Published in final edited form as:

Nanotechnology. 2013 October 4; 24(39): . doi:10.1088/0957-4484/24/39/395101.

Chitosan coating of copper nanoparticles reduces in vitro toxicity and increases inflammation in the lung

Kristan L.S. Worthington^{a,b}, Andrea A. Dodd^c, Amaraporn Wongrakpanich^b, Imali A. Mudunkotuwa^d, Kranti A. Mapuskar^e, Vijaya B. Joshi^b, C. Allan Guymon^a, Douglas R. Spitz^e, Vicki H. Grassian^d, Peter S. Thorne^c, and Aliasger K. Salem^{a,b,*}

^aDepartment of Chemical and Biochemical Engineering, College of Engineering, University of Iowa, Iowa City, Iowa 52242, USA

^bDepartment of Pharmaceutical Sciences and Experimental Therapeutics, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242

^cDepartment of Occupational and Environmental Health, College of Public Health, University of Iowa, Iowa City, Iowa 52242, USA

^dDepartment of Chemistry, College of Liberal Arts and Sciences, University of Iowa, Iowa City, Iowa 52242, USA

^eFree Radical and Radiation Biology and Toxicology Programs, Department of Oncology, Holden Comprehensive Cancer Center, Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242, USA

Abstract

Despite their potential for a variety of applications, copper nanoparticles induce very strong inflammatory responses and cellular toxicity following aerosolized delivery. Coating metallic nanoparticles with polysaccharides, such as biocompatible and antimicrobial chitosan, has the potential to reduce this toxicity. In this study, copper nanoparticles were coated with chitosan using a newly developed and facile method. The presence of coating was confirmed using x-ray photoelectron spectroscopy (XPS), rhodamine tagging of chitosan followed by confocal fluorescence imaging of coated particles, observed increases in particle size and zeta potential. Further physical and chemical characteristics were evaluated using dissolution and x-ray diffraction (XRD) studies. The chitosan coating was shown to significantly reduce the toxicity of copper nanoparticles after 24 and 52 hours and the generation of reactive oxygen species as assayed by DHE oxidation after 24 hours *in vitro*. Conversely, inflammatory response, measured using the number of white blood cells, total protein, and cytokines/chemokines in the bronchoalveolar fluid of mice exposed to chitosan coated versus uncoated copper nanoparticles, was shown to increase, as was the concentration of copper ions. These results suggest that coating metal nanoparticles with mucoadhesive polysaccharides (e.g. chitosan) could increase their potential for use in controlled release of copper ions to cells, but will result in a higher inflammatory response if administered via the lung.

Keywords

Chitosan; Copper; Coatings; Metal Oxide Nanoparticles; Nanoparticle Toxicity; Lung Inflammation; Copper Nanoparticles

CORRESPONDING AUTHOR: Aliasger K. Salem, Ph.D., Division of Pharmaceutics and Translational Therapeutics, College of Pharmacy, the University of Iowa, S228 PHAR, 115 S. Grand Ave. Iowa City, IA 52242, Phone: (319)-335-8810, Fax: (319)-335-9349, aliasger-salem@uiowa.edu.

Nanotechnology plays an increasingly central role in technological innovation in a broad range of fields ranging from cosmetics to sustainable energy. Nanoparticles (NPs) often combine useful properties of bulk materials such as magnetism, conductivity and stability with a very high surface to volume ratio, increasing reactivity. Thus, many important applications for metal NPs are receiving research attention – attention that could impact society in a variety of important ways. For example, the magnetic properties of iron oxide NPs lend themselves well to biomedical applications such as targeting, imaging, and hyperthermia treatments.¹ Due to their high conductivity, copper (Cu), Cu NPs are most traditionally used for applications such as nanofluids^{2,3} to facilitate increased heat transfer and for high throughput catalysis applications^{4,5} due to increased reactivity with higher surface area. More recently, however, studies have demonstrated the antimicrobial activity of Cu NPs against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* in suspension⁶ and *Saccharomyces cerevisiae*, *Escherichia coli*, and several other microbes when released in a controlled manner from polymer films.⁷ Other potential biomedical applications have been demonstrated with other types of metal nanoparticles, including photo-thermal ablation of tumor cells⁸ and medical imaging.^{9,10} Furthermore, the release of copper ions from NPs could prove useful in diseases associated with abnormally low accumulation of copper ions in certain regions of the body, such as Menke's disease.^{11,12}

Despite their potential use, copper and other metal oxide NPs are limited by their widely demonstrated toxic properties. Indeed, metal oxide NPs have been shown to negatively affect the reproduction and embryonic development of white worms¹³ and zebra fish embryos.¹⁴ Likewise, the effects of metal oxide NPs on mammalian cells and on whole organisms can be severe, affecting the central nervous system,¹⁵ and especially the lungs upon inhalation.¹⁶ In a murine model, host defense against bacterial infections was shown to be significantly lowered by exposure to copper oxide NPs in a dose-dependent manner.¹⁷ Another study conducted comparing the toxicity of iron (Fe) and copper nanoparticles (Cu NPs) using a murine model by Pettibone *et al.* clearly shows greater inflammatory responses trigger by Cu NPs than Fe NPs.¹⁶ Furthermore, a study by Yang *et al.* has shown that Cu NPs with greater surface oxidation have higher ROS generating capacity.¹⁸ These researchers also observed a correlation between the surface ligand chain length and the extent of oxidation, which consequently affects the ROS generation and toxicity of the Cu NPs. Therefore, research to understand how to overcome this inherent toxicity is critical, since addressing the issue is necessary for future applications of metal oxide NPs.

Coating metallic NPs with polysaccharides can overcome the drawbacks mentioned above by increasing stability, improving size distributions, increasing biocompatibility and introducing chemical groups that allow for further functionalization of the NPs.¹⁹ Furthermore, these long chain carbohydrate molecules can potentially inhibit surface oxidation of the nanoparticles that may lead to increased toxicity.¹⁸ Chitosan, an attractive polysaccharide for coating Cu NPs, is a naturally occurring polysaccharide that is directly obtained by deacetylation of chitin, the main component of crustacean shells and fungal cell walls. Additionally, chitosan is cationic in solutions of dilute acid and one of the most abundant biopolymers on earth. Many chitosan properties lend themselves well to wound-healing applications, including its biocompatibility in mammals^{20,21} and antimicrobial^{22,23} properties. These properties have led to a diverse number of medical applications, including haemostatic wound dressings,^{24–26} direct wound filling,²⁷ drug^{28–31} and gene^{32–35} delivery, and tissue engineering.^{36–39} Although some have investigated various chitosan coating methods for metal-oxide NPs⁴⁰ and even studied their biocompatibility,⁴¹ no study to date has shown the impact of chitosan coating of Cu NPs on *in vitro* toxicity and *in vivo* inflammatory responses. Such information is critical to allow application involving polysaccharide coatings of metallic NPs.

