

Understanding the influence of Cu-based nanoparticle properties on interactions with plant systems and disease suppression

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Abstract

As the global population continues to grow, innovation in agriculture is needed to increase yield. The inefficient delivery of fertilizers and pesticides in agriculture reduce the yield potential and lead to the environmental accumulation of agrichemicals. Nanomaterials have been proposed to increase the productivity of agrichemical delivery. Nanomaterials have tunable composition, surface chemistry and are small, with at least one dimension under 100 nm. In agriculture, nanomaterials can be used to adhere to the leaf surface and slowly dissolve to release the active agent, or nanomaterials can be internalized to transport the active agent throughout the plant system.

We proposed the use of nanomaterials to deliver Cu for disease suppression. Cu is an essential micronutrient for plant health and has been traditionally used in agriculture as an antifungal agent. Previous research has shown that Cu-based nanoparticles can suppress fungal pathogens and increase biomass growth. We wanted to understand how nanoparticle properties influence disease suppression and interactions with plant systems. The materials properties that we studied include nanoparticle shape, composition, and surface chemistry.

First, we investigated the impact of nanoparticle composition on disease suppression. Nanoparticle composition can influence Cu delivery by tuning the nanoparticle solubility. We compared $\text{Cu}_3(\text{PO}_4)_2$ nanoparticles, a relatively insoluble salt that dissolves to release Cu, to commercial CuO nanoparticles that require a protonation step to dissolve. We developed a

synthesis method to produce $\text{Cu}_3(\text{PO}_4)_2$ nanosheets. We studied the nanomaterial dissolution profiles and found that $\text{Cu}_3(\text{PO}_4)_2$ releases Cu rapidly, while CuO has a slower Cu release profile. In addition, plant matrices may stimulate ion release once nanoparticles are internalized. We studied disease suppression of *Fusarium oxysporum* in watermelon (*Citrullus lanatus*) and showed that $\text{Cu}_3(\text{PO}_4)_2$ suppresses disease at 100 times lower concentrations than CuO nanoparticles, and that a single application of nanoparticles protected plants for the duration of our experiment. This study showed that the nanoparticle composition significantly impacts the disease suppression efficacy.

We investigated the effect of nanoparticle surface chemistry and aspect ratio on disease suppression and plant growth. In this study, we developed a synthesis method to prepare positively charged CuO nanospikes, negatively charged nanospikes, and negatively charged nanosheets with exposed reactive (001) crystal faces. We used X-ray photoelectron spectroscopy to show that an increase in surface oxygen correlates to a negative zeta potential. The synthesized nanoparticles were then used to treat *F. oxysporum* in tomato plants (*Solanum lycopersicum*). The negatively charged nanoparticles significantly reduce the disease progression and increase the biomass, while the positively charged nanoparticles and a CuSO_4 salt control do not impact plant growth or plant health. Elemental analysis of the tissues at the end of the experiment showed that the positively charged nanoparticles increase the foliar Cu concentration when compared to the negatively charged nanoparticles, that showed increase the amount of Cu in the root. To understand the intermolecular interactions between the plant system and the nanoparticles we used self-assembled monolayers to mimic the leaf surface, which showed that nanoparticle electrostatics and hydrogen bonding interactions play an important role in adsorption.

Finally, we investigated interactions between CuO nanoparticles and pumpkin (*Curcubita pepo*) xylem fluid. Xylem fluid is the matrix that carries nutrients and water from the soil to the foliage and contains carbohydrates, amino acids, small organic acids, proteins and ionic species. We showed that the nanoparticles acquire a corona that consists mostly of irreversibly bound proteins, despite the complexity of xylem fluid. Using a combination of X-ray photoelectron spectroscopy and polyacrylamide gel electrophoresis we showed that the corona is selective for specific proteins. In addition, the corona altered the nanoparticle electrophoretic mobility and diffusion coefficient, indicating that corona formation may impact transport through the plant system.

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Chapter 1: Background and Introduction

1.1 Challenges facing global agricultural system

As the global population is projected to reach 9.8 billion by the year 2050, to meet the needs of the growing population innovations in agriculture need to increase yields.¹ The productivity of the global agricultural system is limited by inefficiencies in crops growth, transport, and processing. When growing crops, the allotment of land has a yield potential (the projected amount of food that should be produced), however, factors like draught, flooding, nutrient deficiencies and disease limit the amount produced.²⁻⁴ To compensate for crop losses the agrichemical industry develops fertilizer and pesticide formulations. Fertilizers compensate for nutrient deficiencies including nitrogen, phosphorus, potassium, magnesium, calcium, sulfur and micronutrients(zinc, copper, boron, iron, manganese and boron).⁵ Pesticides can target weeds, insects, bacteria, viruses, and fungi, and can be composed of organic or inorganic molecules that control or kill harmful pest populations.⁶ Agrichemical delivery is inefficient, for instance the conversion efficiency of applied N and P is only 10%.⁷ Furthermore, the use of agrichemicals can be detrimental to environmental health. Excess nitrogen and phosphorus contribute to eutrophication and low oxygen conditions in water bodies.⁸ Excess micronutrients in the environment have been linked to changes in soil microbial communities.⁹ Organic pesticides can have harmful effects on living organisms are found in ground water, soil, and vegetation.¹⁰ Nanotechnology has been proposed to efficiently deliver pesticides and fertilizers to increase crop yields and reduce the environmental impacts of agrichemicals.^{4, 11}

1.2 Nanotechnology

Nanoparticles are small with at least one dimension between 1 and 100 nm.¹² Nanomaterials can be formed naturally through biological or geochemical process or can be engineered in the lab for applications.¹³ Nanomaterials are defined by their size and can therefore be composed of a variety of materials including polymers, semiconductors, metals, liposomes, and clays.¹⁴⁻¹⁸

Nanomaterials are tunable and can be incorporated in many different forms of technology. In the medical field, the small size of nanomaterials facilitates translocation through cells and tissues to deliver targeted molecules. Nanomaterials can also act directly to kill cancer cells or pathogens.¹⁹⁻²¹ Outside of the medical field, nanomaterials are increasingly used to enhance consumer goods. The small size of nanomaterials can be used to incorporate antibacterial silver in to exercise clothing, reduce the white cast of ZnO and TiO₂ mineral sunscreens, and increase the tensile strength of carbon bicycles and tennis rackets.²²⁻²⁴ In technology and industry, the high surface area of nanomaterials increases the number of active sites available by increasing the surface area-to-volume ratio.²⁵ The increased reactivity of nanomaterials has been used to develop more active catalysts, decrease the diffusion length in lithium-ion batteries, and provide sensitive surfaces for sensors.²⁶⁻²⁹ The enhanced reactivity and ability to translocate through biological systems make nanomaterials attractive for agricultural applications like nutrient delivery and pesticides.

1.3 Nanoparticles for agricultural applications

Nanomaterials have been used as a platform for the delivery of small molecules, genetic material, and ions in agriculture.^{4, 11} The nanomaterials can either be functionalized, loaded with, or composed of the active agent to target disease, deliver nutrients or alter plant properties.

³⁰⁻³² Nanomaterials can increase efficiency in agriculture because the small size facilitates uptake by plants, the surface chemistry is tunable, and the active agents can be slowly released.³³⁻

³⁴

1.3.1 Nanoparticle uptake and translocation

Interactions of nanoparticles with plants can include adsorption to the leaf or root, uptake, and translocation from where it is applied to other plant tissues. Where nanoparticles are applied influences the route of uptake. Nanoparticles can be applied to the plant foliage, plant seeds, in the soil, or in a hydroponic solution.³⁵⁻³⁸ Nanoparticles applied to the foliage interact with the leaf cuticle, which is a heterogeneous composite of waxes and biopolymers that create a surface with hydrophobic character and a slight negative charge.³⁹ On the leaf surface there are small hydrophilic pores, trichomes that secrete compounds for plant defense, hydathodes, and stomatal openings that are responsible for gas exchange.⁴⁰⁻⁴² Nanoparticles can be internalized by diffusion across the cuticle or by entering the hydrophilic pores or stomatal openings.³³ Once the nanoparticles have entered the leaf tissue, they can transport either through the cytoplasm, in symplastic transport, or through the extracellular space between the cell wall and cell membrane, in apoplastic transport.⁴³ Nanoparticles can also undergo long range transport through the plant vasculature. Transport can occur through the phloem vasculature from the shoot to the root, or through the xylem vasculature from the root to the shoot.³⁸

Plants interact differently with nanomaterials because of the relative insolubility and tunable properties. For instance, the Cu-based nanoparticles reduced the incidence of rinse off and increased the Cu loading when compared to a Cu salt control.⁴⁴ Tuning the nanoparticle surface chemistry alters the nanoparticle uptake and transport. Gold nanoparticles coated in amphiphilic

PVP and hydrophilic citrate, showed that the amphiphilic coating increased penetration through the cuticle, however, the hydrophilic coating promoted transport in wheat.³⁵ The zeta potential can also be tuned to alter the localization of nanomaterials. Studies performed with charged polylactic acid nanoparticles showed that the trend of adsorption to leaf surfaces was positively charged > neutral > negatively charged.⁴⁵ Similarly, positively charged dextran-coated CeO₂ nanoparticles had increased root adsorption and negatively charged nanoparticles increased translocation efficiency to the foliage.⁴⁶⁻⁴⁷

The translocation of nanoparticles is also affected by nanoparticle shape and size. Smaller nanoparticles have increased mobility in plants.^{35, 48} The nanoparticle shape influences mechanical interactions with plant systems, for instance high-aspect ratio nanoparticles have been shown to increase penetration and transport.^{31, 49-50} For crystalline nanomaterials, the shape also influences the distribution of crystal faces and affects the nanoparticle reactivity. In a study of CeO₂ nanoparticles, rod-shaped nanoparticles with reactive crystal faces were shown to translocate and transform more than less reactive spherical nanoparticles.⁵¹

The composition of nanomaterials influences nanoparticle reactivity and solubility. A study of Cu-based nanoparticles showed that Cu from more soluble Cu(OH)₂ traveled through the plant system over the course of an hour, while less soluble CuO and CuS delivered Cu consistently over the course of 48 hours.⁵² Recently, targeted delivery of nanoparticles to chloroplasts, stomata, and trichomes has been achieved using molecular recognition proteins.⁵³⁻⁵⁴ The nanoparticle properties: surface chemistry, size, shape, and composition all influence nanoparticle uptake and translocation in the plant system.

1.3.2 Nanoparticle transformations in plant matrices

Nanomaterials in biological and environmental matrices undergo chemical transformations of the core material or adsorb molecules on the nanoparticle surface, termed corona formation.⁵⁵ Nanoparticle transformations can alter the nanoparticle size, mobility, surface charge, and ultimately affect how nanoparticles interact with an organism.⁵⁶⁻⁵⁷ For plants, the matrices include root exudate, xylem fluid that transports water and nutrients from the soil to the foliage, phloem fluid that transports synthesized sugars from the foliage to the roots, and cytosol that fills the cells.⁵⁸⁻⁶⁰ Nanoparticles have been identified in xylem, phloem, at the root, and within cells.^{31, 38, 47} In a hydroponic system, exposure to root exudates reduced CuO nanoparticle aggregation and increased uptake of CuO nanoparticles.⁶¹ While, in a soil containing system root-exudates increased the amount of free Cu in the soil and decreased the amount of Cu found in the plant tissues.⁶² Though these results are contrasting, they both indicate that transformations in plant matrices alter translocation. Nanoparticle transformations within plants have been studied using X-ray absorption near edge spectroscopy (XANES). XANES studies show that metal oxides often transform through redox reactions, the formation of small organic acid-metal complexes, or the formation of metal-phosphates.⁶³⁻⁶⁵ The information from XANES relies heavily on matching experimental data to reference spectra and only provides information about the binding environment of the metal. To get molecular level information, leaf extracts were incubated with TiO₂ nanoparticles which showed adsorption of lipids, flavonoids, and metabolites.⁶⁶ A study of gold nanoparticles incubated with plant proteins showed a selective adsorption of proteins related to energy-yielding pathways.⁶⁷ Understanding the role of

nanoparticle-corona formation and surface transformations within plants can help inform nanoparticle design.

1.3.3 Nanoparticles for nutrient and pesticide delivery

Nanomaterials in agriculture have been proposed increase the efficiency of fertilizers and pesticide delivery.^{4, 11} The use of nanomaterials increased the median efficacy of a treatment by 20-30% when compared to commercial products. The nanomaterials that are used as fertilizers are either a) composed of the macronutrient, b) composed of the micronutrient, or c) a nanocarrier loaded with the active ingredient.³⁴ The biomass, yield, pigment analysis, photosynthetic ability, growth rate, root length, and shoot length are used to measure the efficacy of a fertilizer treatment. For macronutrient delivery, phosphorus containing hydroxyapatite nanoparticles increased the growth rate by 32% and the above ground biomass by 18%, when compared to a traditional fertilizer.⁶⁸ Hydroxyapatite nanoparticles have also been coated with urea for nitrogen delivery, which showed comparable yield to traditional fertilizers at half the amount of applied nitrogen.⁶⁹ Nanoparticles composed of ZnO, CuO, Fe₂O₃, and MgO have been explored for micronutrient delivery in plants.^{32, 70} Generally, the micronutrient nanoparticles increase plant growth and plant health.⁷⁰ Cu-based nanoparticles have been studied extensively for micronutrient delivery.

Cu-based nanoparticles act as a micronutrient and pesticide. Cu has been traditionally used in orchards, vineyards, and vegetable gardens as an antifungal agent. Cu acts directly by inhibiting spore germination and is toxic to fungi.⁷¹⁻⁷³ Cu as micronutrient upregulates plant defensive pathways.⁷⁴⁻⁷⁵ However, the long term use of soluble Cu in agriculture has led to accumulation in soil and negative effects on the soil microbial community.^{72, 76} Cu-based

nanoparticles have been proposed to increase Cu delivery and reduce the environmental exposure to Cu. Cu-containing chitosan nanoparticles have been shown to upregulate plant defense pathways and increase biomass and yield in maize infested with curvularia leaf spot.⁷⁷ Cu nanoparticles with different oxidation states showed increased disease suppression efficacy of *Phytophthora infestans* in tomato when compared to commercial Cu-fungicides.⁷⁸ In watermelon infested with *Fusarium oxysporum*, a single 1-2 mg dosage of CuO nanoparticles during the seedling stage increased fruit yields by 40-50%, decreased disease progression, and increased plant biomass. Furthermore, the disease suppression was associated with an increased expression of polyphenol oxidase pathway.⁷⁹ Disease suppression efficacy of CuO was also observed in tomato and eggplant infested with *F. oxysporum*.³² Cu-based nanoparticles are promising as a fertilizer and antimicrobial agent, further research could be performed to understand how nanoparticle properties promote disease suppression.

1.4 Synthesis of metal oxide nanomaterials

Metal oxide nanoparticles have been developed for applications in sensors, solar cells, catalysts and electronics.⁸⁰⁻⁸¹ Nanomaterials can be prepared by bottom-up or top-down syntheses. Top-down synthesis takes a larger version of a material and breaks it in to nanoscale pieces.⁸²⁻⁸³ Nanomaterials can also be prepared by bottom-up synthesis that uses a chemical reaction between precursor molecules. Bottom-up synthesis can be performed in the solid, vapor or solution phase or a combination of phases.⁸⁴⁻⁸⁵ In the solution phase, the methods that can be used to synthesize nanomaterials include hot-injection synthesis of metal-organic precursors into very hot solvents, polyol synthesis in high-boiling and reducing glycol-based solvents, and hydrothermal or solvothermal synthesis in a sealed pressurized vessel with the solvent heated

over the boiling point.⁸⁶⁻⁸⁹ Bottom-up synthesis methods are driven by chemical reaction in a saturated environment. The synthesis method selected tunes the kinetics and thermodynamics of the reaction to alter the nanoparticle properties including nanoparticle shape, size, surface chemistry, and crystallinity.

La Mer growth and Ostwald ripening have been used as models to describe the synthesis of monodisperse nanoparticles. The La Mer model states that when monomers in solution reach a critical saturation the nanoparticles will spontaneously nucleate, following nucleation the concentration of monomers is low enough that no other nucleation events occur. The particles will then continue to grow from diffusion driven growth.⁹⁰ The nanoparticle polydispersity can then be refined through a process called Ostwald ripening. Ostwald ripening is the dissolution of small particles due to their relatively high surface area, and the diffusion of the released monomers to larger particles in solution. Ultimately, conditions that lead to Ostwald ripening create larger nanoparticles overall.⁹¹ Nanoparticles can also grow by coalescence or oriented attachment of adjacent nanoparticles.⁹² La Mer growth and Ostwald ripening are general processes that may occur for a variety of nanomaterials.

Specifically for metal oxides, a metal under basic conditions forms a metal hydroxide coordination complex in solution. The coordination complexes undergo a condensation reaction to eliminate water and form chains of metal-hydroxo bridges. At higher temperatures, the metal hydroxide dehydrates to form a metal oxide.⁹²⁻⁹⁴ Parameters like monomer concentration, reaction temperature, and solvent viscosity all play an important role in the properties of synthesized nanoparticles.

The nanoparticle properties can also be affected by the reagents used in the synthesis. Often, nanoparticle synthesis includes a capping or a stabilizing agent. Stabilizing agents associate with the nanoparticle surface and increase the colloidal stability by separating particles through electrostatic repulsion or a physical barrier. Capping agents can influence the nanoparticle shape by preferentially adsorbing to specific crystal faces and preventing further nanoparticle growth in that direction. Examples of capping and stabilizing agents include larger organic molecules like oleic acid or small ionic species like OH.^{81, 95-96} The selection of different capping or stabilizing agents can tune the nanoparticle shape and surface chemistry.

The nanoparticle shape and surface chemistry impact the catalytic ability and environmental interactions. The nanoparticle shape for nanocrystals is often influenced by the exposed crystal faces. The crystal faces can have a different distribution of atoms, for instance the (111) crystal face of CuO is more close-packed while the (001) crystal face is more open.⁹⁷ The distribution of crystal faces can also impact reactivity, in CuO nanobelts with exposed (001) faces were more reactive to CO oxidation than nanoparticles with exposed (111) faces.⁹⁷ Similarly, a study comparing ZnO nanomaterials with different distributions of crystal faces showed that nanoparticles that had a higher fraction of exposed (0001) faces had increased photocatalytic activity.⁹⁸ In a biological system, a study comparing CuO nanosheets to nanoparticles showed that the enhanced reactivity of nanosheets was linked to enhanced bacterial toxicity.⁹⁹ Understanding the influence of nanoparticle surface chemistry on interactions with plant systems and disease suppression could facilitate the design of more active agrichemicals.

1.5 Scope of thesis

In this thesis we study the impact of Cu-based nanoparticle properties on interactions with plant systems and disease suppression. Nanoparticle factors like composition, surface chemistry, and shape may influence molecular level interactions and disease suppression efficacy in plant systems.

In chapter 2 of this thesis, we study the effect of Cu-based nanoparticle composition on nutrient delivery and disease suppression of *Fusarium oxysporum* infested watermelon (*Citrullus lanatus*). We compared the efficacy of $\text{Cu}_3(\text{PO}_4)_2$ to commercial CuO nanoparticles and showed that the $\text{Cu}_3(\text{PO}_4)_2$ nanosheets suppressed disease at significantly lower concentrations. The $\text{Cu}_3(\text{PO}_4)_2$ nanosheets also increased the foliage wet mass. The disease suppression may be influenced by the ion release profile. We selected $\text{Cu}_3(\text{PO}_4)_2$ because it is a low solubility salt, while Cu release from CuO depends on a protonation step to form $\text{Cu}(\text{OH})_2$. We developed a synthetic protocol to prepare $\text{Cu}_3(\text{PO}_4)_2$ nanosheets and characterized the materials properties using X-ray diffraction and scanning electron microscopy. Dissolution experiments showed that $\text{Cu}_3(\text{PO}_4)_2$ releases Cu rapidly, while CuO has a more sustained release profile. In addition, the dissolution experiments were performed in both nanopure water and a pH 6 matrix containing malic and citric acid to mimic plant xylem fluid. The presence of small organic acids increased the dissolution of both nanoparticle types significantly, indicating that internalization into the plant system may stimulate ion release. This study shows that nanoparticle composition impacts disease suppression and interactions with plant matrices may impact nanoparticle properties.

In chapter 3, we discuss the influence of CuO nanoparticle surface charge on disease suppression of *F. oxysporum* infested tomato (*Solanum lycopersicum*). We developed a synthesis

method to prepare CuO nanospikes with a positive or a negative zeta potential by tuning the reaction pH. The synthesis method can also be used to synthesize CuO nanosheets by increasing the OH concentration in solution. X-ray photoelectron spectroscopy measurements showed that the variation in zeta potential at the nanoparticle surface can be attributed to the relative amounts of O and Cu. We use self-assembled monolayers to show that the nanoparticle charge influences adsorption, and that negatively charged CuO nanoparticles are capable of hydrogen bonding with surfaces while the positively charged nanoparticles are not. The nanoparticle surface charge influenced adsorption and translocation through the plant system, with positively charged nanoparticles adhering more to the leaf surface and negatively charged particles qualitatively increasing the amount of Cu in root tissues. The nanoparticle charge also influenced disease suppression, with negatively charged nanosheets and nanospikes significantly suppressing disease when compared to positively charged nanospikes and a CuSO₄ control. The negatively charged nanoparticles also increased the foliage biomass, when compared to the positively charged nanoparticles. Ultimately, this study shows that the nanoparticle charge is an important parameter to consider when designing nanoparticles for disease suppression.

