, 1501. [PubMed: 28824605]
- (40). Heiligtag FJ; Niederberger M The fascinating world of nanoparticle research. *Materials Today* 2013, 16 (7-8), 262–271.
- (41). Rogers KR; Navratilova J; Stefaniak A; Bowers L; Knepp AK; Al-Abed SR; Potter P; Gitipour A; Radwan I; Nelson C; Bradham KD Characterization of engineered nanoparticles in commercially available spray disinfectant products advertised to contain colloidal silver. *Sci Total Environ* 2018, 619-620, 1375–1384. [PubMed: 29723948]
- (42). Khan I; Saeed K; Khan I Nanoparticles: Properties, applications and toxicities. *Arabian Journal of Chemistry* 2019, 12 (7), 908–931.
- (43). Duncan TV The communication challenges presented by nanofoods. *Nature Nanotechnology* 2011, 6 (11), 683–688.
- (44). Chang Y-N; Zhang M; Xia L; Zhang J; Xing G The Toxic Effects and Mechanisms of CuO and ZnO Nanoparticles. *Materials* 2012, 5 (12), 2850–2871.
- (45). Yugantak Raj Gupta DS, Kannan Monica, Deepa Seetharam, Senthilkumaran Balasubramanian, Basavaraju Yaranguntappa. Effect of copper nanoparticles exposure in the physiology of the common carp (*Cyprinus carpio*). *Biochemical, histological and proteomic approaches Aquaculture and Fisheries* 2016, 1, 15–23.

- (46). Singh J, K. G., Rawat M A Brief Review on Synthesis and Characterization of Copper Oxide Nanoparticles and its Applications. *J Bioelectron Nanotechnol* 2016, 1 (1), 1–9.
- (47). Hinman JG; Hinman JJ; Janicek BE; Huang PY; Suslick KS; Murphy CJ Ultrasonic Nebulization for TEM Sample Preparation on Single-Layer Graphene Grids. *Nano Lett* 2019, 19 (3), 1938–1943. [PubMed: 30742445]
- (48). Jones EW; Zubenko GS; Parker RR Pep4 gene function is required for expression of several vacuolar hydrolases in *saccharomyces cerevisiae*. *Genetics* 1982, 102 (4), 665–677. [PubMed: 6764901]

Figure 1. Characterization of synthesized copper oxide nanoparticles.

A. UV/Vis absorbance of reactants (L-ascorbic acid and CuCl_2) compared to the product after heat (CuONP solution dilution). **B.** IR spectrum of untreated L-ascorbic acid compared to the CuONP solution with inlaid reaction scheme. **C.** TEM imaging of CuONP solution showing nanoparticles of varied shape and size less than 10 nm with crystalline copper lattices (scale bar: 10 nm).

Figure 2. CuONP solution shows toxicity in bacteria, human kidney cells, and rat neuronal cell lines.

A. Growth curves for bacteria treated with 3, 6, 12, or 15 ppm (w/w) unpurified CuONP solution. **B.** Cells grown in the presence of unpurified CuONP in **A** were then plated on agar to determine the cell viability after long-term (33h) exposure. Results are shown as (+) for the appearance of colonies and (-) for no colony formation. **C.** Effects of unpurified CuONP on yeast growth measured by OD at 600 nm. (N=3). **D.** HEK293T cells were treated with unpurified CuONP solution, and cell viability was determined by PI (dead cells) and calcein-AM (live cells) staining. (N=3 except for the 6 ppm and 12 ppm conditions where N=5). **E.** Representative staining images for **D**. **F.** PC12 cells were treated with unpurified CuONP solution, and cell viability was determined by PI (dead cells) and calcein-AM (live cells) staining. **G.** Representative staining images for **F**.

Figure 3. Unpurified CuONP solution has additional toxicity from unreacted reactants.

A. HEK293T cells were treated with the solution of each reactant at low concentrations relative to the starting concentration and cell viability was determined by PI (red, dead cells) and calcein-AM (green, live cells) staining. **B.** Representative images PI and calcein-AM overlay.

Figure 4. Characterization for purified CuONP.

A. Schematic depicting purification of CuONP from solution in an agarose gel. **B.** Photograph of CuONP solution that has traveled from IP to FP before the CuONP is collected from the FP well. **C.** UV/Vis Spectrum of purified CuONP. **D.** TEM images of purified CuONP. **E.** Quantification of the size of the CuONP from TEM imaging.

Figure 5. Determination the cytotoxicity of purified CuONP to mammalian cells.

A. Endpoint growth for bacteria treated with purified CuONP or an equal volume of buffer (N=3). **B.** HEK293T cells were treated with either purified CuONP or buffer and cell viability was determined by propidium iodide (dead cells) and calcein-AM (live cells) staining (N=3). **C.** HEK293T cells were treated with either purified CuONP or buffer and cell proliferation was determined via MTS assay (N=3).

Figure 6. Characterization of the cytotoxicity of purified CuONP to zebrafish embryos.

A. Schematic depicting the determination of CuONP on the embryonic developmental process of zebrafish. Hours post fertilization: hpf. **B.** Representative image of wild-type AB strain embryos incubated in buffer at 48 hpf. The majority (84%) of embryos show normal dark pigmentation across the body. The number of embryos examined: 49. **C.** Representative image of identical AB strain treated with 85 ppm of purified CuONP at 48 hpf. The majority (61%) of embryos show significantly reduced pigmentation across the body, in particular around the retina pigmented epithelium (arrow). The number of embryos examined: 57.