In this study, the effect of chitosan coatings on Cu NP physical properties and toxicity were evaluated. The coated particles were optimized and thoroughly characterized using dynamic light scattering, fluorescent tagging, microscopy, and surface analysis. The impact of the chitosan coating on the level of toxicity induced *in vitro* was assessed using human adenocarcinomic alveolar (A549) cells, as well as the *in vivo* inflammatory response of mice following nasal instillation of coated particles, characterized by differential white blood cell counts and cytokine/chemokine concentration analysis. The information gained through this work provides a basis for understanding the effect of chitosan on NP toxicity and how polysaccharide coatings on NP surfaces alter their characteristics and behavior in biological settings.

RESULTS AND DISCUSSION

A chitosan coating was applied to Cu NPs to study the effect of the polysaccharide on NP physical properties, toxicity *in vitro*, and inflammatory response *in vivo* upon nasal instillation. The physical properties were first examined visually using transmission electron microscopy (TEM, Figure 1). The copper particles alone (Cu NPs, Figure 1a) appear to have a smooth, round morphology. The particles aggregate in water to some degree, making applications using copper particles in water difficult. A direct coating method was first attempted by mixing Cu NPs with a solution of chitosan in acetic acid and buffer. This method, however, caused even more significant aggregation and dissolution of copper ions from the particles. Additionally, the solution appeared to gel, trapping the Cu NPs in a semi-solid matrix. To stabilize the Cu NPs in the aqueous environment and protect them from dissolution, a pre-coating of surfactant (Tween[®] 80) was applied prior to coating the particles with chitosan. The hydrophobic chains of the Tween[®] 80 molecule adsorb via Van der Waals forces (physisorption) to the hydrophobic Cu NPs,⁴² leaving a hydrophilic external layer to the coating. It has been proposed that the π orbital associated with the carbon-carbon double bond in the surfactant's alkyl chain also contributes to its strong interaction with metal NP surfaces and increases its ability to stabilize them.⁴³ Thus, this initial coating process should help to solubilize Cu NPs, as has been shown for other NPs.⁴⁴ The copper particles coated only with Tween[®] 80 (Cu NPs + Tw, Figure 1b), however, exhibited a rough morphology and were heavily aggregated, potentially because the concentration of Tween[®] 80 used was above the critical micelle concentration of only 10 μ M.⁴⁵

We hypothesize that charged, hydrophilic chitosan molecules can adsorb to the externally presented hydrophilic groups of the Tween[®] molecules by physisorption, or Van der Waals interactions. Once the chitosan coating was applied (Cu NPs + Tw + Ch, Figure 1c), these particles demonstrated a smooth, spherical morphology and aggregated to a lesser extent than the copper particles alone or the Tween[®]-coated particles, likely due to increased repulsive forces between the positively charged nanoparticles. Further study of chitosan's interaction with polysorbate surfactants that are coated on NPs would certainly be beneficial for understanding the processes involved in the coating process described herein.

To provide a visual confirmation of chitosan coated on the NP surface, Rhodamine B conjugated chitosan derivatives were prepared and used to coat Cu NPs in the same manner as regular chitosan. Because Rhodamine B is fluorescent and Cu NPs are not, the chitosan on the particles' surface could now be detected using fluorescent microscopy (Figure 2a). To further characterize the coated particles, the hydrodynamic diameter, charge, and surface composition were determined using dynamic light scattering (DLS) and x-ray photoelectron spectroscopy (XPS). For ease of comparison, the size and charge information is summarized in Table 1. The measured hydrodynamic diameter of the Cu NPs before coating, after pre-coating with Tween[®] 80, and after chitosan coating were 187 ± 10 nm, 495 ± 66 nm, and

260 ± 25 nm, respectively. Although these measured sizes are much larger than those observed using TEM, the overall size trends were consistent; chitosan-coated particles were slightly larger than Cu NPs alone and the Tween® 80 coated NPs were highly aggregated and difficult to measure. The charge measurements showed a significant increase in positive charge (from 15.4 ± 3.2 mV to 43.4 ± 1.7 mV) when Cu NPs were coated with chitosan. Since chitosan is a cationic molecule, these results give further evidence that the Cu NPs were, in fact, coated with chitosan. As a final confirmation of the chitosan coating, the surface composition of the NPs was analyzed using XPS. All three NP formulations (Cu NPs, Cu NPs + Tw, Cu NPs + Tw + Ch) exhibited peaks at three corresponding bonding energies standard for copper, confirming the presence of Cu. A scanning trace specific to nitrogen (Figure 2b) showed no representative signals for the Cu NPs or Cu NPs + Tw samples. On the other hand, a very distinct peak at a binding energy of 390 mV was observed for the Cu NPs + Tw + Ch, indicating the presence of nitrogen, the source of which was from the chitosan free amine groups. The relative ratios of various atoms on the NP surface were also estimated by calculating the area under the XPS curves. The number of carbon and oxygen atoms relative to the number of copper atoms on the surface increased dramatically (~550% and ~680%, respectively) between the Cu NPs and Cu NPs + Tw + Ch groups, likely due to the addition of the high molecular weight polysaccharide with large quantities of both carbon and oxygen. Coated and uncoated NPs were also characterized by XRD for their bulk composition to identify any changes taking place during the process of coating. The Cu NPs oxidize during the treatment process into CuO NPs (see Figure 2c). The diffraction patterns indicate that the unprocessed Cu NPs also contain CuO, likely due to surface oxidation of Cu NPs. As noted by the manufacturer, these NPs are in fact “partially” passivated with oxygen. This process can leave behind a surface coating that contains CuO and Cu₂O phases as discussed previously.¹⁶ Additionally, over time this oxide layer can become even thicker under ambient storage conditions.⁴⁶ However, here it is noted that upon treatment, a complete oxidation of the CuO is observed. It is possible that when the NPs are sonicated, the increase in temperature and breaking of the aggregates increases the exposed surface area, facilitating the oxidation process. Dissolution studies indicated that uncoated and Tween® 80 coated NPs showed similar extents of copper dissolution (Table 1). Coating with chitosan, however, resulted in a two-fold increase in copper dissolution. Furthermore, solutions containing Cu NPs + Ch were darker blue in color than those containing Cu NPs, Cu NPs + Tw, or Cu NPs + Tw + Ch, which is due to dissolved Cu²⁺ ions. These characteristics suggest that chitosan enhances dissolution when in direct contact with the Cu NP surface. Upon coating with the Tween® 80, chitosan assisted dissolution is inhibited. Therefore, Tween® acts as a protective coating that inhibits nearly complete dissolution of Cu NPs. It should be noted that because all of the particles examined have a significant oxide layer, as demonstrated in Figure 2c, the deliberate coatings applied (i.e. Tween® 80 and chitosan) can be considered independently with regard to the resulting toxicity and inflammatory response.