In chapter 4, we study the formation of a xylem fluid corona on the surface of CuO nanoparticles. Xylem fluid transports nutrients and water from the soil to the foliage. Xylem fluid is a matrix containing carbohydrates, small organic acids, polyatomic ions, amino acids, and proteins. We showed that in the complex xylem matrix, the corona is formed primarily of proteins. Using *in Situ* attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) we show that carbohydrates weakly adsorb to the nanoparticle surface while proteins form irreversible interactions. Through a combination of X-ray photoelectron

spectroscopy and polyacrylamide gel electrophoresis we show that there is a selective adsorption of proteins from solution. The acquisition of a corona also reduced the nanoparticle diffusion coefficient and electrophoretic mobility, indicating that corona transport may impact nanoparticle interactions and translocation in plants.

Chapter 5 concludes this research and discusses potential future directions.

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Chapter 2. Copper Based Nanomaterials Suppress Root Fungal Disease in Watermelon (*Citrullus lanatus*): Role of Particle Morphology, Composition and Dissolution Behavior

The following chapter is adapted from the article published in *ACS Sustainable Chemistry and Engineering*, **2018**, 6, 11, 14847–14856, with the following co-authors Chuanxin Ma, Natalie Hudson-Smith, Wade Elmer, Cristian David Plaza Pérez, Nubia Zuverza-Mena, Christy L. Haynes, Jason C. White, and Robert J. Hamers. My contributions to the work include synthesis and characterization of copper phosphate nanoparticles, dissolution experiments, and a lab exchange for one of the greenhouse study replicates. Disease suppression, biomass, tissue analysis and *in vitro* studies were performed at the Connecticut Agricultural Experiment Station.

2.1 Introduction

As the global population continues to grow toward 9.7 billion by 2050,¹ new agricultural production strategies must be explored to achieve sustainable food security, especially as water and arable land become more limited resources.² A number of innovations in agricultural practices and chemical additives, described here as amendments, have been proposed to increase crop yields with minimal additional input of resources. These practices include using sensors to monitor crop quality, prescriptive planting, genetic modification, and the development of new agrichemical delivery systems.³⁻⁵ Crop losses from disease and pests remain a significant problem, leading to 20-40% decreases in yield and subsequent economic losses, thus threatening overall food security.⁶ One area of active focus has been on micronutrients, which are known to be critical to plant response against disease by acting as enzyme co-factors to promote the

formation of defensive secondary metabolites.⁷ However, micronutrient availability in soil is often low due to either alkaline conditions or complexation with organic matter.⁸⁻⁹ In addition, translocation from leaves to roots is restricted for many nutrients, meaning that foliar applications are largely ineffective for root pathogens such as fungi and nematodes.

Nanomaterials have been proposed as a more effective micronutrient delivery system than traditional methods due to their high surface area and reactivity, as well as potential enhanced transport within plant tissues.³ Furthermore, there is great interest in tuning material characteristics to customize micronutrient release for optimum success.^{3, 8, 10}

There have been several recent investigations on the use of copper nanomaterials based on the known antimicrobial and antifungal properties of the metal.^{4, 11-14} Traditionally, copper has been applied to crops in either salt or bulk hydroxide form for pathogen control.¹⁵⁻¹⁶ Although somewhat effective for the intended purpose, this application of solubilized copper salts can lead to the accumulation of copper in the field, as well as leaching to fresh water sources.¹⁷ The long term influence of copper accumulation on the soil microbiome and on aquatic species, as well as potential hazards to human health, are not known.^{8, 18-19} To efficiently supply plants with antifungal copper, several strategies based on different nanomaterials have been explored, including CuO, CuS and Cu(OH)₂, and even commercially available products such as Kocide (approximately 46% copper hydroxide particles ranging in size from nanoparticles to micron sized particles).^{10, 12, 14, 20-22} These materials have been shown to reduce disease incidence at lower concentrations than required using soluble salts. For example, a previous study reported that tomato bacterial spot was reduced using Cu composite nanomaterials at Cu concentrations nearly 10-fold lower than the concentration of commercial

Kocide or Cu Mancozeb. Similarly, nanomaterials have been found to reduce disease in cucumber and citrus plants.^{4, 23} While it is possible that the nanomaterial interact directly with the plant system, several researchers have suggested that the mode of action of copper-based nanomaterials is due to the enhanced release of ions, more effectively stimulating the plant defenses and promoting the formation of secondary metabolites.²³⁻²⁴ Furthermore, copper amendments under certain treatment regimens may directly reduce bacterial and fungal growth.^{12, 20, 25-26} In spite of the obvious importance of particle dissolution, little research has been directed at the controlled release of copper ions from nanomaterials for agricultural applications.

Traditionally, metal phosphates have been used as inorganic coatings to tune the release of ions through the generation of insoluble solids.²⁷ Phosphate in the form of buffered phosphate solutions, phosphorous acid salts, or organic-phosphate compounds has shown the reduction of bacterial and fungal infections. In addition, there was enhanced antimicrobial activity when phosphate compounds were applied concurrently with solubilized copper salts.^{4, 28-29} In a previous study, researchers synthesized nanostructured copper phosphate protein composites that showed antifungal properties in vitro.^{12, 30-35} However, the application of copper phosphate nanomaterials to suppress disease and enhance crop yield remains largely unexplored.

In this manuscript, we investigate the use of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets, commercially available CuO nanoparticles (NP), and CuSO_4 solution (consisting of free Cu^{2+} and SO_4^{2-} ions) as amendments on watermelon to improve resistance to the soilborne fungus *Fusarium oxysporum f. sp. niveum* and to increase yield. In two separate greenhouse experiments, different

application strategies (foliar spray and foliar dip) and a broad range of amendment concentrations were investigated to gain a better mechanistic understanding of disease suppression and yield enhancement potential. In addition, field experiments at two different farm locations were conducted to assess if observed successful results in the greenhouse translate to a field application. The findings of this study demonstrate that aqueous suspensions of $\text{Cu}_3(\text{PO}_4)_2$ nanosheets are more effective than suspensions of commercial CuO nanoparticles and aqueous solutions of CuSO_4 at reducing fungal disease, while also increasing total wet mass, an important measure of plant growth. These results suggest that nanoparticle composition, nanoparticle shape, and the associated differences in Cu^{2+} release rate may be important factors controlling the beneficial agricultural effects of nanomaterials.

2.2 Materials and Methods

2.2.1 Materials and synthesis

CuO nanoparticles (30 nm diameter; powder) were obtained from U.S. Research Nanomaterials (Houston TX). Copper chloride dihydrate, ammonium phosphate monobasic, and diethylene glycol were obtained from Sigma Aldrich (St. Louis MO). All reagents were used without further purification. We synthesized $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets using a polyol method.³⁶⁻³⁷

Nanoparticles of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ form in a two-dimensionally sheet-like morphology; we refer to these as “nanosheets”. First, 4 mL of 2 M $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ were added to 20 mL of diethylene glycol, and the mixture was heated to 140 °C under reflux for an hour. A 4-mL aliquot of 1.3 M solution of $\text{NH}_4\text{H}_2\text{PO}_4$ was then rapidly injected into the reaction mixture, and the reaction proceeded for 5 h. The nanosheets were then isolated by centrifugation at 5000 rpm using a Thermo Fischer Sorvall Legend X1R centrifuge and were then rinsed with ethanol (2x) and

water (1x), following by drying overnight under vacuum. For all reported experiments, the mass concentration expressed as mg/L of nanoscale CuO and $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ materials were used. However, through ICP-OES we determined the elemental Cu contribution (described below) in CuO nanoparticles is $85 \pm 6\%$, whereas for $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets the value is $44 \pm 4\%$, consistent with the chemical stoichiometry. Therefore, for each treatment concentration approximately half the amount of copper is added for $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ then CuO.

2.2.2 Nanomaterial characterization

Samples for nitrogen physisorption characterization with Brauner Emmet Teller (BET) analysis were degassed overnight at 120°C under vacuum. The samples were analyzed using a Micrometrics Gemini VII 2390 surface analyzer (Norcross, GA) to measure nitrogen adsorption isotherms. Samples for X-ray powder diffraction were prepared by pressing the nanomaterials into a thin layer of Dow Corning vacuum grease (Midland, MI) on a zero-diffraction plate (B-doped Si, MTI Corporation). X-ray diffraction methods were collected using a Bruker Advance powder diffractometer (Spring TX) fitted with a $\text{Cu K}\alpha$ source and a Lynxeye detector. The diffraction patterns were modified by subtracting the background and stripping the $\text{K}\alpha_2$ peaks using Bruker software DIFFRAC.EVA (Spring TX). Samples were prepared for scanning electron microscopy (SEM) by drop-casting a dilute solution of nanoparticles suspended in isopropanol on a conductive boron-doped silicon wafer. A LEO Supra55 VP field-emission scanning electron microscope (Zeiss, Germany) was used to obtain micrographs of the nanomaterials. Cu concentrations of nanomaterials were determined by digesting approximately 3 mg of the powders in 2 mL of aqua regia overnight. After diluting the samples to a final

volume of 10 mL, they were analyzed using inductively coupled optical emission spectroscopy (ICP-OES, Perkin Elmer 4300 Dual View, Shelton CT).

2.2.3 Dissolution experiments

We evaluated Cu metal release in suspensions of three different compositions: (1) deionized water (2) deionized water with a surfactant, and (3) a simulated xylem sap solution. Suspensions in (1) deionized water were prepared using of 100 mg/L CuO NP or $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets prepared in deionized (DI) water (18 M Ω , Barnstead nanopure systems) and adjusted to pH=7 by adding HCl and NaOH until the desired pH was reached. Suspensions in (2) deionized water were performed in the same manner as those in deionized water, with the addition of 1 mL/L of Regulaid® non-ionic surfactant (Kalo Inc, Overland Park TX) adjusted to pH 7 through the addition of HCl and NaOH. Suspensions in (3) simulated xylem sap were performed by using a solution containing 890 μM malic acid and 1.71 mM citric acid, adjusted with NaOH to pH=6. In some cases 1 mL/L of Regulaid® was added.³⁸ The simulated xylem sap contained chelating small organic acids and a lower pH to determine the impact of the plant environment on nanomaterial dissolution. To conduct ion release studies, triplicate samples of each CuO and $\text{Cu}_3(\text{PO}_4)_2$ nanomaterials suspended in matrix (1), (2), (3), and (4) were placed under constant agitation at 100 rpm using New Brunswick C24 incubator shaker (Marshall Scientific, Cambridge MA) held at 30°C and aliquots were sampled at specific intervals (1 h, 12 h, 24 h, 7 d, 21 d). Nanoparticles were removed from suspension by centrifugation at 5000 rpm, and the supernatant was then analyzed for Cu by inductively coupled plasma optical emission spectroscopy (ICP-OES, Perkin Elmer 4300 Dual View, Shelton CT). Comparable studies were

separately conducted at particle concentrations of 1000 mg/L in the pH 6 organic acid solution and in pH 7 DI water.

2.2.4 *In vitro* toxicity

The toxicity of the two Cu nanomaterials to the pathogen *F. oxysporum f. sp. nivuem* was examined in shake culture. Fifty mL of sterile potato dextrose broth (Difco Laboratories, Livonia, MI) was added to 125 mL Erlenmeyer flasks containing CuO NP or $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets at concentrations of 50 and 500 mg/L, along with nanoparticle-free control samples. These Flasks were seeded with a colonized agar plug of *F. oxysporum f. sp. nivuem* and were set on a platform shaker at 125 rpm for 5 d at 22°C (INNOVA 2000, New Brunswick Scientific CO., INC. Edison, New Jersey, USA). Mycelial mats were harvested under vacuum onto pre-weighed Whatman #1 filter paper that had been dried at 50°C for 18 hr. The mycelia-containing filter papers were re-dried at 50°C for at least 18 h and weighed. There were two replicate flasks per nanoparticle type and concentration. In addition, to determine the contribution of NP to the mass, additional controls were run without the fungus. These final weights were averaged by treatment and subtracted from the fungal weights to isolate the actual effect of NP on the pathogen.

2.2.5 Greenhouse experiments

Nanoparticle exposure

We selected watermelon infested with *F. oxysporum* as our model system to determine the disease suppression efficacy of CuO and $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanomaterials. To perform these greenhouse studies, watermelon seeds (*Citrullus lanatus Thunb. cv Sugar Baby*, Harris Seed Co., Rochester, NY) were germinated in soilless potting mix (ProMix BX, Premier Hort Tech,

Quakertown, PA, USA) and fertilized once after three weeks with 40 mL of Peter's soluble 20–10–20 (N–P–K) fertilizer (R. J. Peters Inc., Allentown, PA). When plants reached the 3- to 4-leaf stage, uniform medium size plants were selected for NP exposure. Different concentrations of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets or CuO NP (both in suspension) were prepared in DI water (10–1000 mg/L). For CuO NP, the suspension was sonicated using a probe sonicator for 2 min in an ice bath. For $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets, the suspension was sonicated in a bath sonicator for 5 min; this less intense sonication method was used out of concern over possible breakage of the nanosheets. Perhaps surprisingly, bath sonication of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets resulted in a more stable suspension than that achieved by more intense probe sonication CuO nanoparticles. Prior to exposure, one-to-two drops (1 ml/L) of Regulaid® nonionic surfactant were added into the suspensions and a polyvinylidene chloride film (Saran™ wrap) was fitted around the stem to prevent the NP suspensions from entering the soil. In the greenhouse experiments, bulk CuO and CuSO_4 (Sigma, St. Louis MO) were used to determine the effects of the nanomaterials independent of composition and Cu ion release. Four different treatment methods were used to determine the importance application method in delivering nanomaterials to the watermelon plant. The treatments were as follows: (1) dipping the foliage in to nanomaterial suspensions, (2) spraying foliage with the nanomaterial suspension (3) injecting the nanomaterial solution directly into the root ball, and (4) repeated foliar applications of nanomaterials. Foliar application prevents against interactions between the nanomaterial and the soil that contains complexing organic acids, bacterial and fungal cultures. To study the influence of foliar application a “dip application” was used, where plants were inverted as shown in **Figure A2.1**. Each plant received approximately 0.6–0.8 mL of suspension as calculated by the volume difference from before and

after exposure. The plants were then inverted and air dried for one hour prior to transplant. We note that the solutions were re-dispersed after treatment of 5 plants. Second, for foliar application, seedlings were sprayed with approximately 2 mL/plant of NP suspension. Third, at transplant to the infected growth media, select plants received a 5 mL amendment of one of the two NP solutions at either 500 or 1000 mg/L into the root ball/zone. Fourth, select plants received two separate foliar applications of the NP solutions at 20 or 200 mg/L; once at the initial transplant as above and a second after approximately 7 d of growth. The intent of these multiple applications was to compare repeated doses to roughly equivalent single treatments (i.e.; two 20 mg/L doses vs a single 50 mg/L dose or two 200 mg/L doses against a single 500 mg/L dose).

Evaluation of *Fusarium oxysporum* resistance

F. oxysporum f. sp. niveum was prepared on Japanese millet as previously described. The isolate of *Fusarium oxysporum f. sp. niveum* was cultured from infested watermelon seeds in Connecticut in 2014, and a colony originating from a single spore was stored at 4°C. This isolate is considered highly virulent and has been used previously in our research. 39 NP-exposed and control seedlings were transplanted into potting mix infested with millet inoculum (0.75 g/L potting mix). There were 12 replicates in each NP treatment; 9 were infested with *F. oxysporum* and the remaining three were in pathogen-free soilless mix as non-inoculated controls. All plants received 50 mL of a complete fertilizer solution (Peter's soluble 20–20–20 N, P, K) once per month and one application of imidacloprid (according to label instructions) (Bayer, Rhein, Germany) for insect control. As symptoms of fungal disease developed, plants

were rated for severity on a scale of 1 to 5 where 1 = no disease, 2 = slightly stunted, 3 = stunted and or partially wilted, 4 = completely wilted, and 5 = dead. The progress of disease as represented by the cumulative ratings of disease on replicate plants was plotted over time and the area-under-the-disease-progress curve (AUDPC) was calculated using the trapezoid rule (equation 1): $AUDPC = \sum [Y_i + Y_{(i+1)}] / 2 \times (t_{(i+1)} - t_i)$, where Y_i = the disease rating at time t_i .⁴⁰ After 6 to 8 weeks, depending on disease progress, the experiment was terminated. At harvest, the fresh shoot and root mass was separately recorded. All tissues were dried in an oven and acid digested for nutrient analysis (described below).

2.2.6 Field experiments

Field experiments were conducted to evaluate if successful greenhouse results would translate to a field application. The following six treatments were established at two field locations (Hamden, CT -Location 1 and Griswold, CT - Location 2) to evaluate their effect on growth and Fusarium wilt of watermelon: (1) CuO nanoparticles, (2) $Cu_3(PO_4)_2 \cdot 3H_2O$ nanosheets, (3) commercially available CuO powder, (4) commercially available $Cu_3(PO_4)_2$ powder, (5) $CuSO_4$, and (6) untreated controls. Again, the primary purpose of this work was to compare the efficacy of the two nanoscale materials, and the bulk/salt materials were included to show the benefit of nanomaterials when compared to other Cu delivery approaches. Watermelon seeds ('Sugar Baby' Harris Seed Co. Rochester NY) were germinated in 36 cell liners (1 plant/cell) filled with ProMix BX potting mix and were fertilized three weeks later with 40 mL of Peter's soluble 20–10–20 (N–P–K) fertilizer. Four-week-old transplants received a foliar application in the greenhouse with 1-2 mL of one of the six Cu treatments at 400 mg/L in distilled water that was amended with Regulaid® (1 ml/L). All treatment solutions had been

sonicated for at least 5 min in a FS20H Ultrasonic cleaner (Fisher Scientific Inc., Pittsburgh, PA) prior to application, except for CuO nanoparticles which were probe sonicated (Fisher Scientific, FB505) at 50% amplitude for 2 min.

Field microplots were prepared in early June 2017. Fertilizer (10-10-10, NPK) was spread over a 0.9 m wide rows at 112 kg/ha. The rows were set 6 m apart, covered in black plastic mulch and lined with irrigation drip tape. Rows were partitioned into 30 microplots (5.6 m²). On June 30th (Location 2) and July 5th (Location 1), two transplants were set 30 cm apart in the center of each microplot. There were six replicate microplots/treatment. Planting holes were each infested with approximately 2 g of millet inoculum and hand mixed into the soil immediately before transplanting. In addition, another 30 microplots were planted immediately adjacent to these microplots and prepared the same way, but were not infested with the millet fungal inoculum. Plants were rated for disease three times on July 18th, August 11th, and Sept 7th for Location 2 and on July 19th, Aug 2nd and Aug 30th for Location 1, using the same scale (from 1-5) described above. Total plant biomass and fruit mass was determined at harvest; select tissues were dried for analysis by ICP mass spectrometry (ICP-MS) as noted below.

2.2.6 Tissue elemental analysis

Root and shoot tissue from greenhouse and field experiments (4-6 replicates per treatment, depending on experiment), as well as fruit tissue from field studies, were dried in an oven at 50°C, ground in a Wiley mill, and passed through a 1 mm sieve. One medium watermelon fruit from each field plot was assayed to determine if the edible flesh portions had greater accumulation of Cu and other elements. Digestion of ground samples (0.5 g) was done in 50 mL polypropylene digestion tubes with 5 mL of concentrated nitric acid at 115°C for 45 min

using a hot block (DigiPREP System; SCP Science, Champlain, NY). The Ca, Cu, Fe, K, Mg, Mn, P, S, Si and Zn content was quantified using inductively coupled plasma optical emission spectroscopy (ICP-OES) on a Thermo Fisher iCAP 6500 (Thermo Fisher Scientific, Waltham, MA) or ICP with mass spectrometry (ICP-MS) on an Agilent 7500ce (Agilent, Santa Clara, CA); element content is expressed as $\mu\text{g/g}$ (dry weight) plant tissue.

2.2.7 Pigment analysis

Chlorophyll content was determined by the method of Lichtenthaler, with modification.⁴¹ Briefly, 50 mg fresh tissue was harvested, cut into pieces (< 1 cm), and added to 15 mL centrifuge tubes amended with 10 mL 95% ethanol. The tubes were kept in the dark for 3-5 d and the chlorophyll content was measured by a UV-Vis spectrophotometer (Spectronic Genesis 2). Concentrations of chlorophyll a (Chla), chlorophyll b (Chlb) and total chlorophyll were determined by the following equations: $\text{Chla} = 13.36A_{664.2} - 5.19A_{648.6}$, $\text{Chlb} = 27.43A_{648.6} - 8.12A_{664.2}$ and $\text{Total chlorophyll} = \text{Chla} + \text{Chlb}$.⁴¹ Five replicates per treatment were analyzed.