After fully characterizing the physical properties of the coated Cu NPs, the *in vitro* toxicity was analyzed in human alveolar epithelial (A549) cells using a standard MTS cell viability assay. To confirm that the differences in toxicity observed in A549 cells could be extrapolated to other cell lines, the treatments were also tested in HEK-293 (human embryonic kidney) cells. The cells were very sensitive to Cu NPs and Cu NPs + Tw and less-so to their chitosan-coated counterparts (Cu NPs + Tw + Ch) after 24 and 52 hours of exposure (Figure 3a). Cells treated with 5 µg Cu NPs + Tw + Ch were four times more viable after 24 hours and five times more viable after 52 hours than those treated with 5 µg Cu NPs. The median lethal dose (LD₅₀) of Cu NPs + Tw + Ch was the highest of all treatments for both exposure times, while the chitosan-coated Cu NPs had the lowest LD₅₀ values in each case. Similar results were observed in the HEK-293 cells (data not shown). This decrease in toxicity is also correlated with a two-fold decrease in the cells' generation

of reactive oxygen species (Figure 3b) when treated with Cu NPs + Tw + Ch compared to Cu NPs. The presence of chitosan coating on Cu NPs apparently lowers the environmental stress for cells, increasing their viability relative to other Cu NP exposures. These dramatic differences in dose-related toxicity *in vitro* demonstrate that coating Cu NPs with chitosan protects exposed cells from Cu NPs and significantly reduces their toxicity, thus increasing their biocompatibility and perhaps suitability for various biomedical applications.

The effect of chitosan coating of Cu NPs on the inflammatory response upon nasal instillation was also investigated. Twenty-four hours after nasal instillation of Cu NPs, Cu NPs + Tw, or Cu NPs + Tw + Ch, exposed mice had lost an average of 12% of their starting body weight (average weight loss of 2.81 ± 0.41 , 2.56 ± 0.32 and 2.59 ± 0.43 g, respectively). After necropsy, analyses of cellularity and concentration of cytokines/chemokines in the bronchoalveolar lavage (BAL) fluid and copper in the excised lung tissue were performed.

The concentration of Cu (NPs and ions) deposited in the lung tissue of all exposed animals (adjusted for the Cu in control mice) was about 30 $\mu\text{g/g}$ lung dry weight (Table 2). The concentration of Cu ions in the BAL fluid supernatants was also measured. This analysis revealed a higher copper ion concentration in the BAL fluid of lungs exposed to chitosan-coated Cu NPs than the other treatments (Table 2). This result corresponds well with the increased dissolution of copper from chitosan-coated Cu NPs *ex vivo* (Table 1).

The total number of white blood cells recovered in the BAL fluid (Table 2) was the highest in the group of animals exposed to chitosan-coated copper NPs ($1,920 \pm 777 \times 10^3$ cells/mouse), and while the number of cells from the animals exposed to uncoated Cu NPs was much lower ($765 \pm 102 \times 10^3$ cells/mouse), this difference was not shown to be statistically significant. Similarly, the concentration of total protein and activity of LDH in the supernatants of BAL fluid were higher in the chitosan-coated Cu NP group compared to the Cu NPs group (Table 2, $p < 0.01$ and $p = 0.063$, respectively). The number of neutrophils (key inflammatory cells in the lung) in the BAL fluid (Figure 4a) was 3.5 times higher in the chitosan-coated Cu NPs group ($1,580 \pm 799 \times 10^3$ cells/mouse) than the Cu NPs alone group ($456 \pm 108 \times 10^3$ cells/mouse). Correspondingly, the percentage of neutrophils in the BAL fluid was highest in the chitosan coated Cu NPs group followed by Cu NPs alone and Cu NPs + Tw groups (71%, 56% and 51%, respectively).

Out of six selected inflammatory cytokines/chemokines that were analyzed in the supernatants of BAL fluid (Figure 4b), IL-6 and KC had higher levels in chitosan-coated Cu NPs than uncoated Cu NPs, corresponding well with the higher neutrophil counts in Figure 4a. The concentration of IFN- and TNF- were below the lower limit of detection (LLOD, 1.03 and 2.02 pg/mL, respectively) in all experimental groups. These *in vivo* results show that chitosan-coated Cu NPs elicit higher pulmonary inflammatory responses than their uncoated counterparts. The higher response is demonstrated by the increased total cell and neutrophil numbers in the BAL fluid as well as increased concentrations of inflammatory cytokine/chemokines (IL-6 and KC) in mice exposed to Cu NPs + Tw + Ch as opposed to Cu NPs and Cu NPs + Tw. Likewise, pulmonary cytotoxicity represented by increased activity of LDH in the BAL fluid was higher in groups exposed to chitosan-coated Cu NPs. Chitosan-coated Cu NPs are more likely to have a high residence time in the lungs due to the well-known mucoadhesive properties of chitosan.^{47,48} Chitosan has been shown, in fact to enhance pulmonary delivery of calcitonin from PLGA nanospheres by this phenomenon coupled with the opening of tight junctions.⁴⁸ The inability to clear these coated NPs causes a higher concentration of copper (from NPs and ions) to remain stagnant during the first 24 hours after exposure, eliciting a higher inflammatory response than non-mucoadhesive Cu NPs that can be cleared relatively quickly from the lungs. Furthermore, chitosan has been

shown to have a powerful effect on the tight junctions and permeability of mucus-rich epithelial cells;⁴⁹ an effect that is enhanced when the chitosan is in NP form.^{50,51}

CONCLUSIONS

The addition of a chitosan coating to Cu NPs changed physical properties and toxicity *in vitro* and *in vivo*. The size of the NPs increased by roughly 50%, but the morphology of the chitosan coated particles was smooth and round, in contrast to aggregated and rough Tween® 80 coated Cu NPs. The presence of chitosan coating was confirmed using fluorescent imaging of Cu NPs coated with Rhodamine B conjugated chitosan. The presence of cationic chitosan on the particle surface also caused the particle charge to increase significantly. XPS analysis demonstrated the presence of chitosan on the surface of the coated NPs via the clearly detectable presence of nitrogen. The *in vitro* toxicity of chitosan coated Cu NPs was significantly lower than uncoated Cu NPs for two different cell types, two time points, and a range of doses. LD50 values were highest for chitosan-coated NPs and lowest for Cu NPs + Tw, indicating that coating with chitosan protects cells in culture from the toxic effects of Cu NPs. Conversely, an increase in inflammatory response was observed for mice exposed to chitosan-coated Cu NPs versus uncoated Cu NPs. These results suggest that coating metal NPs with mucoadhesive polysaccharides (e.g. chitosan) decreases their ability to be cleared from the lungs, prolonging the exposure of cells and tissue to toxic metal oxides and producing a dramatic acute inflammatory response. In future studies, coating of Cu NPs with chitosan and its effect on toxicity should be evaluated by intramuscular, intravenous and subcutaneous routes of administration. Despite the differences in toxicity *in vitro* and *in vivo* in the lung, chitosan-coated Cu NPs for the controlled release of copper ions in the treatment of copper deficiency diseases such as Menke's disease could still be beneficial, if a more appropriate route of administration could be identified. Although chitosan coating was, in fact, shown to increase the inflammatory response of Cu NPs administered via nasal instillation, it was also shown to dramatically decrease toxicity *in vitro*. Thus, this study demonstrates the importance of not only a thorough analysis of NP physical properties, but also a careful analysis of toxicity, especially regarding making inferences about *in vivo* exposures and effects based on *in vitro* studies.

MATERIALS AND METHODS

Reagents

Cu NPs (25 nm, partially passivated with 10% oxygen, Nanostructured and Amorphous Materials, Inc., Houston, TX), Tween® 80 (Fisher Scientific, Hampton, NH), sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), ethylenediaminetetraacetic disodium salt (EDTA, Fischer Scientific), and sodium dodecyl sulfate (SDS, Research Products International Corporation, Mount Prospect, IL) were used as received.

Chitosan (2 g, low molecular weight, 96.1% deacetylation, 1% w/v in 1% v/v acetic acid 35 cps, Sigma Aldrich) was dissolved in dilute acetic acid (200 mL, 1% v/v), vacuum filtered, and precipitated using 1 N sodium hydroxide. The precipitate was then mixed with 500 mL of purification buffer (0.1 M sodium bicarbonate, 20 mM EDTA, 0.5% w/v SDS) for 30 minutes, filtered, rinsed, and dialyzed using a Snakeskin® dialysis tube (MWCO 10,000) against nanopure water for 2 days (water was replaced approximately every 12 hours). After dialysis, the chitosan was vacuum filtered again, resuspended in a small amount of nanopure water, frozen overnight at -20 °C, and lyophilized.

Cu NP Coating

Cu NPs (50 mg) were placed in a 20 mL glass vial and suspended in 10 mL of a dilute solution of Tween[®] 80 (5 mg/mL nanopure water) by mixing overnight with a magnetic stir bar. The resulting solution was then dialyzed (Snakeskin[®] dialysis tube, MWCO 3500) against nanopure water for 6 hours (water was replaced approximately every 90 minutes). Meanwhile, a solution of chitosan was prepared by mixing chitosan (50 mg) with 1% v/v acetic acid (10 mL) to homogeneity, adjusting the pH to ~5.8 with 1 N sodium hydroxide, and then adding acetate buffer (50 mM, pH 5.5) to a final volume of 20 mL. To accomplish the final chitosan coating, 1 mL of dialyzed particles was mixed with 9 mL of the chitosan solution overnight. The finished particles were then centrifuged at 4000 rpm for 15 minutes, the supernatant was decanted, and the particles were resuspended to the desired concentration with nanopure water.

Particle Size Analysis

The size of coated and uncoated Cu NPs was measured by JEOL JEM-1230 transmission electron microscope equipped with a Gatan UltraScan 1000 2kx2k CCD acquisition system. A small drop (10 μ L) of sample solution was left on a 400-mesh TEM copper grid that was pre-coated with a Formvar[®] 0.5% solution in ethylene dichloride film (Electron Microscopy Sciences, Hatfield, PA) for 2 minutes. Whatman[®] filter paper was then used to remove any excess liquid and the grid was air dried. The TEM images were processed using ImageJ (Image Processing and Analysis in Java, Version 1.46b). The hydrodynamic diameter (number-weighted) and surface charge of particles in solution were measured in distilled water at 25 °C using dynamic light scattering (Zeta Sizer Nano ZS, Malvern Instruments, Southborough, MA).

Composition Analysis

X-ray diffraction (XRD) patterns were collected using a Rigaku Miniflex II Diffractometer with a Co source. XRD analysis was conducted according to the following protocol. The solutions containing Cu NPs, Cu NPs + Tw and Cu NPs + Tw + Ch were centrifuged at 22,000 rpm for 1 hour in air tight centrifuge vials and the supernatant carefully removed, leaving ~200 μ L in the vial. Then using a micropipette, the solid was mixed well with the remaining solution and placed on the XRD slides, dried overnight in a dessicator, and diffraction patterns were collected. The unprocessed sample of Cu NPs was also analyzed to investigate the effects of oxidation during the treatment process.

Surface Analysis

The surface functionality of the coated Cu NPs was analyzed using x-ray photoelectron spectroscopy (XPS, Axis Ultra XPS, Kratos, Chestnut Ridge, NY) and confocal microscopy. For XPS analysis, sample preparation followed a standard procedure. Briefly, 20 μ L droplets of each solution were carefully pipette onto a small square of clean, heavy duty aluminum foil. The droplets were allowed to dry, and then the resulting spot was analyzed using XPS. The resulting data were calibrated and analyzed using XPS software (CasaXPS 2.3.15, Casa Software Ltd., Estepona, Spain). The area under the curve for each atomic signal was calculated using an "area under the curve" function of another data analysis software (GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego, CA) and used to estimate ratios of the number of atoms on the surface of the particles. The confocal microscopic analysis was conducted using rhodamine-conjugated chitosan prepared according to the following protocol. A 5 mg/mL chitosan solution was prepared as previously described. 100 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC, Thermo Scientific, Rockford, IL), 50 mg N-hydroxysulfosuccinimide (sulfo-NHS, Thermo Scientific), and 5 mg Rhodamine B (Sigma-Aldrich) were dissolved in 0.1 M 2-(N-

mopholino)ethanesulfonic (MES) buffer (1 mL) with the pH adjusted to 6. After mixing for two hours, this solution (1 mL) was added to a 5 mg/mL chitosan solution (9 mL), prepared as described previously, and allowed to react for six hours. The resulting product was dialyzed (Snakeskin[®] dialysis tube, MWCO 10,000) against nanopure water for 2 days and lyophilized. The rhodamine-conjugated chitosan was used to coat Cu NPs using the same method as described above. For imaging, two coverslips were placed on a glass slide spaced about 3 cm apart to form a thin chamber. A drop of copper particles coated with fluorescent-labeled chitosan was added between coverslips. Another coverslip was placed on top of this chamber to facilitate uniform thickness and to spread the drop. The arrangement was sealed using clear fingernail polish. Images were acquired using a Bio-Rad Radiance 2100 multi-photon microscope (Bio-Rad Laboratories, Hercules, CA). The images were processed using ImageJ (Image Processing and Analysis in Java, Version 1.46b).