2.2.8 Statistical analysis

All data are presented as mean $\pm$ SE (standard error). A one-way analysis of variance (ANOVA) with the Student Newman Keuls (SNK) multiple comparison test was performed using SPSS (IBM SPSS Statistics version 20) to evaluate statistical significance. Differences were considered statistically significant when $p < 0.05$. A Student's t-test was used for select analyses to determine statistical significance within a 95 % confidence interval.

2.3 Results and Discussion

2.3.1 Materials characterization of CuO and $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$

$\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ material had sheet-like morphology consistent with the anisotropic growth observed in $\text{Cu}_3(\text{PO}_4)_2$ protein inorganic complexes (**Figure 2.1B**).³¹⁻³² The sheet-like morphology resulted in a relatively high specific surface area of $40 \pm 1 \text{ m}^2/\text{g}$. A total of 165 exposed nanosheets were measured; the dimensions ranged from 53-608 nm, with a median value of 151 nm. The average nanosheet thickness as determined by atomic force microscopy (AFM) was 13 nm (± 4 ; four replicate measurements). **Figure 2.1C** shows the powder diffraction pattern, which is well matched to JPSD00-022-0548, indicating the composition is $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets.⁴² The synthesis of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets allows for compositional comparison of the phosphate coordinating anion to the oxide. The commercial CuO nanoparticles aggregated in solution and showed an irregular morphology (**Figure 2.1A**), with a surface area of $12 \pm 2 \text{ m}^2/\text{g}$; the X-ray power diffraction pattern is consistent with literature for a tenorite crystal structure (**Figure 2.1D**).⁴³

2.3.2 Dissolution experiments

Dissolution experiments for the two nanomaterials were conducted (1) deionized water, (2) deionized water and Regulaid®, (3) simulated xylem sap, and (4) simulated sylem sap and Regulaid® (**Figure 2.2**). **Figure 2.2A** shows the concentration of Cu^{2+} ions produced from dissolution of the different nanoparticles. **Figure 2.2A** shows that the $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets had a rapid initial release of ions across the media studied, reaching a constant value after 12 h. The slight decrease over time in **Figure 2.2A, B** may be due to minimal Cu ion re-precipitation as both copper phosphate or copper hydroxide species. Conversely, the commercial

CuO nanoparticles showed continual ion release from the particles over the five-week study period. After one week, $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets had released more Cu ions in to solution than CuO nanoparticles, with values of $15 \pm 2.0 \mu\text{M}$ and $7.0 \pm 0.6 \mu\text{M}$, respectively (**Figure 2.2A**). For CuO nanoparticles, a similar slow release profile of extractable Cu ions from nanomaterials incubated in soil has been previously reported.⁴⁴ The presence of Regulaid® in the solution reduced ion release from both CuO nanoparticles and $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets, with similar maximum copper concentrations in solution of $9 \pm 2 \mu\text{M}$ and $8.7 \pm 0.5 \mu\text{M}$, respectively (**Figure 2.2B**). The addition of the organic acids at concentrations approximating plant xylem levels dramatically increased the dissolved copper content by two orders of magnitude, reaching $1110 \pm 30 \mu\text{M}$ and $1010 \pm 40 \mu\text{M}$ for CuO nanoparticles and $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets after one week, respectively. Furthermore, there was significantly more Cu leached from CuO under these conditions (**Figure 2.2B**). We hypothesize that the Regulaid® prevents particle dissolution by stabilizing the nanomaterial dispersion within the solution. Alternatively, the organic acids in the simulated xylem sap mat chelate the copper ions in solution and drive the dissolution process in the forward direction, given the reported favorable binding constants of 1.299×10^4 and $1.801 \times 10^4 \text{ M}^{-1}$ between copper nanomaterials and malic and citric acid, respectively.¹¹ Notably, the presence of Regulaid® did not significantly reduce ion release in the presence of simulated xylem sap (**Figure 2.1D**). Previously, the dissolution behavior of CuO films has been characterized in acidic citric acid solutions which showed Cu concentrations of $<10 \text{ mg/L}$ near $\text{pH}=7$, increasing at lower pH .⁴⁵ The addition of soluble proteins and yeast extract has also been shown to enhance Cu ion release for CuO.⁴⁶ To determine whether the solution was saturating, comparable dissolution studies were performed at

1000 mg/L in deionized water and the organic acid matrix (**Figure A2.2**). When compared to the 100 mg/L experiment, the 1000 mg/L study showed an increase in the ion release from $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets by at least a factor of two in both matrices, while CuO showed relatively small changes in the profile observed, indicating that equilibrium may be established at lower concentrations (**Figure A2.2**). To our knowledge, the dissolution characteristics of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets have not been previously reported. The results of these studies show that $\text{Cu}_3(\text{PO}_4)_2$ and CuO nanomaterials have different ion release profiles, with $\text{Cu}_3(\text{PO}_4)_2$ initially releasing more ions than CuO. The ion release profile of the materials may be related to the disease suppression efficacy reported in later sections. In addition, the results of dissolutions studies show that ion release from both CuO and $\text{Cu}_3(\text{PO}_4)_2$ nanomaterials may be stimulated after the nanomaterials enter the plant tissues and contact xylem sap.

2.3.3 *in vitro* toxicity

To determine whether the nanomaterials could directly influence the health of *F. oxysporum*, a direct assay of fungal toxicity was performed. In the fungal toxicity test, pathogen biomass was not significantly impacted by the presence of CuO nanoparticles at concentrations of 50 and 500 mg/L in the growth medium. Conversely, fungal biomass was significantly reduced by $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets at concentrations of 50 and 500 mg/L by 20 and 40%, respectively. These findings in conjunction with the dissolution data may suggest that the more rapid release of ions from the $\text{Cu}_3(\text{PO}_4)_2$ nanosheets results in greater direct disease suppression. However, using foliar application of the nanomaterials on plants requires that the particles must translocate through the plant to have a direct interaction with the fungal culture. Therefore, *in vitro* studies may not reflect the observations noted in the greenhouse or field.

2.3.4 Greenhouse study dip and root application of nanomaterials

To compare the disease suppression of Cu-based nanomaterial treatments at 10-1000 mg/L applied concentrations to an infected control that was not treated with nanomaterials, a “dip” method was used on the seedlings. This exposure method enabled a more controlled and homogenous application of the nanomaterials to the entire shoot system. To assess the progress of Fusarium infection during plant growth, the area under the disease progress curve (AUDPC) was calculated, with higher values representing greater disease progression and vegetative damage. In **figure 2.3A**, the commercial CuO NP showed a downward trend in disease progression with increasing concentration but statistically significant Fusarium suppression (50.6%) was only achieved at a dose of 1000 mg/L. Conversely, a 10 mg/L treatment of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets exhibited a statistically significant 58% reduction in AUDPC, with higher doses providing no additional effect (**Figure 2.3A**). Based on the stoichiometry and as determined experimentally, CuO is $85 \pm 6\%$ Cu by mass, while $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ is $44 \pm 4\%$, respectively. Therefore, each applied dose of $\text{Cu}_3(\text{PO}_4)_2$ contains roughly half the amount of Cu when compared to CuO and shows disease suppression efficacy at 100 times lower concentrations. Importantly, the repeated application of CuO nanoparticles produced a significant reduction in the AUDPC; dual doses of 20 and 200 mg/L resulted in 46.5 % and 40.3 % reductions in disease (**Figure 2.3C**). For the $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets, the repeated application regime yielded similar resistance to disease as did the single dose at 10 mg/L.

The wet mass of the plant was also used to assess plant health. The root, shoot, and total plant wet mass was determined as a function of Cu treatment. Fusarium infection reduced watermelon biomass by 80.9 %. For the commercial CuO nanoparticles, increasing dose showed

an increase in plant biomass but unlike AUDPC, these values were not statistically significant, even at a dose of 1000 mg/L (**Figure 2.3B**). Conversely, treatment with $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets at 10 mg/L resulted in a statistically significant increase in the biomass of plants infected with *Fusarium*. These findings were consistent with the AUDPC data. Although the higher doses of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets consistently produced greater biomass values, a high degree of replicate variability occasionally confounded statistical significance. In terms of biomass, the repeated applications at 20 or 200 mg/L of either particle type offered no additional resistance to infection.

The nanomaterials were also applied directly into the root zone. Here, we observed a similar lack of effect of CuO nanoparticles at both doses and of the nanosheets at 1000 mg/L for the root application, with respect to biomass (**Figure 2.3D**). However, the 500 mg/L root treatment with $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets resulted in a dramatic 261% increase in total plant biomass. These results demonstrate that the $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets effectively reduce disease at low particle concentrations, without the need for repeated applications, whereas the commercial CuO NP were only partially effective at much higher doses.

2.3.5 Greenhouse nutrient analysis

The content of both macro- (Ca, P, S, K, and Mg) and micro-nutrients (Cu, Fe, Zn, Mn, and Si) were measured in watermelon shoots and roots across all treatments (**Figure 2.4, Table A2.1 and A2.2**). As shown in **Figure 2.4A**, the shoot Cu content in diseased plants increased in a dose-dependent fashion with increasing treatment of Cu-based nanomaterials. The consistently higher levels of shoot Cu for the commercial CuO NP are the result of the greater mass fraction of this element in the molecule relative to the $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets. If the data were

normalized to amount of Cu applied, minimal differences between the two materials would be observed. For all plants exposed to *F. oxysporum*, the addition of Cu based nanomaterials lead to a decrease in the root content of Cu when compared to the *F. oxysporum* infested control (**Table A2.2**), although not all changes were of statistical significance. Our findings do not align with previous studies on CuO nanomaterials with a primary particle size of 30 nm.^{13, 39} While the reasons for these differences remain unknown, it is possible that the larger aggregate size of our current CuO nanoparticles may limit translocation from the shoot to the root. In the healthy plants, the shoot Cu content increased in a dose-dependent manner, which was similar to the *Fusarium* infested treatments (**Table A2.2**). Regarding other mineral elements, it is interesting to note that the root P content in the *Fusarium* infested treatments with both types of Cu nanomaterials was decreased as compared to the control (**Table A2.1**). For example, exposure to 50, 100, and 500 mg/L CuO nanoparticles greatly decreased the root P content by 14.2-21.4% relative to the *Fusarium* control. Similarly, the presence of CuO nanoparticles significantly decreased the *Fusarium* infested root K and S content (**Table A2.1**). For micronutrient content, exposure to both $\text{Cu}_3(\text{PO}_4)_2$ nanosheets and CuO nanoparticles at lower levels significantly elevated the shoot Mn content in the *Fusarium* infested treatments as compared to the *Fusarium* control (**Figure 2.4B**). In addition, the presence of CuO nanoparticles increased the shoot Si content upon infection (**Table A2.2**). Si is known to play an important role in plant defense by interacting with the plant stress signaling system and controlling the timing of disease responses.⁴⁷ The lack of Si change under $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ treatment suggests an overall alleviation of stress in the diseased condition relative to the CuO NP exposure.

2.3.6 Greenhouse foliar spray of nanomaterials

Growth and disease suppression

In greenhouse trials, a foliar spray method of CuO NP and $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets was undertaken to investigate the impact of different application methods on disease response and yield. In the absence of disease, the nanomaterial amendments had no impact on total plant biomass; the total plant wet mass across all treatments ranged from 10.8-12.2 g (**Figure A2.3**). The CuO nanoparticles slightly reduced the vine length of the watermelon plants by approximately 15%. Under disease conditions, total biomass and vine length of the nanomaterial-free control was reduced by more than 55%. Although treatment with CuO NP and CuSO_4 had no impact, $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets significantly alleviated the decrease in vine length, resulting in values that were statistically similar to the controls (**Figure A2.4**). Under disease conditions, the CuO and $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanomaterials significantly reduced the AUDPC by 23 and 25%, respectively, as compared to the controls, bulk and ionic copper treatments (**Figure A2.5**). Both $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets and CuO NP increased plant mass by approximately 40% relative to the diseased controls, although the change was only statistically significant for the CuO (**Figure A2.3**). There was greater consistent variability in both biomass and AUDPC in this experiment with the foliar spray. It is likely that the foliar application method resulted in less uniform and incomplete coverage of Cu materials on the plant shoot system as compared to the dip method, thereby resulting in greater variability in treatment efficacy. In addition, another reason for the variability between the AUDPC and biomass is that the AUDPC was generated from ratings taken during growth when significant delays in the onset of symptoms were evident in the $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets treatment, whereas total biomass was recorded at final harvest

when the disease had progressed in all inoculated treatments, thus reducing the likelihood of any differences being detected.

Pigment analysis

In this experiment, the impact of disease and treatment on pigment production was also assessed; changes in pigment concentrations can be an indicator of plant stress.⁴⁸ In the absence of nanomaterials, the total chlorophyll and carotenoid content significantly increased from 0.7 ± 0.02 mg/g to 0.9 ± 0.2 mg/g in response to disease (**Figure A2.6**). For uninfected plants, the addition of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets, CuO nanoparticles and CuSO_4 significantly increased total chlorophyll and carotenoid content. Under disease conditions, CuSO_4 and CuO NP did not significantly change the chlorophyll content when compared to the 0.9 ± 0.2 mg/g of the diseased control, while significantly less chlorophyll was observed for $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets with a value of 0.70 ± 0.08 mg/g. For the healthy plants, the increased pigment concentration may be an indication of decreased plant stress and robust plant health. Conversely, in the diseased plants, the decreased chlorophyll in response to $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets may indicate that the rapid dissolution of ions interferes with chlorophyll biosynthesis.^{26, 49}

2.3.7 Field study

Full life cycle studies were performed at two separate field sites to determine whether the results of the greenhouse studies would translate to a practical field agricultural application. At both field locations, the CuO and $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanomaterials significantly reduced the AUDPC when compared to the diseased control, although the two chemical compositions yielded results that were statistically equivalent to each other. At location 1, **Figure 2.5** shows

that the CuO and $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanoparticles reduced disease by 29.9 % and 39.2 %, respectively. Similarly, measurements at location 2 showed that treatment with $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ reduced disease by 34%; no CuO NP treatment was established at location 2. The nanomaterial results were compared to Cu ions produced by a CuSO_4 salt control; these two treatments were shown to be statistically equivalent.

Fruit productivity

At both locations, the presence of disease reduced crop yield (total wet mass of fruit per replicate plot) by 34.8% (from 10.9 ± 1.1 kg to 7.1 ± 1.1 kg) and 57.1% (from 12.6 ± 1.8 kg to 5.4 ± 1.6 kg), respectively. At location 1, in the presence of disease the CuO nanoparticle treatment increased yield by 29% when compared to the diseased control, resulting in a value that was statistically equivalent to the untreated controls (**Figure 2.5B**). The $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets and CuSO_4 salt control offered no such benefit on total yield. Disease reduced the average mass of per fruit from 2.30 kg to 1.90 kg; under disease conditions, treatment with $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ restored the average per fruit mass to 2.70 kg. At location 2, the $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ and CuSO_4 salt control treatments increased fruit yield by 38.2%, although large replicate variability resulted in a lack of statistical significance (**Figure 2.5C**).

Elemental analysis

Elemental analysis was conducted on the roots, shoots and fruit of plants from both field locations (**Table A2.3-6**). In general, changes with nanoscale treatment were minimal. For the fruit from the field study at both locations, there appeared to be no increase in fruit Cu content with respect to nanoparticle treatment; which may be due to the fact that nanomaterials were

applied to the foliage several months before harvest. In addition, at location 1 the fruit treated with the CuO nanoparticles showed reduced content of Zn, Si, S, P, Mn, Mg, K, and Fe. Conversely, the fruits of plants treated with $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets showed increased concentrations of Si, Mn, Mg, Fe and Ca and had reduced concentrations of Zn, S, P, Cu, and K. Furthermore, in response to disease, plants at location 1 showed decreased shoot Mg and Fe; increases in the root Cu and Ca concentration; and decreases in root Si and Fe. Under disease conditions, the nanomaterials increased the Mn content and CuO NP increased the Fe content. At location 2, shoot Cu, Mg, and S content decreased and shoot Mn increased with respect to disease. No noticeable changes were observed in the roots. In response to $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets, there was an increase in the shoot Zn content.

2.4 Environmental implications

Taken together, the greenhouse and field studies demonstrate the significant potential for nanoscale Cu treatments to suppress fungal root infection and sustainably promote overall plant health and perhaps yield. Cu has been widely used in traditional agricultural practices to repress disease. However, the high volumes of Cu currently used has led to concerns about environmental accumulation of this metal, as well as the development of resistance among pathogens. The development of nanoscale Cu delivery systems that could suppress disease and increase yield at lower active nutrient concentrations would be a significant achievement. Foliar application of commercial nanoscale CuO has been shown to suppress *Fusarium* and *Verticillium* wilt of tomato and eggplant, respectively, and increase the biomass and fruit yield in controlled laboratory settings.¹³ Furthermore, foliar application of CuO nanoparticles in the presence of disease was correlated with an up-regulation of gene expression of polyphenol oxidase, which is

involved in the non-specific defense reactions that protect plants against pathogens.³⁹ Nanoscale properties are clearly important to the efficacy of Cu treatment, with the materials showing effectiveness with a variety of species, including the 53% reduction of red root rot disease in tea.⁵⁰ To further enhance the effectiveness of nanomaterial treatments, effort should be focused on optimization of the nanomaterial composition, structure, and activity. For instance, Cu nano-composites, including core-shell Si-Cu, multivalent Cu, and quaternary ammonium stabilized Si-Cu composites, have shown increased disease suppression on tomato bacterial spot (*Xanthomonas perforans*) when compared to commercially available Kocide.⁴ Similarly, the findings of the current study show the significance of nanomaterial formulation. The $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets are significantly more effective than CuO NP, with 100x less mass needed to produce the same result in greenhouse studies, and nearly half the amount of Cu delivered on a per gram basis. The results of these studies should stimulate investigations to optimize efficacy as a function of elemental structure, and as well as to understand nanoparticle-plant surface interactions and the particle dissolution profile.

2.5 Conclusions

In summary, $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets and commercial CuO NP were applied to watermelon plants infected with Fusarium wilt to determine the role of ion release profile and nanomaterial composition/structure on disease suppression in greenhouse and field studies. The greenhouse studies indicate that the $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets reduce disease at a significantly lower mass concentration of particles as compared to CuO NP. The efficacy of the materials may be attributed to the reduced overall particle size, unique particle structure (sheets) and more rapid initial release of ions from $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets compared to the CuO NP. Both Cu

nanomaterials exhibited similar efficacy in field trials involving full life cycle plant growth, in spite of the fact that the amendment occurred only once at the seedling stage. These results indicate that the $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets and similar materials hold great promise for agricultural applications. However, the present variability in the particle morphology, surface area, and size motivates mechanistic studies to understand how these materials interact with the plant-pathogen system.

2.6 Figures

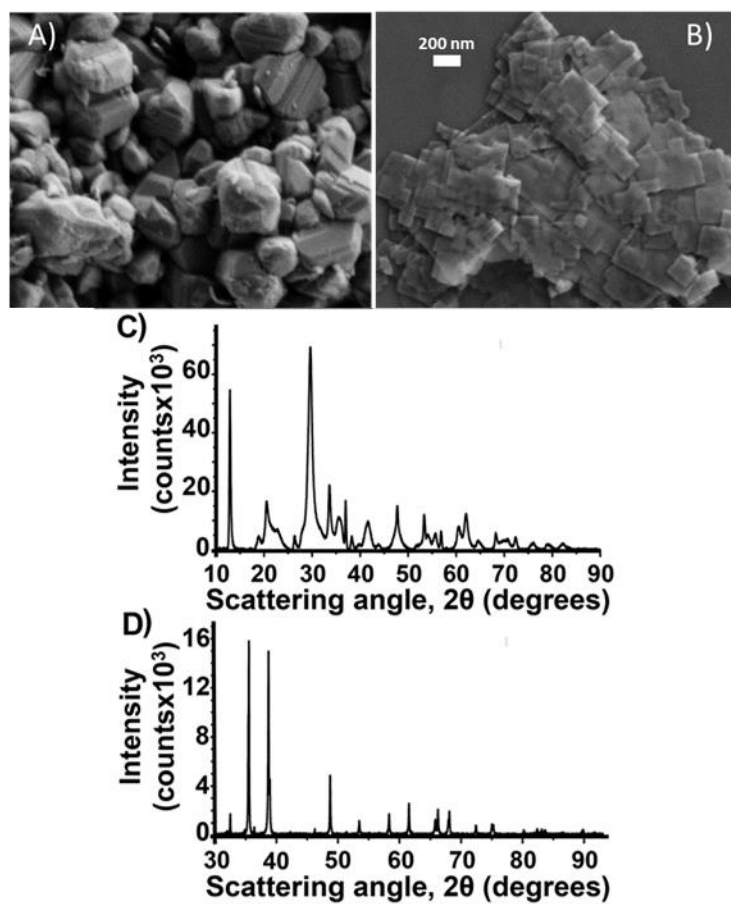


Figure 2.1 Micrographs and PXRD patterns of Cu₃(PO₄)₂•3H₂O nanosheets (B, C) and commercial CuO nanoparticles (A, D). The scale bar is for both A and B.