Dissolution of the Coated and Uncoated Particles

The dissolution studies were conducted using a Varian inductively coupled plasma optical emission spectrophotometer (ICP-OES). The experiments were conducted according to the following protocol. Cu NPs, Tween + NPs and Cu NPs + Tw + Ch containing solutions were sonicated for 5 minutes and 1.5 mL aliquot was filtered using 0.2 μ m filters (Xpertec) into centrifuge vials. The filtered samples were centrifuged at 22,000 rpm for 45 minutes to ensure complete removal of any remaining Cu NPs in the filtered solutions. From the supernatant 0.5 mL was carefully transferred into 4.5 mL of 2% HNO₃ followed by the analysis by ICP-OES with a calibration curve of 5, 10, 25 and 50 ppm. A total of five replicates were conducted for each sample.

Cytotoxicity Analysis in vitro

The cytotoxicity of chitosan coated Cu NPs was determined using an MTS assay. A549 (adenocarcinomic human alveolar basal epithelial) cells were seeded at a density of 1×10^4 cells/well in RPMI-1640 medium (Gibco[®], Life Technologies Corporations, NY) at 37 °C and 5% CO₂ in a 96-well plate. The media were supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, GA), 10 mM HEPES (Gibco[®]), 50 μ g/mL gentamycin sulfate (Cellgro, VA), 1 mM sodium pyruvate (Gibco[®]), and 1 mM Glutamax[™] (Gibco[®]). After 24 hours, the medium was discarded and replaced with 100 μ L fresh media in each well. Various concentrations (0.01 – 0.09 μ g/mL) of each treatment in phosphate buffered saline (PBS, Invitrogen) were then added to each well at a volume of 100 μ L. The cells were incubated for either 24 or 52 hours, the treatment removed, and fresh media (100 μ L) added. 20 μ L of CellTiter 96[®] Aqueous One solution reagent (Promega Corporation, Madison, WI), which changes from MTS tetrazolium to formazan in proportion to the number of live cells present, was added to each well. The cells were incubated between one and four hours, then the formazan product was quantified by spectrophotometric analysis (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA) using the absorbance at 490 nm, according to the company protocol.

Reactive oxygen species (ROS) production was estimated by measuring the oxidation of dihydroethidium (DHE, Molecular Probes, Eugene, Oregon), which becomes fluorescent upon oxidation with superoxide. A549 cells ($2-4 \times 10^5$ cells per 60 mm Petri dish) were cultured 24 hours, exposed to one of the three NP treatments (2 mL at 50 μ g Cu NPs per mL) for 24 hours, and then tested for DHE oxidation as described previously.⁵² Briefly, the cells were washed once with PBS and labeled at 37 °C for 40 minutes in PBS containing 5 mM pyruvate with DHE (10 μ M; in 1% DMSO). Culture plates were placed on ice to stop the labeling, trypsinized, and resuspended in ice-cold PBS. Samples were analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Inc., Mountain View, CA; excitation 488 nm, emission 585 nm band-pass filters). The mean fluorescence

intensity (MFI) of 10,000 cells was analyzed in each sample and corrected for auto-fluorescence from unlabeled cells. The MFI data were normalized to levels from A549 cells treated with PBS only.

Animal Models

An inflammation mouse model was used in these studies. Mice (C57Bl/6, males, 6 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). The instillation protocol was approved by the Institutional Animal Care and Use Committee and complied with the NIH Guidelines. Animals were housed in a vivarium in polypropylene, fiber-covered cages in HEPA-filtered Thoren caging units (Hazelton, PA, USA) in the Pulmonary Toxicology Facility at the University of Iowa. They were acclimatized for 10 days prior to the exposures. Food (sterile Teklad 5% stock diet, Harlan, Madison, WI, USA) and water (via an automated watering system) was provided *ad libitum* and a 12-hr light-dark cycle was maintained in the animal room.

Nasal Instillation Exposure

Animals in each experimental group (n = 6): Cu NPs, Cu NPs + Tw, and Cu NPs + Tw + Ch, were exposed to tested material by nasal instillation. Each mouse was exposed twice with 50 μ L (Cu NPs and Cu NPs + Tw groups) and with 100 μ L (Cu NPs + Tw + Ch) of exposure solution with a 1 hour interval between each dosing. The total dose of Cu NPs in each experimental group, as measured by inductively coupled plasma-mass spectrometry (ICP-MS, X Series, Thermo Scientific, Waltham, MA, USA) was approximately 30 μ g/mouse (33.5, 34.3, and 29.0 μ g/mouse in Cu NPs, Cu NPs + Tw, and Cu NPs + Tw + Ch group, respectively). Suspensions of tested materials were prepared, as described above and vortexed immediately before the instillation exposure. Nasal instillation was conducted under anesthesia by inhalation of isoflurane (3 %) using a precision Fortec vaporizer (Cyprane, Keighley, UK). Naïve mice (mice with no exposure) served as a control group. All mice were euthanized 24 hours post exposure by overdose inhalation of isoflurane, cervical dislocation and exsanguinations, after which bronchoalveolar lavage (BAL) fluid and lung tissues were collected.

Bronchoalveolar Lavage (BAL) Fluid

The lungs from 6 mice in each group were lavaged *in situ* three times with approximately 1 mL of sterile saline (0.9% sodium chloride solution, Baxter, Deerfield, IL, USA). The lavage fluid was centrifuged (800 x g for 5 min at 4 °C), the total white blood cells were counted using a hemocytometer and the supernatants were stored at -80°C for later analyses. For differential cell counts, resuspended cells in Hank's balanced salt solution and fetal calf serum, were spun (800 x g, 3 min, Cytospin 4, Thermo Shandon, Thermo Scientific, Waltham, MA, USA) onto microscope slides and air-dried. The cells were then stained using HEMA 3[®] stain set (PROTOCOL[®], Fisher Scientific Company LLC, Kalamazoo, MI) and the number of macrophages, neutrophils and lymphocytes (total of 400 total cells per each animal) were counted.

A Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to measure total protein levels in BAL fluid supernatants and lactate dehydrogenase (LDH) activity was determined by a Cytotoxicity Detection Kit (Roche Diagnostics, Penzberg, Germany). Concentrations of selected inflammatory cytokines/chemokines (tumor necrosis factor [TNF]- , interferon [IFN]- , interleukin [IL]-6, IL-12(p40), keratinocyte-derived cytokine [KC], and macrophage inflammatory protein [MIP]-1) were measured in the BAL fluid using a multiplexed fluorescent bead-based immunoassay (BioRad Laboratories, Inc., Hercules, CA). The lowest limit of detection (LLOD) for cytokine assays was calculated by

dividing the lowest detected point on the standard curve by 2. (IL-6 = 0.52, IL-12(p40) = 0.72, IFN- γ = 1.03, KC = 1.42, MIP-1 β = 1.47 and TNF- α = 2.02 pg/mL)

Copper Analysis in Exposure Solutions, Lung Tissue, and BAL Fluid

The lungs from mice in each group were harvested at the time of necropsy and stored at -80°C . Tissues were dried using a freeze dryer for approximately 16 hrs at 1.3×10^{-1} mBar and -50°C (Labconco Corp., Kansas City, MO, USA) and subsequently weighed. Dried lungs were placed in 50 mL digestion tubes and high purity nitric acid and hydrochloric acid were added (Optima grade, Fisher Scientific, Pittsburgh, PA, USA). Tissues were then digested in 36-well HotBlockTM (Environmental Express, Mt. Pleasant, SC, USA) at 95°C for 6 hrs. The concentration of Cu ions was determined by inductively coupled plasma-mass spectrometry (ICP-MS, X Series, Thermo Scientific, Waltham, MA, USA). Each sample was spiked with an internal standard at 20 $\mu\text{g/L}$.

Statistical Analysis

The results were analyzed using the statistical analysis package included in the analytical software (GraphPad Prism 5.02). For the *in vitro* results, a two-way analysis of variance (ANOVA) was performed, followed by Bonferroni post-tests between each group. When the data could not be assumed to be normal in distribution (ROS and *in vivo* results), a Kruskal-Wallis one-way analysis of variance on ranks test was performed, followed by Dunn's multiple comparison tests. Data are expressed as a mean $\pm$ standard error. *P*-values less than 0.05 were considered significant.

Acknowledgments

The authors gratefully acknowledge support from the Environmental Health Sciences Research Center (NIH P30 ES005605). Other sources of support include the American Cancer Society (RSG-09-015-01-CDD), the National Cancer Institute at the National Institutes of Health (1R21CA13345-01/ 1R21CA128414-01A2/UI Mayo Clinic Lymphoma SPORE), and the National Science Foundation (CBET-0933450). K. Worthington thanks the University of Iowa Graduate College for additional support. The authors would also like to gratefully acknowledge Dr. David Peate for his generosity with ICP-MS, the Central Microscopy Research Facility at the University of Iowa for the use of TEM, confocal microscopy, and XPS equipment, Jonas Baltrusaitis for technical support in performing the XPS analyses, and the Radiation and Free Radical Research Core Lab and P30-CA086862 for technical support in measuring DHE oxidation.

References

1. Pankhurst QA, Thanh NTK, Jones SK, Dobson J. Progress in applications of magnetic nanoparticles in biomedicine. *J Phys D-Appl Phys*. 2009; 42:224001.
2. Chang M, Liu H, Tai CY. Preparation of copper oxide nanoparticles and its application in nanofluid. *Powder Technol*. 2011; 207:378–386.
3. Yu W, Xie H. A Review on Nanofluids: Preparation, Stability Mechanisms, and Applications. *J Nanomater*. 2012:435873.
4. Ramu VG, Bordoloi A, Nagaiah TC, Schuhmann W, Muhler M, Cabrele C. Copper nanoparticles stabilized on nitrogen-doped carbon nanotubes as efficient and recyclable catalysts for alkyne/aldehyde/cyclic amine A(3)-type coupling reactions. *Appl Catal A-Gen*. 2012; 431:88–94.
5. Ranu BC, Chattopadhyay K, Adak L, Saha A, Bhadra S, Dey R, Saha D. Metal nanoparticles as efficient catalysts for organic reactions. *Pure Appl Chem*. 2009; 81:2337–2354.
6. Ren G, Hu D, Cheng EWC, Vargas-Reus MA, Reip P, Allaker RP. Characterisation of copper oxide nanoparticles for antimicrobial applications. *Int J Antimicrob Agents*. 2009; 33:587–590. [PubMed: 19195845]
7. Cioffi N, Torsi L, Ditaranto N, Tantillo G, Ghibelli L, Sabbatini L, Blevè-Zacheo T, D'Alessio M, Zambonin P, Traversa E. Copper nanoparticle/polymer composites with antifungal and bacteriostatic properties. *Chem Mat*. 2005; 17:5255–5262.