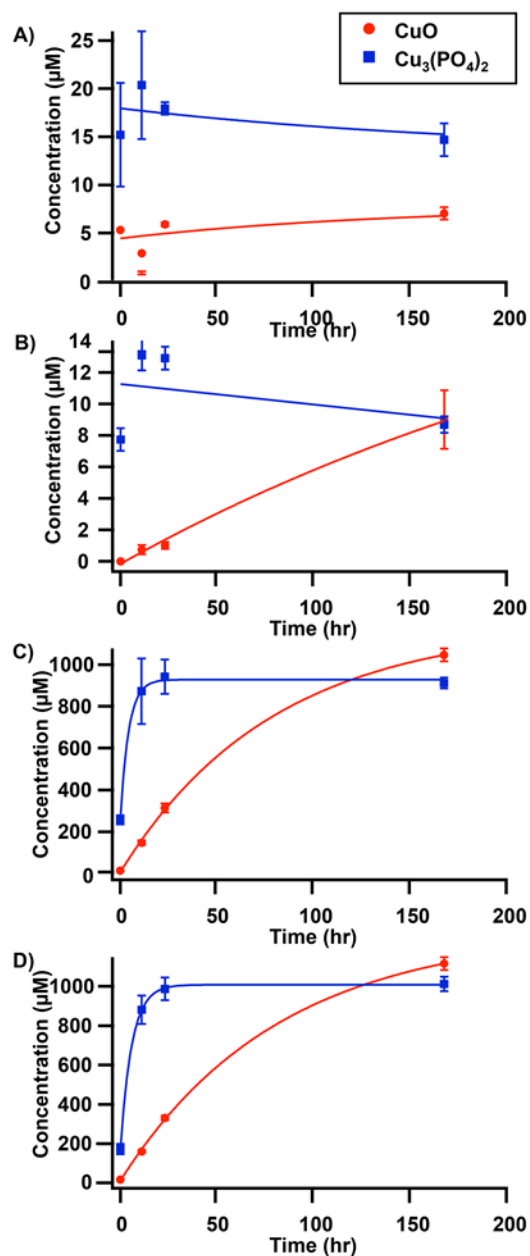


Figure 2.2 Concentration of Cu^{2+} in solution vs. time after the indicated nanomaterials was introduced into different media at a mass concentration of 100 mg/L of. Media shown are: A) deionized water B) Regulaid® C) malic and citric acid mixture and D) malic and citric acid mixture with added Regulaid®. Lines were generated from an exponential fit to the data.

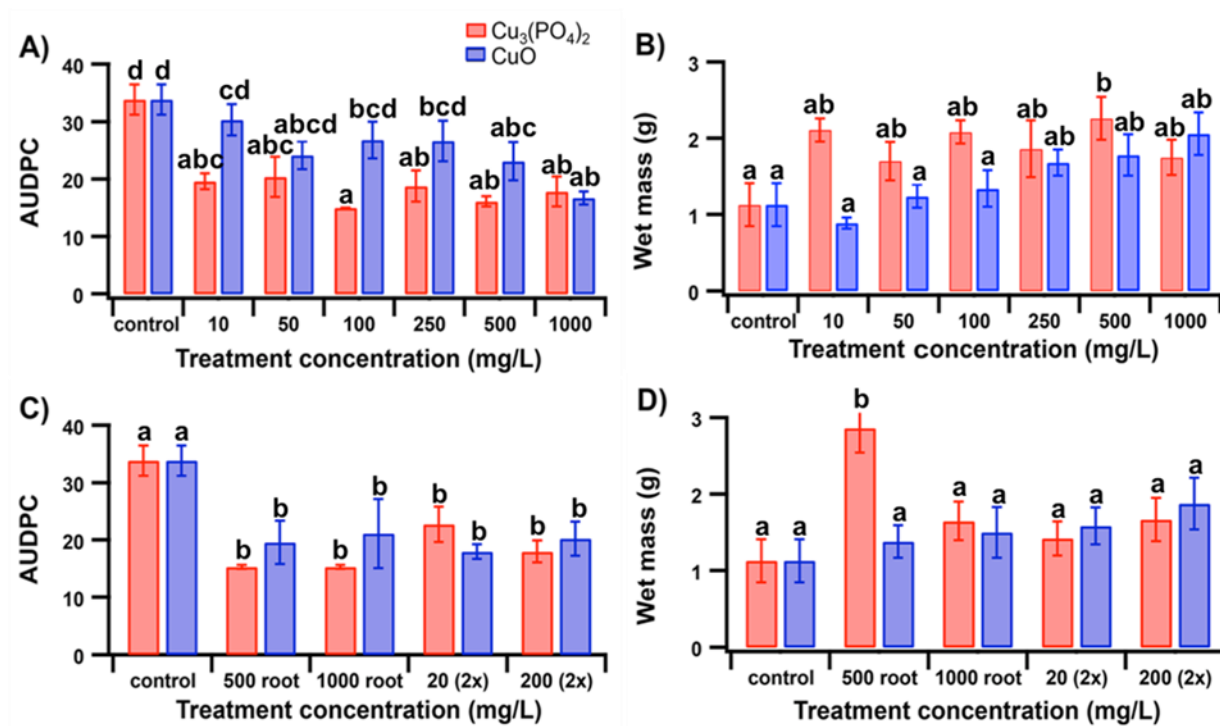


Figure 2.3 Disease rating and fresh weight of *Fusarium* infested watermelon upon exposure to $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets and CuO NP: A) The reduction of the area under the disease progress curve (AUDPC) with respect to foliar nanoparticle treatments; B) changes in total wet mass with respect to foliar treatment; C) the change in the AUDPC with respect to repeated foliar treatment and root applications of CuO NP and $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets D); changes in total wet mass with respect to repeated foliar applications and root exposure. For the repeated applications, “2x” indicated two separate foliar treatments of either 20 mg/L or 200 mg/L. A one-way ANOVA with a SNK multiple comparison test was used to determine significance across all the treatments. Values in each panel followed by different letters are significantly different at $p < 0.05$.

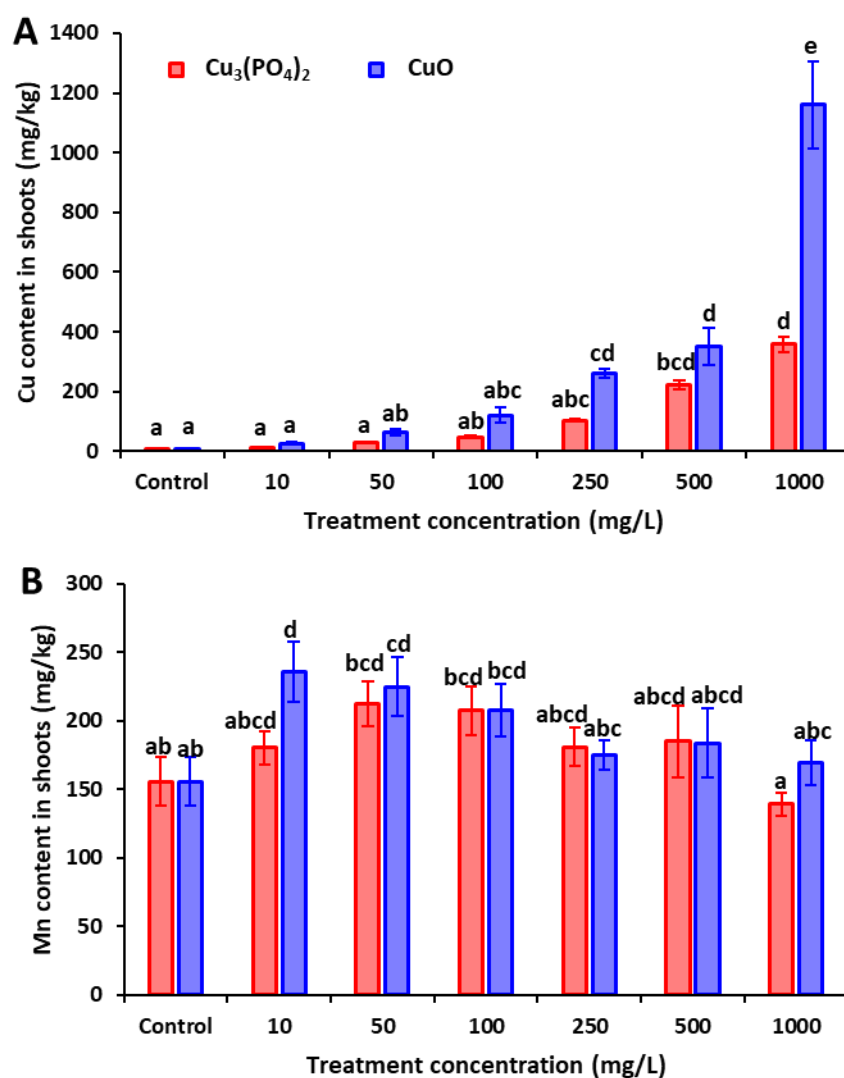


Figure 2.4 The Cu (A) and Mn (B) content in *Fusarium* infected watermelon shoots upon foliar exposure to different concentrations of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanosheets and CuO nanoparticles. A one-way ANOVA with an SNK multiple comparison test was used to determine significance across all the treatments. Values in each panel followed by different letters are significantly different at $p < 0.05$.

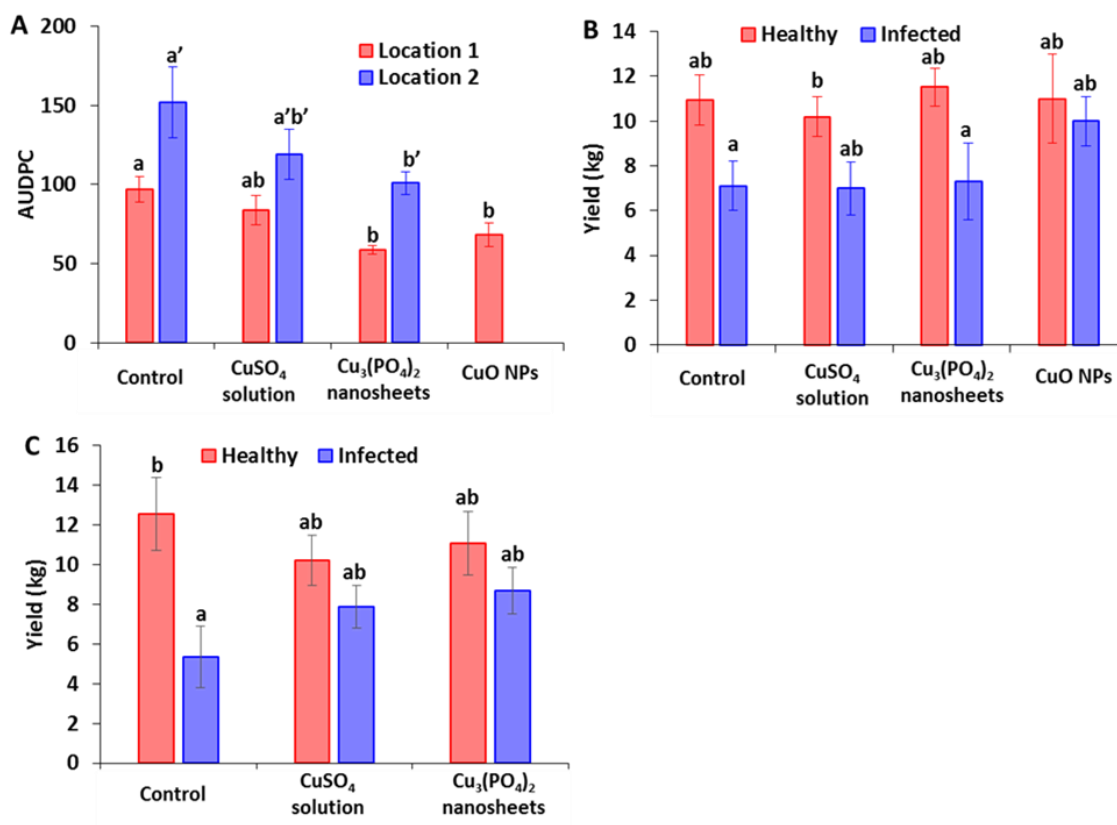


Figure 2.5 Disease progress and yield of Fusarium infested watermelon in Lockwood and Griswold field studies. Figure A represents disease progress for the Lockwood (Location 1) and Griswold (Location 2) using applications of CuO NP and Cu₃(PO₄)₂•3H₂O nanosheets compared to a CuSO₄ salt control. CuO NP were not used in Griswold field. Figure B and C represents the total yield of Cu-based nanomaterial treated watermelon w/ or w/o Fusarium infection for the Lockwood and Griswold locations, respectively. A one-way ANOVA with a SNK multiple comparison test was used to determine significance across all the treatments. For panel A, the analysis was within a location. For panels B and C, the analysis was across are treatments.

Values in each panel followed by different letters are significantly different at $p < 0.05$.

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Chapter 3: Influence of CuO nanoparticle aspect ratio and surface termination on disease suppression in tomato (*Solanum lycopersicum*)

This research was performed in collaboration with Yu Shen, Carlos Tamaz, Curtis Green, Jenny Orbeck, Wade Elmer, Jason White, and Robert J. Hamers. My contributions to the work include synthesis and characterization of the CuO nanomaterials, SAMs studies, and dissolution studies. TEM data collection and analysis was performed by Curtis Green. Biological data was collected at the Connecticut Agricultural Experiment Station.

3.1 Introduction

Innovation in agricultural practices is needed to meet the demands of the growing global population. Nanomaterials have been proposed to deliver agrichemicals to increase crop yields and growth efficiency while reducing the amount of agrichemicals that enter the environment. Only 30-40% of applied nutrients are used by crops with the excess accumulating in soils or bodies of water.¹⁻² The excess agrichemicals can have negative impacts on the environment affecting aquatic systems and the soil microbiota.³ Nanoparticles can tune the delivery rate of nutrients, increase interactions with plant tissues, and their small size allows for unique uptake mechanisms into plant systems.⁴⁻⁶ For instance, Cu-based nanoparticles applied to leaf surfaces were more resistant to rinse off than a Cu-salt solution.⁷ Nanomaterials applied to the foliage can transport through the plant system and enter other tissues.⁸ The nutrient containing nanoparticles that have been studied include Fe₂O₃, ZnO, liposome encapsulated Fe, hydroxyapatite, mesoporous silica, Cu₃(PO₄)₂, and CuO.⁹⁻¹⁴ Copper-containing nanomaterials are of particular interest because of their ability to suppress fungal diseases. Copper-based nanoparticles have been shown to suppress fungal pathogens in soybean, tomato, eggplant, and watermelon plants, where similar

concentrations of Cu-salt solution have no significant impact on disease.^{9, 15-17} The use of Cu-based nanomaterials have been related to increased expression of plant protection pathways.^{15-16,}

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The nanomaterial properties: shape, size, composition, and surface chemistry influence how nanomaterials interact with plant systems. The nanoparticle solubility and transport is impacted by the chemical composition. A comparison of CuO and Cu₃(PO₄)₂ showed that the more rapid Cu release profile from Cu₃(PO₄)₂ enhanced disease suppression.⁹ Another study comparing the uptake of CuO, CuS, and Cu(OH)₂ showed that more soluble Cu(OH)₂ nanoparticles were rapidly taken up, while less soluble CuO and CuS provided a sustained release of Cu.¹⁹ For crystalline nanomaterials the morphology is related to the distribution of crystal faces, that can have different surface energies depending on the organization of atoms.²⁰ A study comparing different morphologies of CeO₂ showed that rod-like CeO₂ with reactive crystal faces was more transformed and translocated in cucumber plants when compared to octahedral and cubic nanoparticles.²¹ The surface chemistry of nanomaterials influences nanoparticle adhesion to leaf surfaces and transport. Studies of positively and negatively charged CeO₂ nanoparticles applied at the root showed that positively charged particles adhere more to the root surface while negatively charged particles are able to translocate more efficiently to the foliage.²²⁻²³ In contrast, positively charged carbon dots were shown to target chloroplasts more effectively than negatively charged particles.²⁴ Nanoparticle shape and charge are linked to uptake and translocation, however, to our knowledge there are no studies that show the effect of nanoparticle charge and aspect ratio on disease suppression.

In this study, we developed a synthesis method to prepare CuO nanoparticles with tunable aspect ratios and zeta potentials. We studied the disease suppression efficacy of CuO(+) nanospikes, CuO(-) nanospikes, and CuO(-) nanosheets on *Fusarium oxysporum* infected tomato (*Solanum lycopersicum*) plants to probe the effect of nanoparticle charge and aspect ratio on disease suppression. To understand the intermolecular between the tomato plants and the nanoparticles, we investigated the interaction of the nanoparticles with self-assembled monolayers. In addition, we performed dissolution experiments to determine the influence of simulated plant matrices on ion release.

3.2 Materials and methods

3.2.1 Reagents

We purchased $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{LiOH} \cdot \text{H}_2\text{O}$, citric acid, maleic acid, malic acid, hexadecanethiol, 16-mercaptohexadecanoic acid, and (11-mercaptopundecyl)-N,N,N-trimethylammonium bromide from Millipore Sigma. We purchased 16-mercaptohexadecan-1-ol from VWR. We purchased ACROS Organics 99.5% ethanol from Fisher scientific. All reagents were used without further purification.

3.2.2 CuO nanoparticle synthesis

CuO nanoparticles were synthesized using a microwave assisted-hydrothermal method. A 1.00 mL aliquot of 1 M CuCl_2 and 2.00 ml of 1 M LiOH solution were added to 17 mL of nanopure water (Barnstead systems, 18.2 M Ω). The addition of LiOH resulted in the formation of a vibrant blue precipitate. The solution was stirred for 10 minutes and the pH of the solution was measured, and possibly adjusted by adding LiOH solution. As discussed below, the nanoparticle shape was strongly dependent on the solution pH. The solution was then added to a CEM

Discover microwave synthesizer. In the microwave the solution was pre-stirred for 2 minutes, then reacted at 160 °C for 10 minutes. After the reaction, the black precipitate was cooled to room temperature. The particles were isolated by centrifugation for 5 minutes (x4696g) and the supernatant was removed. To clean the particles, the particles were resuspended in water and isolated by centrifugation. The cleaning procedure was repeated 3 times with water, and the final rinse was performed with ethanol. The isolated particles were dried at 30 °C under vacuum overnight.

3.2.3 Scanning electron microscopy

A dilute solution of nanoparticles was prepared in ethanol (~100 mg/L). The nanoparticles were suspended by ultrasonication for 5 minutes (1.5 W). The nanoparticle suspension was drop cast onto a conductive silicon wafer. Scanning electron micrographs were collected with a Leo Ziess Supra V55 scanning electron microscope. We measured the dimensions of 100 nanoparticles using ImageJ software²⁵⁻²⁶ and then prepared histograms using Igor Pro (WaveMetrics, Lake Oswego, OR, USA). .

3.2.4 Transmission electron microscopy (TEM)

Samples were prepared for imaging via TEM by ultrasonicated a suspension of 1mg/mL CuO nanoparticles in ethanol. An aliquot of the suspension was dripped onto a carbon-backed Cu grid from Ted Pella, Inc. (Redding, CA) and allowed to dry overnight. TEM images and diffraction patterns were collected on a Tecnai TF30 TEM at an accelerating voltage of 300 kV. Images were processed using ImageJ software. Lattice d-spacings were confirmed by analysis of the fast Fourier transform (FFT) of lattice images.

3.2.4 X-ray diffraction

A thick slurry of CuO nanoparticles was prepared in water. The slurry was drop-cast onto a silicon zero- diffraction plate (MTI corporation). The diffraction pattern was collected on a Bruker D8 powder diffractometer, and the data was processed using Bruker Diffrac.Suite EVA (Bruker-AXS, Madison, WI, USA).

3.2.5 Zeta potential measurements

A 50 mg/L suspension of CuO nanoparticles was vortexed and ultrasonicated (21.5 W) for 5 min before the zeta potential measurement was collected. The suspension was analyzed a Malvern Panalytical Zetasizer nanoseries.

3.2.6 X-ray photoelectron spectroscopy

Samples were prepared for X-ray photoelectron spectroscopy (XPS) by drop casting a thick slurry of nanomaterials on a gold substrate. The XPS spectra were collected with a PHI 5000 VersaProbe III. To collect survey spectra the pass energy was 280 eV, and the step size was 2 eV. We collected high resolution spectra in the O1s, Cu(2p), C(1s), and Si(2p) regions using a pass energy of 26 eV, step size of 0.1 eV, and a dwell time of 20 ms. The spectra were processed using CasaXPS and were calibrated to the adventitious carbon peak at 284.8 eV.

3.2.7 Greenhouse experiment

For the greenhouse experiment, tomato seeds (*Solanum lycopersicum* L. cv Bonnie Best; Harris Seed Co., Rochester NY) were germinated in 36 cell ($5.66 \times 4.93 \times 5.66$ cm) plastic liners (1 plant per cell) filled with soilless potting mix (ProMix BX. Premier Hort Tech, Quakertown, PA, USA). After three weeks, the seedlings were fertilized with 40 ml of Peter's soluble 20-10-20 (N-P-K) fertilizer (R.J. Peters, Inc., Allentown, PA). The pathogen inoculum was prepared as

described previously. Japanese millet (*Echinochloa esculenta*) was autoclaved with distilled water (1:1, wt: wt) for 1 h on two consecutive days and was seeded with three agar plugs colonized with *Fusarium. oxysporum f. sp. lycopersici*. After growth for 2 weeks at 22-25 °C, the millet was air-dried and ground in a mill. The inoculum was then hand incorporated into the potting mix Mix BX (without mycorrhizae; Premier Hort. Tech, Quakertown, PA, USA) at 0.75 g millet inoculum/L potting mix. Seedlings were subsequently added to the potting mix.

Plants of uniform size with 3-4 leaves were selected for the greenhouse experiment. There were five treatments in both diseased and healthy soils: 1. untreated control, 2. 274.68 mg/l CuO(-) nanosheets; 3. 125.00 mg/l CuO(-) nanospikes, 4. 125.00 mg/l CuO(+) nanospikes; 5, 393.70 mg/l CuSO₄·5H₂O solution. NP suspensions of CuO nanoparticles were prepared in deionized water amended with a nonionic surfactant (Regulaid® 1 ml/l); this surfactant is typical in commercial agrichemical formulations to facilitate retention on the leaf surface. The CuO suspension was sonicated with probe sonicator for 2 min in an ice bath. Foliar application was used for treatment; each plant was sprayed with approximately 2 ml of the appropriate suspension. The plants were then inverted until the foliage had completely dried (1 hour) and were then transplanted into 10 cm diameter plastic pots (350 ml) containing potting mix that was or was not infested with *Fusarium*. The potted tomato seedlings were arranged on greenhouse benches in a randomized block design. Temperatures averaged 17-22 °C night and 19-25 °C day. After 28 days of growth, the tomato seedlings were harvested. During growth, disease progress was determined and the area-under-the-disease-progress curve (AUDPC) was calculated. At harvest, wet and dry biomass was measured and tissue elemental composition was determined.

3.2.8 Tissue Elemental Analysis

Root and shoot tissue samples were dried in an oven at 50 °C, ground in a Wiley mill, and passed through a 1 mm sieve. Digestion of ground samples (0.5 g) was done in 50 ml polypropylene digestion tubes with 5 ml of concentrated nitric acid at 115 °C for 45 min using a hot block (DigiPREP System; SCP Science, Champlain, NY). The Cu and Si contents were quantified using inductively coupled plasma optical emission spectroscopy (ICP-OES) on a Thermo Fisher iCAP 6500 (Thermo Fisher Scientific, Waltham, MA); element content is expressed as µg/g (dry weight) plant tissue. Yttrium (Yi) was used as an internal standard and a sample of known concentration was read every fifteen samples.

3.2.9 Interaction of CuO nanoparticles with self-assembled monolayers

To prepare self-assembled monolayers we submerged ultra-flat gold films (RMS=0.2 nm Platypus technologies) in 1 mM ethanolic solutions of alkanethiol molecules for 24 hours. The alkanethiol molecules that we used were hexadecanethiol (-CH₃), 16-mercaptohexadecanoic acid (-COOH), 16-mercaptohexadecan-1-ol (-OH), and (11-Mercaptoundecyl)-N,N,N-trimethylammonium bromide (-N(CH₃)₃). The SAMs were cleaned with nanopure water, ethanol, and dried with nitrogen. The self-assembled monolayers were characterized with a Dataphysics OCA 15 plus contact angle measurement system, measured as sessile drops in triplicate.

To expose the nanomaterials to the SAMs, a 100 mg/L suspension of CuO nanoparticles was bath sonicated for 10 minutes. The SAMs were submerged in the nanoparticle suspension and held perpendicular to the ground to minimize gravitational settling of nanoparticles on the SAM surface. After incubating in the nanoparticle suspension for 1 hr, the nanoparticle suspension was diluted using nanopure water until the water flowing out of the beaker was clear. The SAMs were

then removed from the water and dried with nitrogen. Adsorption of nanoparticles to the SAMS was quantified using electron microscopy, counting 7 images per SAM and analyzed using ImageJ. The number of nanoparticles was normalized by the area analyzed, and, three separate experiments were performed.

3.2.10 Dissolution of CuO nanoparticles

We measured the dissolution of CuO nanoparticles in a simulated xylem fluid and nanopure water. In both cases we adjusted the solution pH to 6.0 using NaOH and HCl. We prepared a pH 6 simulated xylem fluid containing 628 μM malic acid, 1206 μM maleic acid, and 301 μM citric acid, using White et. al as a reference for the most abundant small organic acids in tomato xylem fluid.²⁷ The nanoparticles were suspended at a concentration of 100 mg/L. The nanoparticle suspensions were incubated for 18 hours and agitated at 150 rpm using a New Brunswick C24 incubator shaker. The suspensions were then centrifuged for 30 minutes (4696 $\times g$) and the supernatant was extracted, the process was repeated once. The supernatant was then acidified to 2% HNO_3 , and the concentration of Cu in solution was determined using an Agilent 5110 inductively-coupled plasma optical emission spectrometer (ICP-OES).

3.3 Results and discussion

3.3.1 CuO nanoparticle morphology and crystal structure

We developed a synthesis method to control the aspect ratio and zeta potential of CuO nanoparticles by altering the reaction pH. **Figure 3.1** shows the nanoparticles that we synthesized. **Figure 3.1A** shows the positively charged nanospikes synthesized at pH ranges from 11.1-11.3. The positively charged nanospikes had a zeta potential of 23 ± 1 mV. When the pH of the reaction solution was increased to 11.4-11.6 the zeta potential of the nanoparticles switched from positive

to a negative zeta potential of -27.6 ± 0.6 mV, **Figure 3.1B** shows that the negatively charged particles maintained a spike like morphology. **Figure 3.2** shows histograms of the nanoparticle dimensions based on the measurement of 100 nanoparticles, the spike-like nanoparticles had aspect ratios of 7 ± 3 . **Figure 3.1C** shows the nanoparticles synthesized at pH values between 11.6 and 12.1 were negatively charged with a zeta potential of -48.8 ± 0.3 mV and had a sheet-like morphology. The aspect ratio of the nanoparticles was 4 ± 1 .

Figure 3.1D shows that synthesized CuO nanoparticles had diffraction patterns consistent with the monoclinic tenorite reference spectra.²⁸ The drop-casted samples showed an increase in the (002) peak compared to the reference spectrum. In **Figure 3.1A-C**, the nanoparticles are all shown to lay relatively flat on the Si surface, therefore, when the X-ray diffraction patterns were collected the preferential alignment of the CuO nanoparticles increased the sampling of the major (001) face. A similar effect has been reported for preferred orientation of CdS rods which increased the sampling of the (110) face.²⁹ **Figure 3.3** shows transmission electron micrographs (TEM) and selected angle electron diffraction (SAED) patterns used to determine the exposed crystal faces. In **Figure 3.3 (B,E,H)** SAED confirmed (001) zone axes for all samples when imaging the large, flat face, confirming (001) as the primary exposed face of the nanoparticles. In **Figure 3.3 (C,F,J)** high-resolution TEM (HRTEM) images show clear lattice fringes. The corresponding FFT (inset) shows a d-spacing of 0.27 nm, in agreement with the (110) plane of CuO that is normal to the [001] direction.

3.3.2 Surface chemistry of CuO nanoparticles

We used X-ray photoelectron spectroscopy to investigate any differences in surface composition between CuO(+) nanoparticles and CuO(-) nanoparticles. **Figure 3.4** shows data

obtained in the O(1s) and Cu(2p) region. As shown in **Figure 3.4A**, the Cu(2p) region shows a Cu(2p_{1/2}) spin-orbit component at 953 eV and Cu(2p_{3/2}) component at 933 ± 0.1 eV. Each spin orbit peak is accompanied by a satellite feature 8-10 eV higher that is attributed to the O_{2p} → Cu_{3d} ligand-to-metal transition characteristic of CuO.³⁰ In **Figure 3.4A** the Cu(2p_{3/2}) peak show a small shift, from 933.4 for CuO(+) nanospikes to 933.8 ± 0.1 eV for Cu(-) nanospikes, and at 934.0 ± 0.5 eV for nanosheets prepared at higher pH values.

Figure 3.4B shows the O(1s) spectra, the O(1s) spectra have apparent differences between the different CuO nanoparticle types. All samples show a single large peak, at 529.55 ± 0.03 eV, 529.86 ± 0.03 eV, and 530.2 ± 0.3 eV for the CuO(+) nanospikes, CuO(-) nanospikes, and CuO(-) nanosheets, respectively, that we attribute to lattice oxygen of CuO. Curve-fitting of the peak indicated that the higher binding energy region of the O1s spectra for the CuO(+) nanospikes showed one additional component at 531.06 ± 0.02 eV. Fitting of the negatively charged particles reveal two components at higher binding energies, centered at 531.1 ± 0.2 eV and 532.4 ± 0.2 for CuO(-) nanosheets and at 530.70 ± 0.03 eV and 532.70 ± 0.07 eV for the nanospikes. A comprehensive study of metal oxide XPS spectra showed that the features between 530- 531 eV most likely arise from surface OH species, while the features at 532 eV may be attributed to under-coordinated oxygen.³¹

We quantified the relative percent of surface Cu and O using the following equation:

$$\%a = 100 \times \frac{A_a/SF_a}{\left(\frac{A_a}{SF_a} + \frac{A_b}{SF_b}\right)}$$

2(1)

where, A represents the XPS peak area of the element and SF represents the sensitivity factor for each element, with respect to element a and b. **Figure 3.4C** shows the relative percent of O and Cu on the nanoparticle surface calculated using three points measured per sample for the high-resolution O(1s) and Cu(2p) spectra.

In **Figure 3.5** we show the survey spectra of CuO where we noticed the presence of small Si peaks on the negatively charged nanoparticles; upon further investigation we found that this Si arises from the glass vessels used for microwave synthesis. As a result, some of the observed O signal likely arises from SiO_x species. To calculate the surface coverage of Si and O we used the following coverage equation:

$$Coverage = \frac{Area_{Element}}{Area_{Sub}} * \frac{SF_{Sub}}{SF_{Element}} * \frac{Scans_{Sub}}{Scans_{Element}} * \rho * \lambda * \cos(45^\circ)$$

where, sub represents the substrate, element represents the overlayer, SF represents the sensitivity factory, ρ represents the number density, and λ represents the inelastic mean free path. The coverage of Si on the surface was calculated to be 3.9 ± 0.2 atoms/nm² for CuO(-) nanopikes and 3.5 ± 0.3 atoms/nm² for CuO(-) nanosheets. The peak at 532 eV may be attributed to silica, as previous research has reported silica peaks within that region.³² We calculated the coverage of the O component at 532 eV to be 23 ± 5 atoms/nm² for CuO(-) nanopikes and 20 ± 1 atoms/nm² for CuO(-) nanosheets. Assuming that the stoichiometry of the silica layer is 2:1 for O:Si, the silica accounts for 49 and 53 % of the O peak at 532 eV for the nanopikes and nanosheets, respectively. It is possible that the Si adsorb to the polar O terminated face to reduce the surface energy. Ultimately, while Si may contribute to the surface concentration O of CuO(-) nanopikes and CuO(-) nanosheets we prepared positively and

negatively charged CuO nanoparticles that have zeta potentials that correlate to the surface O and Cu concentration.

3.3.3 Influence of CuO shape and charge on fungal disease suppression

We studied the influence of CuO(+) nanospikes, CuO(-) nanospikes, CuO(-) nanosheets, and CuSO₄ on healthy and *F. oxysporum* infested *S. lycopersicum*. **Figure 3.6A** shows the extent of disease progression, assessed by measuring the plant health as a function of time; the resulting Area Under the Disease Progression Curve (AUDPC) is a widely accepted method for assessing plant health, with lower values representing healthier plants.³³ The negatively charged CuO nanospikes and nanosheets significantly reduced the AUDPC when compared to the positively charged nanospikes and CuSO₄ salt control indicating that negatively charged nanoparticles repress disease.

Figure 3.6B,C shows measurements of the wet biomass of healthy plants (**Figure 3.6b**) and of *F. oxysporum*-exposed plants (**Figure 3.6C**) and that were exposed to the CuO nanoparticle types. Also shown are control experiments of plants with no additional treatment, and plants that were exposed to Cu ions as aqueous solution of CuSO₄. **Figure 3.6B** shows that even in the absence of *F. oxysporum* infestation, exposure to CuO nanoparticles increased the overall wet mass, with the negatively charged nanoparticles having the most beneficial effect. **Figure 3.6C** shows that in all cases, exposure to *F. oxysporum* reduced the biomass compared to the healthy plants. The negatively charged nanosheets significantly increased biomass for *F. oxysporum* infested tomato, while negatively charged CuO nanospikes qualitatively increased the wet mass. The positively charged CuO nanospikes and CuSO₄ did not increase the biomass when compared to the diseased control. Overall, the data show that the negatively charged

nanoparticles have the greatest positive impact on both healthy plants and fusarium-infected plants.

3.3.4 Adsorption and transport of Cu

We analyzed the amount of Cu that remained on the plant shoots at the end of the greenhouse study. **Figure 3.7A** shows that under healthy conditions, the control had 10.6 ± 0.8 mg/kg of Cu in the shoot. The negatively charged CuO nanoparticles increased the shoot Cu concentration to 17 ± 2 mg/kg and 18 ± 2 mg/kg for the nanosheets and nanospikes, respectively. The CuO(+) nanospikes and CuSO₄ significantly increased the Cu concentration to 23 ± 2 mg/kg and 29 ± 4 mg/kg, respectively. The positively charged particles and salt control increase the amount of Cu on the leaf surface when compared to the negatively charged treatment. **Figure 3.7B** showed that under disease conditions the Cu concentration in the foliage was higher overall. All the Cu-treatments increased the amount of Cu in the foliage when compared to the control. The positively charged nanoparticles showed significantly more Cu in the foliage than the other nanoparticle types. The negatively charged CuO nanosheets increased the Cu concentration on the leaf surface slightly when compared to the negatively charged spikes.

Figure 3.7C showed that under healthy conditions, none of the nanoparticle treatments significantly increased the amount of Cu in the roots when compared to the control. **Figure 3.7D** showed that under disease conditions, the control had a Cu concentration of 18.5 ± 0.5 mg/kg, which was significantly increased for the CuO(-) nanosheets that had a root concentration of 24.41 ± 0.04 mg/kg.

3.3.5 Impact of CuO surface termination on intermolecular interactions

We prepared self-assembled monolayers (SAMs) to understand the intermolecular interactions that may govern interactions between CuO nanoparticles and plant systems. The leaf surface is hydrophobic, has OH functionality, and bears a negative charge.³⁴ To simplify and simulate interactions at the leaf surface we prepared 16-mercaptohexadecan-1-ol and hexadecanethiol to represent OH terminated and hydrophobic surfaces respectively. We also prepared negatively charged SAMs with 16-mercaptohexadecanoic acid, and a positively charged control with (11-mercaptopundecyl)-N,N,N-trimethylammonium bromide. Contact-angle measurements yielded values of 35 °, 25°, 30°, and 108° for the -N(CH₃)₃, -COOH, -OH, and -CH₃ SAMs, respectively.

We analyzed the nanoparticle attachment to the SAMs after one hour incubation period. **Figure 3.8B** shows the percent coverage of CuO nanoparticles, measured by SEM. The coverage was measured by counting the number of nanoparticles per unit area, and multiplying the particles by the area of a particle. The attachment of nanoparticles to the SAMs was strongly dependent on the charge of the SAM and of the nanoparticles, with negatively charged nanoparticles binding more strongly to positively charged SAMs, and positively charged nanoparticles binding most strongly to the negatively charged SAMs. The data in **Figure 3.8B** show that there almost no binding of charge nanospikes to SAMS of the opposite charge, likely due to electrostatic repulsion. Negatively charged nanosheets and nanospikes exhibited higher binding when compared to the positive nanospikes on both the polar OH terminated SAMs and the non-polar CH₃ terminated SAMs.

3.3.6 Dissolution study

We performed dissolution measurement to determine the influence of CuO shape and surface charge on the overall release of Cu in pH 6 water and in simulated xylem fluid. **Figure 3.9A** shows that in simulated xylem fluid the positively charged CuO(+) nanospikes released more Cu than the other nanoparticles. **Figure 3.9B** shows that in pH 6 water the Cu release was similar for all nanoparticles and was much lower than that in the simulated xylem fluid. The presence of small organic acids may interact through the chelation of metals on the nanoparticle surface or metals free in solution to drive the dissolution process. These results indicate plant matrix likely plays a strong role in controlling the rate of Cu release.

3.3.7 Plant interactions and materials properties

A key finding of this study is that the negatively charged nanosheets showed the highest efficacy for disease suppression and biomass enhancement. The negatively charged nanospikes also significantly impacted plant health, while the positively charged nanoparticles and Cu(II) ions showed no impact. The nanomaterial delivery to plants may influence the disease response, which includes the adsorption of Cu to the leaf surface, nanoparticle uptake, translocation, and ion release. In addition, the nanoparticle charge may influence the plant metabolic or defense pathways leading to the observed differences in disease suppression. A) the amount of Cu delivered to the plants by adsorption to the leaf surface B) internalization into plant tissues, B) the translocation efficiency of nanomaterials through the plant, C) the nanoparticle ion release response, D) the direct toxicity of the nanomaterials to the fungal pathogens, and E) the molecular level interactions between the nanomaterials and the plant biomolecules.

We use self-assembled monolayer studies to understand the intermolecular interactions that drive adsorption of charged CuO nanoparticles to the leaf surface. The ICP measurements give information about the amount of Cu present in the leaf tissue and gives information about the nanoparticle adsorption to the leaf surface. Under diseased and healthy conditions, all Cu-treatments increased the amount of Cu in the foliage. As expected, the studies with SAMs showed that charge-based interactions can be a strong driving force for nanoparticle adsorption or repulsion. Previous studies have shown that the leaf surface carries a negative charge.³⁴ The increased adsorption of the positively charged nanoparticles to the negatively charged leaf surface could be attributed to the electrostatic attraction. The negatively charged nanoparticles may be electrostatically repulsed from the leaf surface which may lead to less adsorption to the leaf surface, however, negatively charged nanoparticles were shown to increase the foliage Cu concentration when compared to the control, which may be attributed to the increased adsorption of negatively charged particles to polar OH and hydrophobic CH₃ terminated SAM surfaces. The increased adsorption of the negatively charged particles to the OH terminated surface may be attributed to the increased O concentration and hydrogen bonding interactions. The initial surface that nanoparticles will come in to contact with is the leaf cuticle. The leaf cuticle consists of cuticular waxes and biopolymers like cutan and cutin that creates a surface that has hydrophobic character and regions of hydrophilicity. The monomer units for cutin have epoxide and alcohol moieties that may contribute to hydrogen-bonding interactions with nanoparticles.³⁴ From this perspective, the negatively charged particles may form interactions with the leaf surface through hydrogen bonding interactions or through hydrophobic interactions.

Despite the higher concentration of Cu from positively charged nanoparticles in the foliage, positively charged nanoparticles do not impact plant health. The ICP measurements give information about the total amount of Cu in the plant tissues, not the speciation and location. Therefore, even though the positively charged nanoparticles have more Cu on the foliage, the nanoparticles may be internalized less or immobilized on the leaf surface by the electrostatic interaction. ICP measurements of the root Cu concentration can provide information about translocation through the plant system but have the same limitations. The root Cu concentration qualitatively increases slightly for all Cu-treatments under diseased and healthy conditions. The negatively CuO nanosheets significantly increased the root Cu concentration, which indicates that negatively charged nanosheets may have increased translocation efficiency. The increased translocation efficiency may contribute to the enhanced disease suppression of negatively charged nanoparticles. Previous studies have shown that positively charged nanoparticles increase the nanoparticle interaction with either the root or leaf surface, and negatively charged nanoparticles have increased translocation through the plant system.^{22-23, 35}

The nanosheets had the greatest impact on plant health. The large flat surface of the nanosheets may increase the contact with the leaf surface to facilitate adsorption and Cu uptake. In addition, the nanoparticle shape is influenced by the nanoparticle crystal faces. Our TEM and XRD data suggest that the major crystal face is the (001). Previous studies have shown that the CuO (001) crystal face has the highest surface activity which increases reactivity.³⁶ The CuO nanosheets have a larger exposed (001) crystal face when compared to the nanospikes which may contribute to increased reactivity. The reactivity may influence molecular level interactions like the adsorption of biomolecules to the nanoparticle surface or redox reactions with

biomolecules which ultimately may impact the plant response. A study that probed the effect of CeO₂ nanoparticle shape, showed that rod-shaped nanoparticles with reactive crystal faces had increased translocation and transformations when compared to less reactive spherical nanoparticles.²¹ The reactivity can also be thought of in terms of the nanoparticle dissolution. It is an interesting result that the negatively charged nanoparticles released less Cu when compared to the positively charged particles, but still had a greater biological response.

We determined that nanoparticle shape and surface charge are important parameters for disease suppression and nutrient delivery. Future studies can be performed to directly probe some of the hypotheses outlined above.