8. Li Y, Lu W, Huang Q, Huang M, Li C, Chen W. Copper sulfide nanoparticles for photothermal ablation of tumor cells. *Nanomedicine*. 2010; 5:1161–1171. [PubMed: 21039194]
9. Na HB, Song IC, Hyeon T. Inorganic Nanoparticles for MRI Contrast Agents. *Adv Mater*. 2009; 21:2133–2148.
10. Tu C, Louie AY. Nanoformulations for molecular MRI. *Wiley Interdiscip Rev -Nanomed Nanobiotechnol*. 2012; 4:448–457. [PubMed: 22488901]
11. Brewer GJ. Copper in medicine. *Curr Opin Chem Biol*. 2003; 7:207–212. [PubMed: 12714053]
12. Sivasubramanian KN, Henkin RI. Behavioral and dermatologic changes and low serum zinc and copper concentrations in two premature infants after parenteral alimentation. *J Pediatr*. 1978; 93:847–851. [PubMed: 101647]
13. Barros Amorim MJ, Scott-Fordsmand JJ. Toxicity of copper nanoparticles and CuCl₂ salt to *Enchytraeus albidus* worms: Survival, reproduction and avoidance responses. *Environ Pollut*. 2012; 164:164–168. [PubMed: 22361055]
14. Bai W, Tian W, Zhang Z, He X, Ma Y, Liu N, Chai Z. Effects of Copper Nanoparticles on the Development of Zebrafish Embryos. *J Nanosci Nanotechnol*. 2010; 10:8670–8676. [PubMed: 21121381]
15. Yang Z, Liu ZW, Allaker RP, Reip P, Oxford J, Ahmad Z, Ren G. A review of nanoparticle functionality and toxicity on the central nervous system. *J R Soc Interface*. 2010; 7:S411–S422. [PubMed: 20519209]
16. Pettibone JM, Adamcakova-Dodd A, Thorne PS, O'Shaughnessy PT, Weydert JA, Grassian VH. Inflammatory response of mice following inhalation exposure to iron and copper nanoparticles. *Nanotoxicology*. 2008; 2:189–204.
17. Kim JS, Adamcakova-Dodd A, O'Shaughnessy PT, Grassian VH, Thorne PS. Effects of copper nanoparticle exposure on host defense in a murine pulmonary infection model. *Part Fibre Toxicol*. 2011; 8:29. [PubMed: 21943386]
18. Shi M, Kwon HS, Peng Z, Elder A, Yang H. Effects of Surface Chemistry on the Generation of Reactive Oxygen Species by Copper Nanoparticles. *ACS Nano*. 2012; 6:2157–2164. [PubMed: 22390268]
19. Dias AMGC, Hussain A, Marcos AS, Roque ACA. A biotechnological perspective on the application of iron oxide magnetic colloids modified with polysaccharides. *Biotechnol Adv*. 2011; 29:142–155. [PubMed: 20959138]
20. De Souza R, Zahedi P, Allen CJ, Piquette-Miller M. Biocompatibility of injectable chitosan–phospholipid implant systems. *Biomaterials*. 2009; 30:3818–3824. [PubMed: 19394688]
21. Mi F, Tan Y, Liang H, Sung H. In vivo biocompatibility and degradability of a novel injectable-chitosan-based implant. *Biomaterials*. 2002; 23:181–191. [PubMed: 11762837]
22. No HK, Young Park N, Ho Lee S, Meyers SP. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *Int J Food Microbiol*. 2002; 74:65–72. [PubMed: 11929171]
23. Du Y, Zhao Y, Dai S, Yang B. Preparation of water-soluble chitosan from shrimp shell and its antibacterial activity. *Innovative Food Science & Emerging Technologies*. 2009; 10:103–107.
24. Pusateri A, McCarthy S, Gregory K, Harris R, Cardenas L, McManus A, Goodwin C. Effect of a chitosan-based hemostatic dressing on blood loss and survival in a model of severe venous hemorrhage and hepatic injury in swine. *J Trauma-Injury Infect Crit Care*. 2003; 54:177–182.
25. Wedmore I, McManus J, Pusateri A, Holcomb J. A special report on the chitosan-based hemostatic dressing: Experience in current combat operations. *J Trauma-Injury Infect Crit Care*. 2006; 60:655–658.
26. Brown MA, Daya MR, Worley JA. Experience with Chitosan Dressings in a Civilian Ems System. *J Emerg Med*. 2009; 37:1–7. [PubMed: 18024069]
27. Paul W, Sharma CP. Chitosan and alginate wound dressings: a short review. *Trends Biomater Artif Organs*. 2004; 18:18, 19, 23.
28. Hombach J, Hoyer H, Bernkop-Schnuerch A. Thiolated chitosans: Development and in vitro evaluation of an oral tobramycin sulphate delivery system. *European Journal of Pharmaceutical Sciences*. 2008; 33:1–8. [PubMed: 17980561]

29. Rekha MR, Sharma CP. Synthesis and evaluation of lauryl succinyl chitosan particles towards oral insulin delivery and absorption. *J Controlled Release*. 2009; 135:144–151.
30. Saravanakumar G, Min KH, Min DS, Kim AY, Lee C, Cho YW, Lee SC, Kim K, Jeong SY, Park K, Park JH, Kwon IC. Hydrotropic oligomer-conjugated glycol chitosan as a carrier of paclitaxel: Synthesis, characterization, and in vivo biodistribution. *J Controlled Release*. 2009; 140:210–217.
31. Ye Y, Yang F, Hu F, Du Y, Yuan H, Yu H. Core-modified chitosan-based polymeric micelles for controlled release of doxorubicin. *Int J Pharm*. 2008; 352:294–301. [PubMed: 18096336]
32. Fernandes, JC.; Tiera, MJ.; Winnik Françoise, M. Polysaccharides for Drug Delivery and Pharmaceutical Applications. Vol. 934. American Chemical Society; 2006. Chitosan Nanoparticles for Non-Viral Gene Therapy;; p. 177-200.
33. Lavertu M, Méthot S, Tran-Khanh N, Buschmann MD. High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation. *Biomaterials*. 2006; 27:4815–4824. [PubMed: 16725196]
34. Kiang T, Wen H, Lim H, Leong K. The effect of the degree of chitosan deacetylation on the efficiency of gene transfection. *Biomaterials*. 2004; 25:5293–5301. [PubMed: 15110480]
35. Park I, Kim T, Kim S, Akaike T, Cho C. Chemical modification of chitosan for gene delivery. *J Dispersion Sci Technol*. 2003; 24:489–498.
36. Crompton KE, Prankerd RJ, Paganin DM, Scott TF, Horne MK, Finkelstein DI, Gross KA, Forsythe JS. Morphology and gelation of thermosensitive chitosan hydrogels. *Biophys Chem*. 2005; 117:47–53. [PubMed: 15905019]
37. Kim S, Nishimoto SK, Bumgardner JD, Haggard WO, Gaber MW, Yang Y. A chitosan/beta-glycerophosphate thermo-sensitive gel for the delivery of ellagic acid for the treatment of brain cancer. *Biomaterials*. 2010; 31:4157–4166. [PubMed: 20185170]
38. Ruel-Gariepy E, Chenite A, Chaput C, Guirguis S, Leroux JC. Characterization of thermosensitive chitosan gels for the sustained delivery of drugs. *Int J Pharm*. 2000; 203:89–98. [PubMed: 10967431]
39. Ruel-Gariepy E, Shive M, Bichara A, Berrada M, Le Garrec D, Chenite A, Leroux JC. A thermosensitive chitosan-based hydrogel for the local delivery of paclitaxel. *European Journal of Pharmaceutics and Biopharmaceutics*. 2004; 57:53–63. [PubMed: 14729080]
40. Osuna Y, Gregorio-Jauregui KM, Gerardo Gaona-Lozano J, de la Garza-Rodriguez IM, Ilyna A, Diaz Barriga-Castro E, Saade H, Lopez RG. Chitosan-Coated Magnetic Nanoparticles with Low Chitosan Content Prepared in One-Step. *J Nanomater*. 2012:327562.
41. Shi S, Jia J, Guo X, Zhao Y, Chen D, Guo Y, Cheng T, Zhang X. Biocompatibility of chitosan-coated iron oxide nanoparticles with osteoblast cells. *Int J Nanomed*. 2012; 7:5593–5602.
42. Aslan K, Perez-Luna VH. Surface modification of colloidal gold by chemisorption of alkanethiols in the presence of a nonionic surfactant. *Langmuir*. 2002; 18:6059–6065.
43. Zhao Y, Wang Z, Zhang W, Jiang X. Adsorbed Tween 80 is unique in its ability to improve the stability of gold nanoparticles in solutions of biomolecules. *Nanoscale*. 2010; 2:2114–2119. [PubMed: 20697612]
44. Sun WQ, Xie CS, Wang HF, Hu Y. Specific role of polysorbate 80 coating on the targeting of nanoparticles to the brain. *Biomaterials*. 2004; 25:3065–3071. [PubMed: 14967540]
45. Sivers U, Tjerneld F. Mechanisms of phase behaviour and protein partitioning in detergent/polymer aqueous two-phase systems for purification of integral membrane proteins. *Biochimica et Biophysica Acta (BBA) - General Subjects*. 2000; 1474:133–146.
46. Mudunkotuwa IA, Pettibone JM, Grassian VH. Environmental Implications of Nanoparticle Aging in the Processing and Fate of Copper-Based Nanomaterials. *Environ Sci Technol*. 2012; 46:7001–7010. [PubMed: 22280489]
47. Zaru M, Manca M, Fadda AM, Antimisariar SG. Chitosan-coated liposomes for delivery to lungs by nebulisation. *Colloids and Surfaces B: Biointerfaces*. 2009; 71:88–95.
48. Yamamoto H, Kuno Y, Sugimoto S, Takeuchi H, Kawashima Y. Surface-modified PLGA nanosphere with chitosan improved pulmonary delivery of calcitonin by mucoadhesion and opening of the intercellular tight junctions. *J Controlled Release*. 2005; 102:373–381.