3.4 Conclusions

We developed a synthesis method to make CuO nanoparticles with exposed (001) crystal faces that have tunable zeta potentials and aspect ratios. The nanoparticle synthesis was tuned by altering the relative ratio of CuCl₂:LiOH in solution, under conditions where Cu was in excess the nanoparticles were positively charged, and when OH was in excess the nanoparticles were negatively charged. Increasing the OH concentration further caused the (001) face of the crystal to expand. The synthesized nanoparticles were used to suppress an *F. oxysporum* infestation in tomato plants. The CuO(-) nanosheets and nanospikes significantly repressed disease and increased plant biomass when compared to the CuO(+) nanospikes. The CuO(+) nanoparticles increased the foliar Cu concentration likely through electrostatic interactions with the negatively charged leaf surface. The CuO(-) nanosheets increased the amount of Cu in the root, and their relatively high mobility through the plant system likely promoted the disease suppression efficacy. The CuO(-) nanosheets increased the plant health slightly more than the CuO(-)

nanospikes, which may be attributed to increasing the exposed area of the reactive (001) crystal face or a shape effect.

The nanoparticle charge and aspect ratio impact the plant health and disease suppression efficacy. While, we believe that transport through the plant system influences the disease suppression efficacy, further studies could be performed to investigate the transport of charged nanoparticles through the cuticle and throughout the plant system as a function of time. To understand the influence of nanoparticle charge on plant disease suppression pathways we could measure the expression of pathogenesis-related genes transcriptional activator, polyphenol oxidase, and plant resistance protein 1A1.³⁷ Other studies have shown that nanoparticle charge influences the identity of the biomolecular corona in plants, further research could be performed to look at how the corona formed in plant matrices is impacted by nanoparticle charge or aspect ratio.³⁸⁻³⁹ In addition, in this study the suite of nanomaterials that we used have reactive crystal faces of CuO nanoparticles exposed, another study could be performed to look at how the reactivity of crystal faces affects disease suppression by using nanoparticles with less reactive crystal faces as a comparison. Understanding how nanomaterial properties impact interactions with plant systems and promote disease suppression can help develop new agrichemicals to meet the needs of the growing population.

3.5 Figures

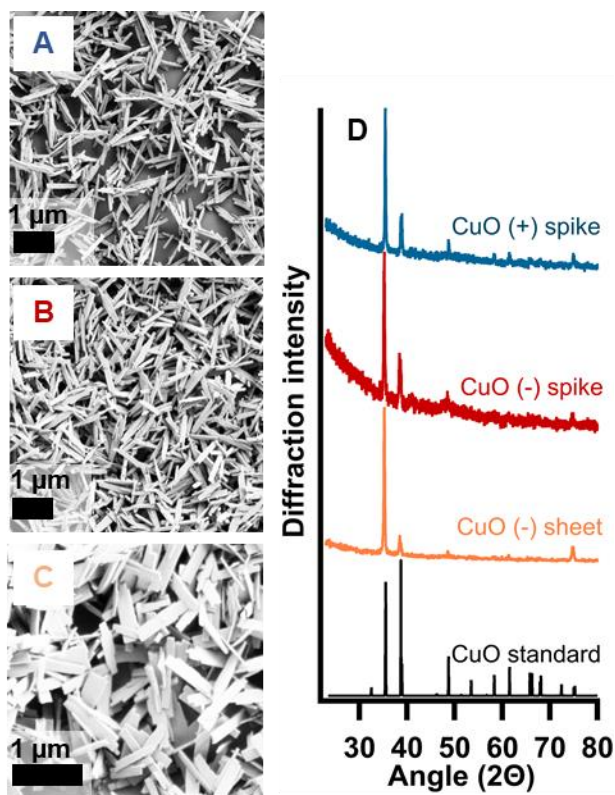


Figure 3.1 Scanning electron micrographs of A) CuO (+) nanospikes, B) CuO (-) nanospikes, and C) CuO (-) nanosheets and D) X-ray diffraction patterns of tenorite CuO nanomaterials.

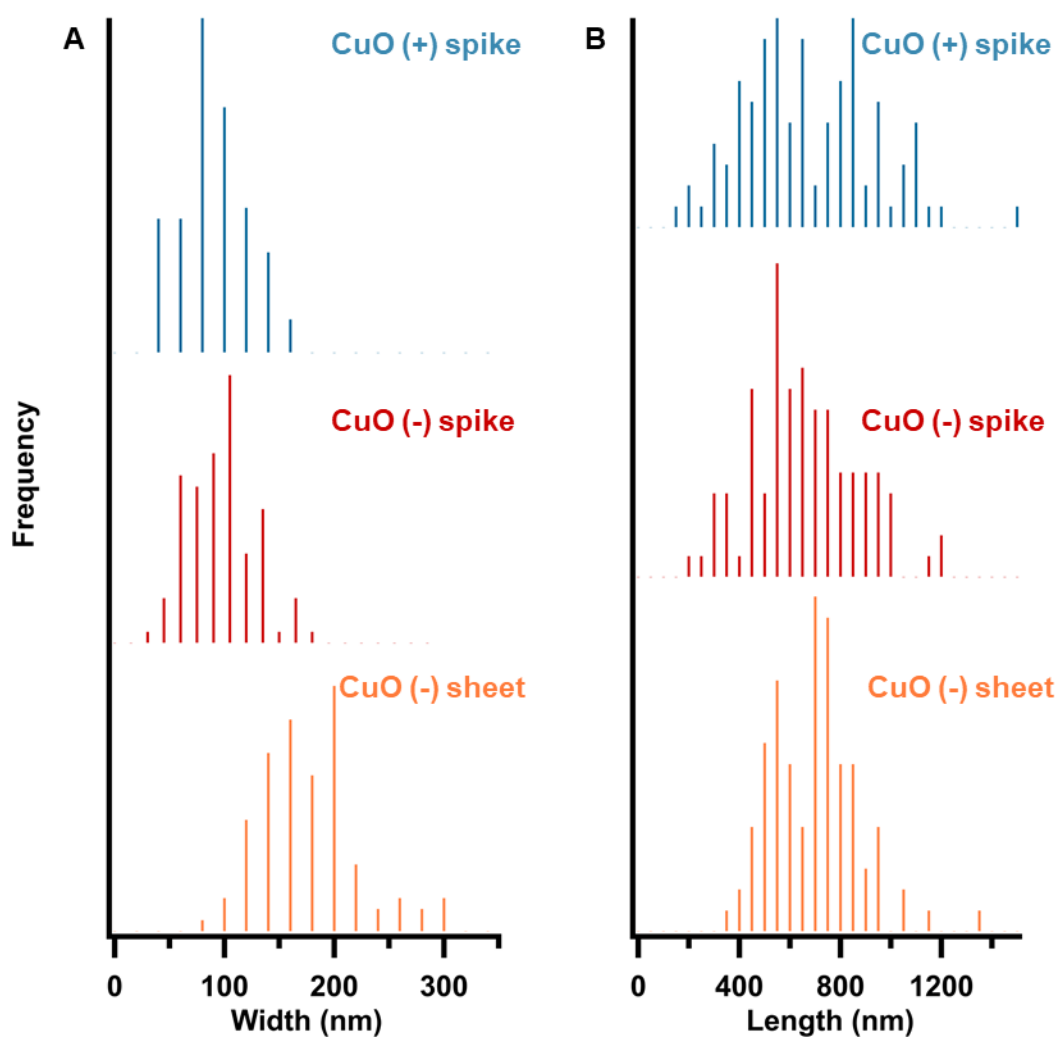


Figure 3.2 A) histogram of CuO nanoparticle width and B) histogram of CuO nanoparticle length. The bin width for the nanoparticle width was 20 and the bin width for the length was 50.

Figure 3.3 TEM image, SAED pattern, and high resolution image of CuO nanoparticles for A-C) CuO(+) nanospike, D-F) CuO(-) nanospike and G-I) CuO(-) nanosheet.

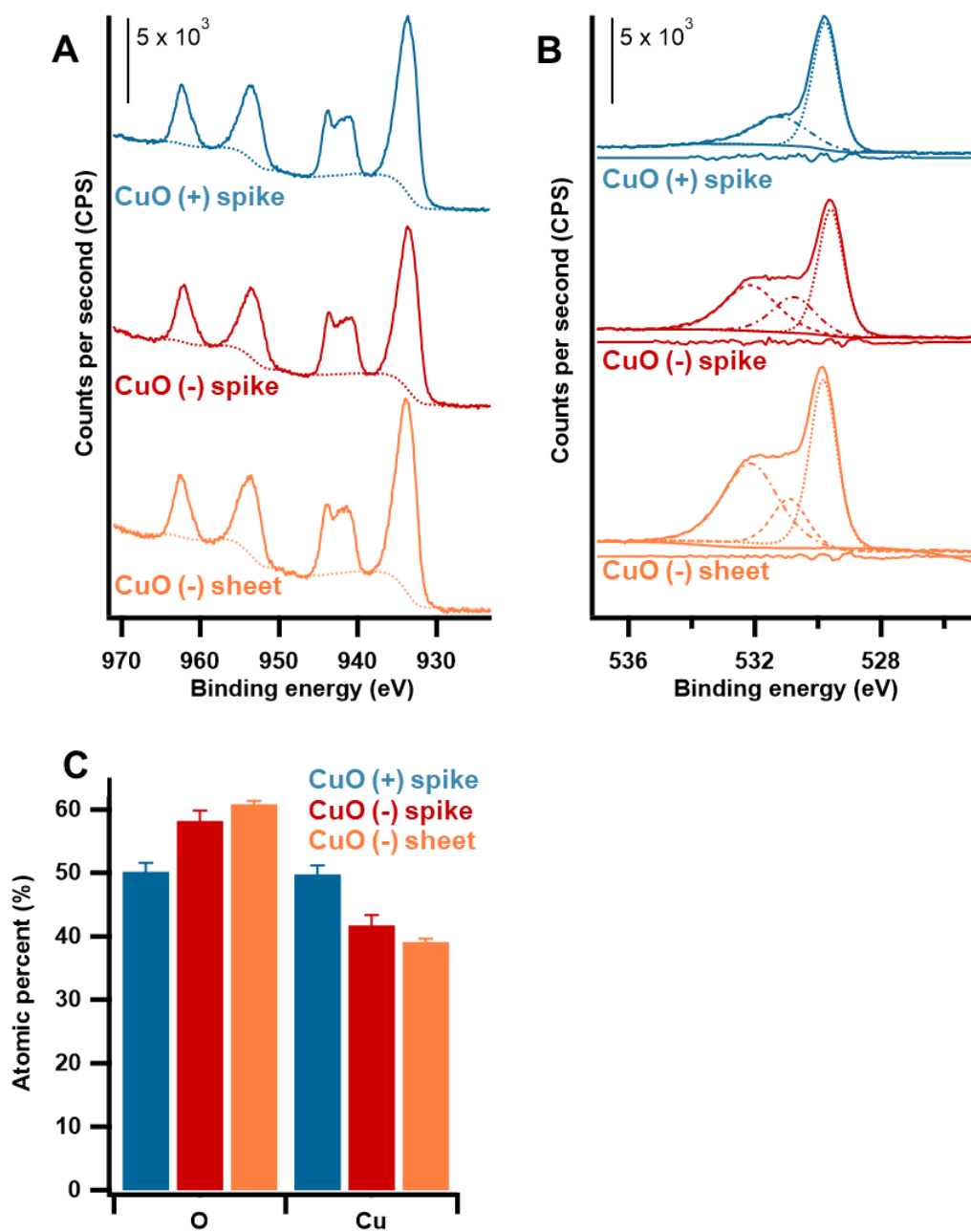


Figure 3.4 A) XPS spectra of CuO nanoparticles in the Cu 2p region, B) XPS spectra in the O1s region, C) atomic percentages of Cu and O at the nanoparticle surface.

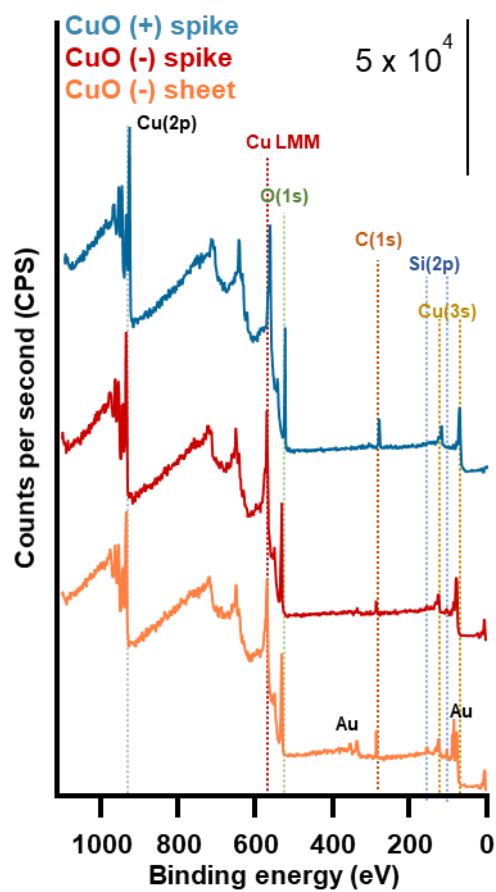


Figure 3.5 XPS survey spectrum of CuO nanoparticles.

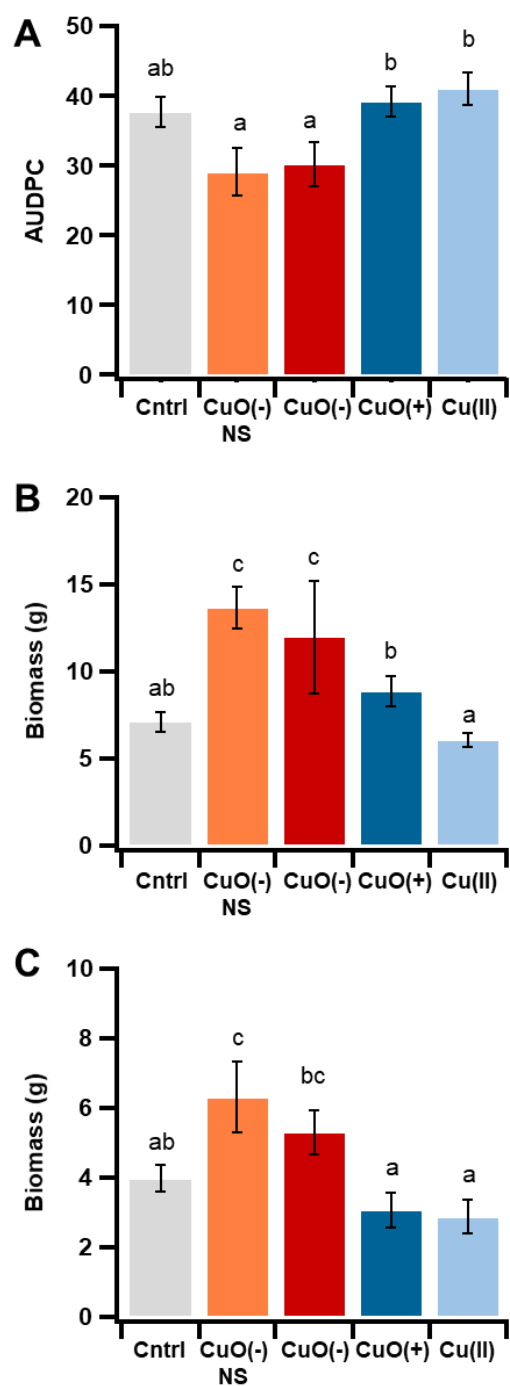


Figure 3.6 a) Area under the disease progression curve measured for CuO nanomaterials and a CuSO₄ salt control b) wet mass of healthy plants exposed to CuO nanomaterials and CuSO₄ salt control and c) wet mass of plants exposed to *F. oxysporum*, CuO nanomaterials, and CuSO₄ salt control.

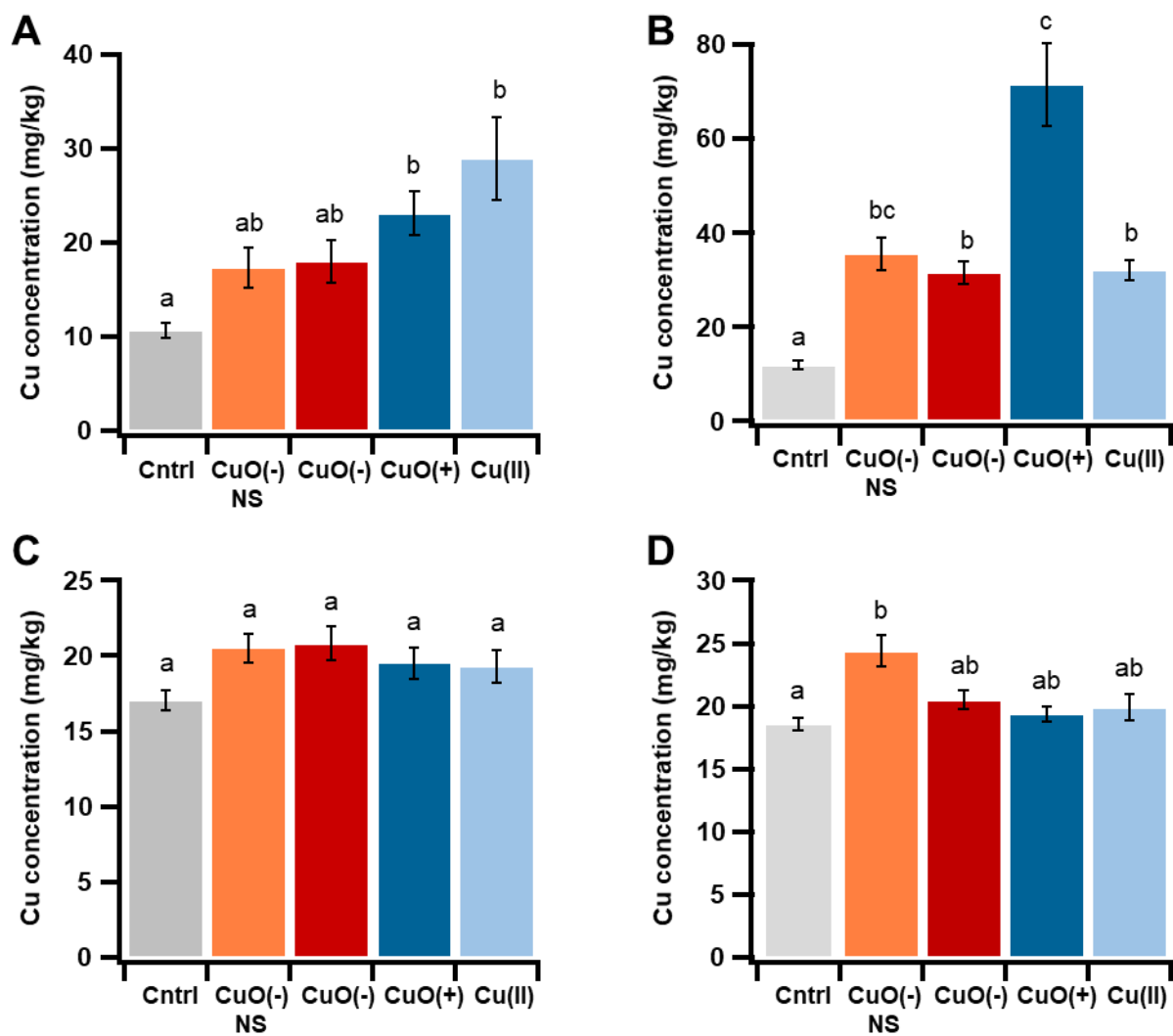


Figure 3.7 A) Cu concentration in the foliage of healthy plants B) Cu concentration in the foliage of diseased plants C) Cu concentration in the root of healthy plants and D) Cu concentration in the root of diseased plants.

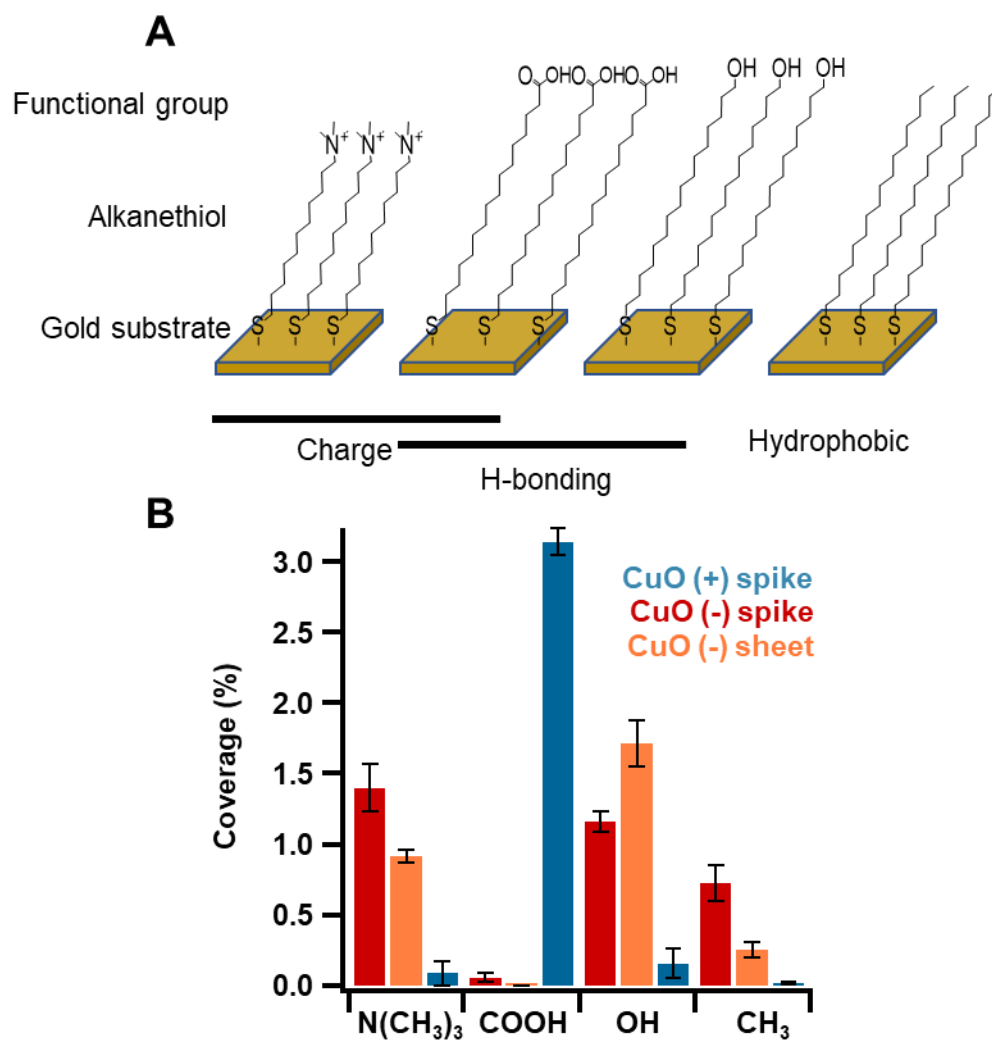


Figure 3.8 A) Visual depiction of self-assembled monolayer surfaces, from left to right: tertiary amine terminated (positively charged), carboxylate terminated (negatively charged), OH terminated (hydrogen bonding), CH₃ terminated (hydrophobic, London dispersion). B) Coverage calculated for CuO nanoparticles on self-assembled monolayer (SAM) surfaces using the number of particles per unit area and the average particle area (n=7).

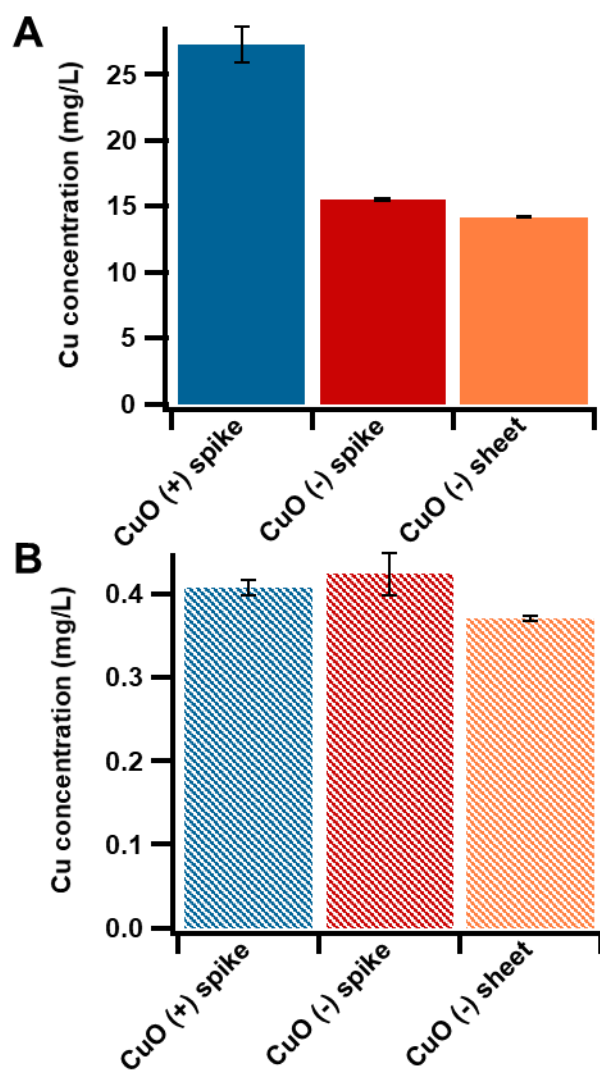


Figure 3.9 CuO dissolution in A) simulated xylem fluid and B) water.

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Chapter 4. Biomolecular Corona Formation on CuO Nanoparticles in Plant Xylem Fluid

The following chapter is adapted from the article published in *Environmental. Science: Nano*, **2021**, 8, 1067-1080. The article was written in collaboration with Christian A. Lochbaum (co-first author), Wade Elmer, Jason C. White, Joel A. Pedersen, and Robert J. Hamers. My contributions to the work include nanoparticle synthesis and characterization, *in situ* ATR-FTIR experiments, and X-ray photoelectron spectroscopy. Electrophoretic mobility, diffusion coefficient, biomolecule assays and polyacrylamide gel electrophoresis measurements were collected by Christian Lochbaum. Xylem fluid was collected at the Connecticut Agricultural Experiment Station

4.1 Introduction

The interaction of nanoparticles (NPs) with bacterial and animal cells can be influenced by chemical and physical transformations of the NPs in environmental and biological media. Nanoparticle interaction with biomolecules – and subsequent formation of a surface coating of biomolecules (referred to as a biomolecular corona) – can alter the hydrodynamic and electrokinetic properties of NPs and influence their distribution within organisms, uptake by cells, and storage in intracellular compartments.¹⁻⁴ The chemical identity and presentation of corona molecules influences NP uptake by cells⁵⁻⁷ and depends on both the initial surface chemistry of the NP and the biomolecules with which the NP interacts. Serum, blood, cytosol, and cell culture medium have been investigated as sources of corona biomolecules and have been shown to alter NP charge, size, and morphology.^{3, 8-11}

Acquisition of biomolecular coronas by nanoparticles has been widely investigated in animal and bacterial cell culture and in the extracellular and intracellular fluids of animals.^{6, 10} In contrast, the formation of biomolecular coronas in plant vascular fluids (xylem, phloem) has not been previously reported. Nanoparticles have been proposed for use in agriculture to deliver nutrients, plant-protection chemicals, and nucleic acids.¹²⁻¹⁸ The nanoparticles can be applied either to the foliage or at the root. After internalization, corona formation may alter nanoparticle long range transport through the plant vascular system (xylem, phloem), crossing of cell walls or membranes, or storage.¹⁹ Two prior reports on corona formation from plant tissues focused on metabolite coronas acquired from intact plant leaves²⁰ and adsorption of biomolecules from leaf tissue extracts.²¹ Corona formation in plant vascular fluids is expected to be important as it may dictate the transformations and spatial distribution of NPs within plants. Recent studies have shown that the initial size and surface properties of NPs influence their spatial distribution within plants.²²⁻²⁶ Nanoparticle size and surface properties have been shown to influence the composition of the biomolecular corona formed in other biological matrices and are thus expected to exert similar influence on coronas acquired in plant fluids.²⁷ Biomolecular composition in the fluid has been shown to impact the formation of the nanoparticle corona.^{2, 28-30} The composition of xylem and phloem fluids differ dramatically from that of the fluids in the vascular systems of animals. Xylem and phloem fluids have a much higher abundance of carbohydrates relative to proteins.^{31, 32} Furthermore, protein abundance in xylem fluid (0.05 to $0.1 \text{ g}\cdot\text{L}^{-1}$)³³ is orders of magnitude smaller than in animal circulatory fluids (2 to $60 \text{ g}\cdot\text{L}^{-1}$).³⁴ An initial step in understanding corona formation in plant vascular fluids is to identify the predominant biomolecules in vascular fluids that are recruited to the nanoparticle surface.

Here, we investigated the composition of the biomolecular corona acquired by CuO NPs in pumpkin xylem fluid. We selected CuO NPs for investigation because prior studies showed that foliar application of Cu-based NPs upregulate plant defense pathways against fungal diseases, and previous studies have shown the presence of CuO NPs in xylem fluid when nanoparticles were applied at the root.^{12, 14-16, 35} We chose pumpkin as a model plant because relatively large volumes of xylem fluid can be isolated from this species, allowing multiple types of analytical measurements to be made on the same stock xylem solution. In situ attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) measurements reveal time-dependent association of carbohydrates and proteins with the CuO NP. X-ray photoelectron spectroscopy (XPS) studies confirm formation of a strongly surface-attached protein adlayer. By isolating NP-corona complexes and analyzing the proteins remaining in solution by gel electrophoresis, we find that protein binding is selective, with a subset of proteins selectively binding to NP surfaces. Our results demonstrate that corona formation in a plant vascular fluid is a chemically selective and dynamic process. These findings represent an initial step toward a mechanistic understanding of how biomolecule-induced changes in surface properties influence NP interactions in plants.

4.2 Materials and Methods

4.2.1 CuO nanoparticle synthesis

All reagents were purchased from Millipore Sigma and used without further purification. We used a microwave-assisted hydrothermal method to synthesize CuO NPs. Stock solutions of 1 M $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ and 1 M $\text{LiOH} \cdot \text{H}_2\text{O}$ were prepared. A 938 μL aliquot 1 M CuCl_2 solution was diluted in 22.2 mL of ultrapure water (Barnstead systems; 18 $\text{M}\Omega \cdot \text{cm}$), and 1800 μL of the 1 M LiOH

solution was then rapidly injected. The solution was stirred for 10 min, yielding a pH of 11.28. The reaction mixture was transferred to a pressurized microwave synthesizer (CEM corporation) and heated to 160 °C for 10 min. The black NPs were allowed to cool to room temperature. The NPs were isolated from the supernatant by centrifugation (5 min, 4696g). The NPs were then cleaned by resuspension followed by centrifugation (10 min, 4696g); this procedure was performed twice using water and once using ethanol. After isolating the pellet by centrifugation, the particles were dried overnight at 30 °C under vacuum.

4.2.2 Scanning electron microscopy

The CuO NPs were suspended in ethanol to make a dilute suspension. The solution was bath sonicated for approximately 10 min. The solution was drop-casted onto a conductive silicon wafer and air-dried. The CuO NPs were imaged using a LEO Supra55 VP field-emission scanning electron microscope (SEM). The SEM images were analyzed using ImageJ to extract information about the size and shape of the NPs; NP dimensions were determined based on ImageJ measurements of 224 particles.

4.2.3 Atomic force microscopy

A high-grade mica substrate (Ted Pella) was freshly cleaved using double-sided tape. A dilute suspension of CuO NPs in ethanol was drop-cast on the mica surface. The sample was then analyzed with a Bruker Dimension Icon atomic force microscope using the tapping mode and TESPA-V2 tips. Atomic force microscopy data were processed using Gwydion software. Height information was obtained by fitting the region around the NP with a plane and extracting a height profile from the image.

4.2.4 Powder X-ray diffraction

Samples were prepared for powder X-ray diffraction by pressing CuO NPs into a thin layer of vacuum grease on a zero-diffraction plate (MTI corporation). The diffraction pattern of the CuO NPs was determined using a Bruker D8 Advanced powder diffractometer.

4.2.5 Xylem fluid collection

Pumpkins (*Cucurbita pepo* “Gladiator”) were seeded at the Lockwood farm in Hamden, CT in late June 2019 in a field mulched with black plastic. Fertilizer (NPK; 10-10-10) was applied before laying the plastic mulch. Seven weeks after seeding, plant stems were cut with razor blades at the base, and xylem fluid was collected from the cut ends with Pasteur pipettes (**Figure A4.1**). The xylem fluid was stored in glass vials and immediately placed on ice until they could be frozen at -80°C and shipped overnight to University of Wisconsin – Madison on dry ice. Xylem fluid was stored at -80°C until use. After warming, the fluid was filtered through $0.22\ \mu\text{m}$ syringe filters to remove particulate matter, and then partitioned into 5 mL aliquots, flash-frozen in liquid nitrogen, and stored at -80°C until use. Each aliquot of xylem fluid was frozen/thawed a maximum of two times.

4.2.6 Dynamic light scattering and laser Doppler electrophoresis

Nanoparticle diffusion coefficients and electrophoretic mobilities were measured by dynamic light scattering and laser Doppler electrophoresis, respectively (Malvern Zetasizer Nano ZS). Nanoparticles were suspended in water or xylem fluid at a concentration of $50\ \text{mg}\cdot\text{L}^{-1}$ immediately prior to measurement. For measurement of NP diffusion coefficients and electrophoretic mobilities after 3 h incubation, two suspensions were prepared with one measured at 0 h and one after 3 h

incubation at 25 °C. Diffusion coefficients and electrophoretic mobilities were measured for five analytical replicates of each sample. The diffusion coefficient depends upon the viscosity of the solution. The viscosity of water at 25 °C is 8.90×10^{-4} Pa·s, and the viscosity of xylem fluid has been estimated to range between 8.90×10^{-4} Pa·s and 1.2×10^{-3} Pa·s.³⁶ The impact of xylem fluid viscosities between 8.90×10^{-4} Pa·s and 1.2×10^{-3} Pa·s on the diffusion coefficient is examined in the Supplementary information.

4.2.7 Biomolecular corona isolation and quantification

Suspensions of CuO NPs (0, 50, 500, or 1000 mg·L⁻¹) were incubated in xylem fluid for 3 h and then centrifuged (10 min, 14 000g) to isolate the NPs with their surrounding corona into pellets, leaving non-bound species in the supernatant. The CuO NP pellets were rinsed once with a volume of water equal to that of the original supernatant and centrifuged again (10 min, 14 000g). The supernatant from this rinse was combined with that of the previous step, and the total amount of protein and carbohydrate in solution was measured. The amount of protein and carbohydrate bound to the NP surfaces was determined by resuspending CuO NP pellets in a volume of water equal to that of the original xylem fluid and measuring the concentration of total protein and carbohydrate using the assays described below.

The amount of carbohydrate in supernatant and resuspended CuO NP pellet samples was quantified using a phenol absorbance assay.³⁷ Briefly, 200 µL 5% w/v phenol in water solution was combined with 250 µL of sample in a glass vial. One mL of 98% sulfuric acid was rapidly added, and the solution was vortexed briefly. The solution was cooled for 1 h, and the absorbance at 460 nm was measured using a BioTek Synergy 2 plate reader. To ensure consistency, a calibration curve was prepared using glucose standards for every phenol solution (**Figure A4.3**).

The amount of protein in supernatant and resuspended CuO NP pellet samples was quantified using a bicinchoninic acid assay. A PierceTM BCA Assay Kit (Thermo Scientific product number 23225) was used to quantify protein content in the xylem fluid and in the supernatants. Briefly, 25 μL of sample was added to 200 μL working reagent in a 96-well plate, incubated for 30 min at 60 °C, and the absorbance at 562 nm was measured using a BioTek Synergy 2 plate reader. Bovine serum albumin calibration curves were prepared separately for every experiment to maximize consistency of results between replicate 96-well plate incubations. **(Figure A4.4).**

4.2.8 X-ray photoelectron spectroscopy (XPS)

Samples for XPS were prepared by suspending CuO NPs at 50 or 1000 $\text{mg}\cdot\text{L}^{-1}$ in xylem fluid with vortexing. Suspensions were agitated on a Thermolyne Rotomix orbital shaker for 3 h, after which the CuO NPs were isolated by centrifugation (10 min, 14 000*g*). To remove loosely adsorbed molecules, the particles were resuspended in ultrapure water and centrifuged again (10 min, 14 000*g*). The NPs remaining after supernatant removal were deposited onto a gold-coated silicon wafer and analyzed using a Thermo K alpha X-ray photoelectron spectrometer. Survey spectra were collected with a pass energy of 200 eV and a dwell time of 0.05 s, and averaging two scans. To analyze elements of interest, higher-resolution spectra were collected for N(1s), O(1s), and C(1s) binding regions using a pass energy of 30 eV and a dwell time of 0.05 s, and averaging 15 scans. The spectra were analyzed using CasaXPS, and binding energies from all spectra were calibrated to carbon at 284.8 eV. The composition and thickness of the NP coronas were determined from an analysis of the relative abundance of these elements, and the thickness of the corona was estimated from the attenuation of the underlying Cu(3p) emission by the corona. Spectra

were quantitatively analyzed using a graphical approach to solve the nonlinear equations, as described in the SI. For each measurement reported here, three experimental replicates were performed and analyzed.

4.2.9 Polyacrylamide gel electrophoresis

We incubated CuO NPs (0, 50, 500, or 1000 mg·L⁻¹) in 1 mL xylem fluid for 3 h and sedimented the CuO NPs from suspension by centrifugation (10 min, 14 000g). After removing the supernatant, the pellets were resuspended in 1 mL of ultrapure water. The proteins in the samples were concentrated by acetone precipitation. In detail, a 1 mL sample was combined with 4 mL acetone at -20 °C. The solution was incubated for 60 min at -20 °C and centrifuged (10 min, 14 000g). Protein pellets were dried for 30 min on the benchtop and resuspended in 200 µL ultrapure water. We performed a recovery study to assess protein loss during the acetone precipitation by measuring protein concentration in xylem fluid and xylem fluid after acetone precipitation with the BCA assay. No difference in total protein was observed between the xylem fluid and xylem fluid after acetone precipitation ($p < 0.05$).

Proteins were fractionated on a Invitrogen NuPAGE™ 10% Bis-Tris Protein Gel (1.0 mm, 10-well; Thermo Scientific product number NP0301BOX) following the manufacturer's protocol. The running buffer (5 µL), reducing agent (2 µL), and sample (13 µL) were combined and incubated at 70 °C for 10 min. A 15 µL aliquot of sample was added to each well. The gel was run at an applied potential of 200 V for 40 min and stained with a Pierce Silver Stain Kit (Thermo Scientific product number 24612). Gels were imaged using a Azure 600 imaging system (Azure Biosystems). Images of the gels were analyzed using Igor Pro software, using custom routines to extract intensity profiles along the separation direction and average over the width of each line.

4.2.10 Preparation of CuO nanoparticle film for *in situ* ATR-FTIR experiments

The CuO film was prepared on a 10-bounce Ge trapezoidal internal reflectance element (IRE, 45° angle, 10 bounce, Specac gateway, **Figure A4.4**). The IRE was cleaned by exposure to UV-ozone and rinsed in ethanol. The CuO NPs were suspended in 4 mL ethanol to make a 1000 mg·L⁻¹ suspension, and the suspension was ultrasonicated for 10 min (repeated cycles of 10 s on, 10 s off). The suspension was drop-cast in 70 µL aliquots on to the surface of the Ge IRE, and the suspension was immediately distributed by spin-coating at 1000 rpm for approximately 10 s (Laurell Technologies Corporation) to produce a thin, uniform layer. The process was repeated until the entire 4 mL NP suspension was deposited on the Ge IRE surface. The film was annealed in a box furnace (Thermolyne) in air at 400 °C for 4 min to remove excess water and to improve adhesion of the film. Scanning electron micrographs were collected to determine the coverage of the CuO film on the surface of the Ge element.

4.2.11 *In situ* ATR-FTIR measurements of biomolecule attachment to CuO nanoparticle surfaces

To perform *in situ* ATR-FTIR measurements, the Ge IRE was mounted in a Specac Gateway flow cell (internal volume 550 µL) that was then installed in a Bruker Vertex 70 FTIR spectrometer. A volumetric flow rate of 0.5 mL·min⁻¹ was used. Each spectrum was collected from 500 to 4000 cm⁻¹ with 2 cm⁻¹ resolution, and 500 scans were averaged (3.75 min·scan⁻¹). Background scans were collected using the same flow cell and ultrapure water. To collect a reference spectrum of xylem fluid, a ZnSe (45° angle, 6 bounce, Specac gateway, **Figure A4.4**) IRE was mounted in the flow cell, a background was collected in water, and 5 mL of xylem was flowed through the system.

To collect experimental data of xylem fluid biomolecules interaction with the CuO NPs, the CuO-coated Ge IRE was mounted in the spectrometer. Then, 5 mL of xylem fluid was flowed through the cell. The flow was stopped, and the xylem fluid remaining in the ATR cell was incubated with the CuO film, for approximately 3 h. The flow was then re-established using ultrapure water for 3 h to rinse away loosely adsorbed molecules. Spectra were continuously recorded through the duration of the experiment (initial reference, xylem exposure, and ultrapure water rinse) to monitor the temporal evolution of the protein corona. To determine the xylem molecules that interact with CuO a series of model molecules were used which included malic acid, dextran to represent carbohydrates, ubiquitin to represent proteins, and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) to represent lipids; more information is provided in the supplementary section.