49. Vllasaliu D, Casettari L, Fowler R, Exposito-Harris R, Garnett M, Illum L, Stolnik S. Absorption-promoting effects of chitosan in airway and intestinal cell lines: A comparative study. *Int J Pharm.* 2012; 430:151–160. [PubMed: 22525084]
50. Vllasaliu D, Exposito-Harris R, Heras A, Casettari L, Garnett M, Illum L, Stolnik S. Tight junction modulation by chitosan nanoparticles: Comparison with chitosan solution. *Int J Pharm.* 2010; 400:183–193. [PubMed: 20727955]
51. Hombach J, Bernkop-Schnürch A. Chitosan solutions and particles: Evaluation of their permeation enhancing potential on MDCK cells used as blood brain barrier model. *Int J Pharm.* 2009; 376:104–109. [PubMed: 19409469]
52. Zhu Y, Kalen AL, Li L, Lehmler H, Robertson LW, Goswami PC, Spitz DR, Aykin-Burns N. Polychlorinated-biphenyl-induced oxidative stress and cytotoxicity can be mitigated by antioxidants after exposure. *Free Radical Biology and Medicine.* 2009; 47:1762–1771. [PubMed: 19796678]

Figure 1. TEM images of Cu NPs with a) no coating (Cu NPs), b) a Tween[®] 80 coating (Cu NPs + Tw), and c) a Tween[®] 80 coating followed by chitosan coating (Cu NPs + Tw + Ch)

Figure 2.

Physical characterization of coated and uncoated Cu NPs. Shown are a) confocal microscopy images of Cu NPs with no coating (inset), and coated with Tween[®] 80 and rhodamine-conjugated chitosan; b) XPS data in the N1s binding energy region for Cu NPs, Cu NPs + Tw, and Cu NPs + Tw + Ch where only the Cu NPs + Tw + Ch show the presence of nitrogen on the surface of nanoparticles; and c) XRD characterization of Cu NPs, Cu NPs + Tw, Cu NPs + Tw + Ch, and unprocessed Cu NPs

Figure 3.

Toxicity of coated and uncoated Cu NPs to A549 cells. Toxicity was measured relative to untreated cells of coated and uncoated Cu NPs on A549 cells exposed for a) 24 and b) 52 hours. Statistical analysis was performed by a two-way analysis of variance followed by Bonferonni post tests. DHE oxidation levels (c) of A549 cells after 24 hours of exposure to coated and uncoated Cu NPs. Statistical analysis was performed by a Kruskal-Wallis test followed by Dunn's post tests. For all panels, the statistical significance shown is relative to Cu NPs. Error bars represent the standard error mean, ** $p < 0.01$, *** $p < 0.001$

Figure 4.

Inflammatory response in the post-necropsy lung BAL fluid 24 hrs after nasal instillation of coated and uncoated Cu NPs. Differential cell counts (a) show the number of macrophages, neutrophils and lymphocytes, while chemokine/cytokine analysis (b) shows the levels of selected inflammatory markers. Statistical analyses were performed by Kruskal-Wallis tests for each cell type or chemokine/cytokine followed by Dunn's post tests. Statistical comparisons shown are relative to the naive group for each cell type or inflammatory marker. The differences between all other groups are not statistically significant. Error bars represent the standard error mean, $n = 5-7$, * $p < 0.05$, *** $p < 0.001$, *** $p < 0.001$

Table 1

Size, zeta potential, and dissolution of Cu NPs with and without coating

	Cu NPs	Cu NPs + Tw	Cu NPs + Tw + Ch
Radius (nm) [†]	187 ± 10	495 ± 66 ^{††}	260 ± 25
Zeta Potential (mV) [†]	15.4 ± 3.2	13.6 ± 0.3 ^{††}	43.4 ± 1.7
Dissolved Cu (ppm)	15.7 ± 0.1	12.4 ± 0.6	27.2 ± 0.4

[†] n=3,^{††} Data quality report indicated poor data quality and high likelihood of aggregation

Table 2

Characterization of lung tissue and fluid after nasal instillation of coated and uncoated Cu NPs. Except where noted, data was obtained from the post-necropsy BAL fluid. Data are expressed as mean $\pm$ standard error.

	Cu in Lung Tissue [†] ($\mu\text{g/g dry weight}$)	Cu [†] ($\mu\text{g/L}$)	Total Cells/Mouse ($\times 10^{-3}$)	Total Protein ($\mu\text{g/mL}$)	LDH Activity (U/L)
Naïve	-	-	168 $\pm$ 15	63.6 $\pm$ 1.8	21 $\pm$ 4
Cu NPs	30.4 $\pm$ 5.3	36.4 $\pm$ 13.4	765 $\pm$ 102	142 $\pm$ 19	31 $\pm$ 5
Cu NPs + Tw	27.9 $\pm$ 5.8	46.2 $\pm$ 13.7	756 $\pm$ 89	172 $\pm$ 24	27 $\pm$ 7
Cu NPs + Tw + Ch	28.8 $\pm$ 6.6	88.5 $\pm$ 26.5	1,920 $\pm$ 777	378 $\pm$ 111 [#]	73 $\pm$ 14 [*]

* $p < 0.01$, activity of LDH in BAL fluid was significantly higher in Cu NPs + Tw + Ch (one-way ANOVA followed by Tukey test) compared to Cu NPs,

[†] Data were corrected for concentration of Cu in the naïve group,

[#] $p = 0.063$