4.3 Results and Discussion

4.3.1 Nanoparticle size, shape and crystal structure

Figure 4.1a shows a representative SEM micrograph of the copper oxide NPs. The NPs exhibit an anisotropic morphology and are in the form of thin, elongated sheets with lateral dimensions of 120 ± 40 nm and lengths of 900 ± 300 nm ($n=224$). The CuO NPs exhibit a powder x-ray diffraction pattern (Fig. 1b) in excellent agreement with that of a reference spectrum of CuO in the tenorite structure.³⁸ Atomic force microscopy measurements of CuO NPs deposited onto flat substrates show a typical height of approximately 50 nm (**Figure A4.5**). The anisotropic shape of the CuO NPs is consistent with the monoclinic crystal structure of tenorite and has been reported in prior studies of CuO NPs grown under conditions similar to those used here.³⁹

4.3.2 Nanoparticle hydrodynamic and electrokinetic properties in xylem fluid

To understand the effect of xylem fluid on hydrodynamic and electrokinetic properties of the CuO NPs, we determined the diffusion coefficients (D , from dynamic light scattering) and electrophoretic mobilities (μ_e , from laser Doppler electrophoresis) of the particles. We report D and μ_e rather than estimated hydrodynamic diameters and zeta potentials to obviate the need to invoke assumptions about particle shape, charge homogeneity, or the permeability of aggregates or biomolecular coatings. Fig. 2a shows the diffusion coefficients and electrophoretic mobilities of CuO NPs suspended in water, xylem fluid, and xylem fluid after 3 h incubation. The CuO NPs suspended in xylem fluid exhibited diffusion coefficients of $0.32 \pm 0.04 \mu\text{m}^2\cdot\text{s}^{-1}$, significantly lower than those obtained in water ($1.18 \pm 0.03 \mu\text{m}^2\cdot\text{s}^{-1}$). After 3 h incubation in xylem fluid, D decreases further to $0.17 \pm 0.03 \mu\text{m}^2\cdot\text{s}^{-1}$ ($p < 0.001$). The lower diffusion coefficient in xylem fluid cannot be attributed solely to the higher viscosity in xylem fluid relative to water. The highest reported viscosity of xylem fluid ($1.2 \times 10^{-3} \text{ Pa}\cdot\text{s}$)³⁶ does not differ enough from water ($8.90 \times 10^{-4} \text{ Pa}\cdot\text{s}$) to lead to such a dramatic decrease in diffusion coefficient (see supplementary information). The decrease in the diffusion coefficient in xylem fluid may be due to aggregation of NPs or association of xylem components with the NPs; either of these processes would be expected to increase the diffusion coefficient of the NPs.

Figure 4.2b shows the corresponding electrophoretic mobilities in these solutions. While CuO NPs suspended in water exhibit $\mu_e = +2.7 \pm 0.6 \text{ cm}^2\cdot\text{mV}^{-1}\cdot\text{s}^{-1}$, CuO NPs suspended in xylem fluid display a much lower mobility, $\mu_e = +0.2 \pm 0.3 \text{ cm}^2\cdot\text{mV}^{-1}\cdot\text{s}^{-1}$. Incubation for 3 h did not produce any further change in mobility ($\mu_e = +0.1 \pm 0.3 \text{ cm}^2\cdot\text{mV}^{-1}\cdot\text{s}^{-1}$; $p > 0.05$). The smaller magnitude of the electrophoretic mobility in xylem fluid vs. water may have arisen from the

association of xylem fluid molecules with the surface of CuO NPs or the higher ionic strength of the xylem fluid relative to water. The ionic strength of xylem fluid has been reported to be 32 ± 14 mM (*Ricinus communis*).⁴⁰ Using this value, the difference in ionic strength between water and xylem fluid is sufficient to cause the observed decrease in electrophoretic mobility (see supplementary information). In addition to the difference in ionic strength, adsorption of molecules to the surface of CuO NPs is consistent with prior studies reporting changes in NP electrophoretic mobility due to adsorption of charged biomolecules²⁷ or small charged molecules⁴¹ which are both present in xylem fluid. Neutralization of NP surface charge by charged organic (macro)molecules in xylem fluid would be consistent with the influence of electrostatic attraction between the CuO surface and components of the xylem fluid. Neutralization of NP surface charge may also lead to loss of particle colloidal stability and help explain the decrease in observed diffusion coefficient.^{42,}

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4.3.3 CuO nanoparticles adsorb proteins from xylem fluid

As an initial step toward determining the composition of the biomolecular corona acquired in xylem fluid, we quantified the amount of carbohydrate and protein associated with the CuO NPs using a phenol-based total carbohydrate assay and the bicinchoninic acid (BCA) assay, respectively. We focused on proteins and carbohydrates because they have been previously reported in xylem fluid at quantifiable concentrations and because these classes of molecules have been shown to form coronas on nanoparticles.^{28, 44} Protein and carbohydrate concentration of fluid varied among plants sampled (90 ± 20 mg·L⁻¹ and 250 ± 50 mg·L⁻¹, respectively). Differences in concentration among samples were accounted for by measuring concentrations of biomolecules for every aliquot to normalize results to starting protein and carbohydrate concentrations. We

incubated CuO NPs at 0, 50, 500, and 1000 mg·L⁻¹ in xylem fluid for 3 h, sedimented the NPs *via* centrifugation and analyzed both the supernatant and isolated CuO NPs for carbohydrate and protein content. **Figure 4.3a** shows the amount of carbohydrate remaining in the xylem fluid supernatant after exposure to the CuO NPs; incubation with CuO NPs did not result in detectable removal of carbohydrate at any NP concentration investigated relative to control samples ($p > 0.05$). **Figure 4.3a** also shows the corresponding measurements of the amount of carbohydrate extracted from the NP (“CuO-bound”). The amount of carbohydrate bound to the NPs is below our estimated limit of detection ($< 10 \text{ mg}\cdot\text{L}^{-1}$).

Figure 4.3b shows the concentration of protein remaining in xylem fluid supernatant after incubation with CuO NPs at three NP concentrations with the corresponding concentrations of protein extracted from sedimented nanoparticles. We note that the total mass concentration of carbohydrates (Fig. 3a, $\sim 270 \text{ mg}\cdot\text{L}^{-1}$) is much higher than that of proteins (Fig. 3b, $\sim 80 \text{ mg}\cdot\text{L}^{-1}$). At the lowest CuO NP concentration investigated (50 mg·L⁻¹), protein removal from the xylem fluid was below the detection limit of the assay ($< 5 \text{ mg}\cdot\text{L}^{-1}$); furthermore, no significant protein was extracted from CuO NPs that were isolated by centrifugation. At higher CuO NP concentrations (500 and 1000 mg·L⁻¹), significant amounts of protein were adsorbed and removed from solution ($p < 0.05$), and equivalent amounts of protein were extracted from the CuO NPs that were isolated by centrifugation. In all cases, the sum of the amount of protein in the supernatant and the amount extracted from the NPs corresponded to the original amount of protein in xylem fluid. The detection of surface-bound protein without detectable surface-bound carbohydrate from xylem fluid indicates that the biomolecular corona composition after 3 h incubation is composed primarily of protein. As the NP concentration increases, the amount of protein detected on the NPs

increases as does the magnitude of the amount removed from the supernatant; this trend is consistent with the increase in surface area available for corona formation at higher nanoparticle concentrations. To investigate the relationship between CuO NP surface area and protein adsorption, we analyzed the thickness of the corona formed.

4.3.4 Dependence of corona thickness on CuO nanoparticle concentration

We used X-ray photoelectron spectroscopy to directly probe the elemental composition of xylem fluid components bound to the CuO NPs as well as their chemical state. X-ray photoelectron spectroscopy is an *ex situ* method that provides quantitative information about the strongly bound corona components that persist after rinsing and drying. Therefore, XPS provides information about the hard corona and associated strongly-bound molecules, while the weakly associated molecules of the soft corona were likely removed before XPS analysis. **Figure 4.4a** shows survey XPS spectra of CuO NPs that had been exposed to xylem fluid for 3 h. Peaks associated with C, O, and Cu are apparent in the spectra for all samples. Spectra of CuO NPs that had been exposed to xylem fluid also exhibit surface nitrogen that is detectable over 3σ of the noise, while no detectable nitrogen is observed in spectra from unexposed CuO samples. After exposure to xylem fluid, the absolute and relative intensities of the peaks change; the absolute intensities of the Cu(2p) and O(1s) peaks decrease, while C(1s) emission intensity increases and a new N(1s) peak appears. The XPS data yield two complementary pieces of information. First, the attenuation of the Cu(2p) and O(1s) emission by the biomolecular corona provides a way to assess the overall thickness of the strongly bound corona components. Second, the relative intensities of emissions from N(1s), C(1s), and O(1s) provide information about the chemical composition of the corona.

The thickness of the corona can be assessed from XPS measurements by quantifying the degree to which the Cu(2p) emission intensity is decreased upon formation of the NP corona, or by measuring the emission intensity from elements specific to the NP core (e.g., Cu) and those that are specific to the corona (e.g., N). In the latter case, the corona thickness can be determined from the equation:

$$\frac{(1 - e^{-t/\lambda_{N,P} \cos \theta})}{e^{-t/\lambda_{Cu,P} \cos \theta}} = \frac{A_N \rho_{Cu,CuO} S_{Cu} \lambda_{Cu,CuO}}{A_{Cu} \rho_{N,P} S_N \lambda_{N,P}} \quad (1)$$

where t is the thickness of the biomolecular corona; S_{Cu} and S_N are the atomic sensitivity factors for Cu and N; $\rho_{Cu,CuO}$ is the atomic number density of Cu atoms in CuO; $\lambda_{Cu,CuO}$, $\lambda_{Cu,P}$, and $\lambda_{N,P}$ are the inelastic mean free paths (IMFPs) of Cu photoelectrons in CuO, Cu photoelectrons passing through the protein layer, and N photoelectrons in the protein layer; and θ is the angle of collection of photoelectrons with respect to the surface normal. Using estimated IMFPs, this equation can be solved to determine an approximate corona thickness (see Supplementary Information). The corona thickness was also estimated in the same manner using the C(1s) signal; the value presented represents an average of the corona thicknesses calculated using both the C(1s) and the N(1s) data, which were similar. Using this approach, we estimated corona thickness to be 5.1 ± 0.2 nm at a NP concentration of $50 \text{ mg} \cdot \text{L}^{-1}$ and 1.9 ± 0.1 nm at a NP concentration of $1000 \text{ mg} \cdot \text{L}^{-1}$. We note that a 20-fold increase in NP concentration decreased the average corona thickness by a factor of approximately two. Rigorously accurate determination of corona thickness from XPS measurements would require inclusion of the uncertainty in the IMFP values. Although we did not include the error in the IMFPs in our analysis, such errors are systematic and would not change the overall conclusion that thinner coronas were found at higher NP concentrations. This

observation suggests that specific proteins are depleted from the xylem fluid. This conclusion is consistent with results from the BCA assay (*vide infra*) showing partial removal of proteins at high NP concentrations.

To assess the consistency between the results of the XPS and protein depletion studies, we estimated the amount of protein that would be removed from the xylem to form coronas with the thicknesses estimated from XPS. We approximated the CuO NPs rectangular prisms with dimensions of $900\text{ nm} \times 120\text{ nm} \times 50\text{ nm}$ based on SEM measurements, and we approximated the corona as a layer of protein of uniform density ($1.35\text{ g}\cdot\text{cm}^{-3}$)⁴⁵ covering the entire CuO NP surface. We estimated that formation of a 1.9 nm thick corona on CuO NPs in a $1000\text{ mg}\cdot\text{L}^{-1}$ suspension would remove $24\text{ mg}\cdot\text{L}^{-1}$ protein from the xylem fluid; this is very close to the protein removal of $28 \pm 5\text{ mg}\cdot\text{L}^{-1}$ measured by BCA assay. We also estimated that formation of a 5.1 nm thick corona on CuO NPs in a $50\text{ mg}\cdot\text{L}^{-1}$ suspension would be accompanied by a removal of $3.2\text{ mg}\cdot\text{L}^{-1}$ protein from the xylem fluid. This value is below the BCA detection limit of $5\text{ mg}\cdot\text{L}^{-1}$ and is consistent with our inability to detect significant protein loss *via* the BCA assay at the $50\text{ mg}\cdot\text{L}^{-1}$ CuO NP concentration.

To obtain more detailed chemical information about the corona, we collected XPS spectra at higher resolution in the Cu(2p), O(1s), N(1s), and C(1s) regions. The high resolution Cu(2p) spectra in Fig. 4b show that CuO NPs exposed to water (but not xylem fluid) exhibit a $2p_{3/2}$ peak at a binding energy (BE) of $932.60 \pm 0.08\text{ eV}$, a $2p_{1/2}$ peak at $952.60 \pm 0.08\text{ eV}$, and shake-up features at $942.1 \pm 0.1\text{ eV}$ and $961.90 \pm 0.03\text{ eV}$.⁴⁶ For the $50\text{ mg}\cdot\text{L}^{-1}$ nanoparticle concentration, the Cu(2p) emission intensity (**Figure 4.4**) is attenuated by a factor of ~ 13 compared to bare CuO samples, but the overall shapes and relative intensities of the individual Cu peaks remain

unchanged. This attenuation factor (F) is also consistent with a corona thickness of ~ 5 nm, as calculated using the following equation:

$$F = e^{(-\frac{t}{\lambda})} \quad (2)$$

where, t is the thickness and λ is the inelastic mean free path approximated at 2 nm. The O(1s) region displayed in Fig. 5a shows a large peak at 529.40 ± 0.04 eV from bulk CuO and a smaller peak at 530.88 ± 0.06 eV that is consistent with Cu–OH groups.⁴⁷ The peak energies for the adsorbates on CuO NPs from the $50 \text{ mg}\cdot\text{L}^{-1}$ and $1000 \text{ mg}\cdot\text{L}^{-1}$ suspensions in xylem fluid were similar; we report the binding energies for only the $50 \text{ mg}\cdot\text{L}^{-1}$ sample here. The C(1s) region shown in Fig. 5b exhibits a peak at 284.80 eV (used as our internal binding energy calibration) due to alkane-like adventitious carbon contamination, along with a smaller peak at 287.38 ± 0.04 eV; the higher BE of this peak is typical for oxidized carbon forms such as carbonyl groups. The CuO NPs that had been incubated with xylem fluid (and then rinsed) exhibit changes in the C(1s) and O(1s) peaks and show a new peak in the N(1s) region (Fig. 5c). In the O(1s) region, particles exposed to xylem fluid exhibit peak at 532.06 ± 0.03 eV that falls within the range of energies typically observed for carbonyl (C=O) oxygens in proteins.⁴⁸ This is consistent with the appearance of a peak at 288.1 eV in the C(1s) region that is consistent with C=O moieties. The C(1s) region in spectra of CuO NPs exposed to xylem fluid also contains a peak at 285.8 eV which is consistent with C–N or C–O moieties in proteins^{49, 50} and C–O–C groups in carbohydrates.⁵¹ The N(1s) region in spectra of CuO NPs exposed to xylem fluid shows a single, sharp peak at 399.9 ± 0.2 eV, similar in appearance to prior reports of the proteins fibrinogen, bovine serum albumin, and soy protein (**Figure 4.5c**).^{49, 52, 53} The binding energies of the C(1s), N(1s), and O(1s) are consistent with functional groups present in xylem fluid including proteins, carbohydrates, small organic acids,

and phospholipids. However, the relative atomic abundances of C, O, and N indicate that proteins are the main biomolecules present.

The changes in surface composition can be expressed as atomic percentages, where the atomic percentage of element X is given by

$$\%x = 100 \times \frac{\frac{I_x}{\text{IMFP}_x S_x \lambda_j}}{\sum_j \left(\frac{I_j}{\text{IMFP}_j S_j \lambda_j} \right)}$$

I_j is the area of the photoemission peak for element j , IMFP_j is the inelastic mean free path for element j , S_j is the atomic sensitivity factor for element j , and λ is the inelastic mean free path at the energy of the photoelectrons for each element. The summation is over photoemission peaks from all elements corresponding to the corona layer (here, C, N, and the high binding-energy component of O). To calculate the atomic percentages in corona constituents, we use only the O(1s) peak component centered at 532.06 ± 0.03 eV that corresponds to the protein layer, thereby excluding the O(1s) signal arising from the underlying CuO.

Figure 4.6 shows that calculated atomic abundances of C, O and N atoms in the corona layer were similar for CuO NPs that had been incubated with xylem fluid at 50 and 1000 $\text{mg}\cdot\text{L}^{-1}$ concentrations. The CuO from the 1000 $\text{mg}\cdot\text{L}^{-1}$ suspension exhibited atomic percentages of $45.7 \pm 0.1\%$, $12.2 \pm 0.2\%$, and $42.1 \pm 0.2\%$ for C, N, and O, respectively. The atomic composition of individual carbohydrates and proteins vary in accordance with their specific molecular structures. Nonetheless, their atomic compositions fall into distinct ranges. Both carbohydrates and proteins contain approximately 50% carbon, but carbohydrates contain significantly more oxygen (45-49% O vs. ~35% O for proteins) and less nitrogen (<3% vs. 15-20% N for proteins).⁵⁴ The compositions

of the coronas on the CuO NPs, particularly the high N content, are similar to those of typical proteins and not consistent with typical carbohydrates. The atomic abundances of molecules in the coronas on the CuO NPs were similar for the 50 mg·L⁻¹ and 1000 mg·L⁻¹ NP suspensions (paired t-test, $p > 0.05$), suggesting that while the corona layer has a different thickness, it is comprised of proteins in both cases. The atomic abundances of C, O, and N also suggest that very little carbohydrates or other biomolecules are found in the corona isolated after a rinsing step.

In summary, the corona formed on CuO NPs from xylem fluid is thinner at the higher (1000 mg·L⁻¹) NP concentration, and atomic abundances of C, N, and O are in the range typical of proteins, for both high and low nanoparticle concentrations. Our data suggest that recruitment of proteins to the CuO surface is selective. At low NP concentrations, strongly binding proteins associate with the NP surface to form a corona with an average thickness of ~5 nm. The xylem fluid appears to contain insufficient amounts of high affinity proteins to form a similarly thick coating on the CuO NPs when the NPs are present at a 20-fold higher concentration. This is due to the larger amount of CuO surface area available to bind proteins at the higher NP concentration, leading to a thinner (~2 nm thick) corona of approximately the same composition.

To confirm that specific proteins from xylem fluid selectively bind to CuO NPs, we examined the proteins remaining in xylem fluid and bound to CuO NPs after 3 h incubation by lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE). After separating the CuO NP-bound proteins from those in solution by centrifugation, we fractionated the proteins in the supernatant and extracted from the pellet by LDS-PAGE and visualized the proteins by silver staining. **Figure 4.7** shows an image of the resulting silver-stained gel which includes xylem fluid

after exposure to 0 1000 mg·L⁻¹ CuO NPs (lanes 2, 3), and corona components extracted from the surface of the NPs (lanes 4, 5). The features in lane 4 (corresponding to proteins extracted pellets from the 0 mg·L⁻¹ CuO NP exposures) are too weak to reliably observe. Lanes 2 and 4 (supernatant and pellet from the 0 mg·L⁻¹ CuO NP exposure) demonstrate that the centrifugation procedure used does not lead to the sedimentation of detectable amounts of proteins in the absence of CuO NPs. The banding pattern produced by corona components extracted from the NPs after exposure to 1000 mg·L⁻¹ CuO NPs (lane 5) differs from that for the starting xylem fluid (lane 2). Specifically, the most intense bands in lane 5 correspond to a molecular mass of approximately 6 kDa, while that in lane 2 occurs at a molecular mass of approximately 9 kDa. Lane 2 contains multiple bands between 28 and 49 kDa, while lane 5 contains no observable bands in this region. This result indicates that the relative proportion of xylem fluid proteins in the extracted protein corona differs from that in the xylem fluid; the most abundant proteins in the extracted corona do not correspond to the most abundant proteins in the xylem fluid.

4.3.5 Corona formation dynamics

Previous studies in non-plant environments have shown that the corona evolves with time as layers formed initially under kinetic control are eventually (partially) replaced by more thermodynamically favored species.⁵⁵ To directly characterize the dynamic evolution of the corona and gain additional chemical information about the adsorbed layers, we used *in situ* ATR-FTIR spectroscopy in a flow cell geometry. We acquired FTIR spectra as a function of time during exposure to xylem as well as while rinsing to provide information about both the kinetics and reversibility of corona.

Figure 4.8 shows a spectrum of xylem fluid measured using ATR-FTIR using a trapezoidal ZnSe internal reflection element. The spectrum exhibits vibrational features typical of proteins.⁵⁶ These include a peak at 1636 cm^{-1} attributed to the peptide backbone Amide I band and the carboxylate asymmetric stretch; a peak 1541 cm^{-1} , consistent with the Amide II band, and a peak at 1231 cm^{-1} , consistent with the Amide III band.⁵⁷⁻⁵⁹ Peaks are also observed at 1394 cm^{-1} and 1348 cm^{-1} , which we attribute to COO^- symmetric stretch and the O–H deformation modes of carboxylic acids.⁶⁰ These carboxylic acid and carboxylate moieties may be attributed to small organic acids or the side chains of asparagine or glutamine residues in proteins or amino acids in xylem fluid.^{60, 61} Peaks at 1150 cm^{-1} and at 1081 cm^{-1} are consistent with C–O stretching modes associated with carbohydrates, or P–O–C stretching modes that may be associated with phospholipids, or C–OH modes associated with small carboxylic acids (**Table A4.1**). The peak near 1080 cm^{-1} is also consistent with spectra previously reported for carbohydrates and phospholipids.⁶²⁻⁶⁴ Prior studies have shown that xylem fluid from the members of the *Curcubita* genus contains proteins, carbohydrates, organic acids, and polyatomic ions such as ammonium and phosphate.^{31, 65-67} The infrared spectrum displayed in Fig. 8 indicates that proteins are present in the xylem fluid as well as phospholipids, carbohydrates, and/or carboxylate-containing molecules. Indexing the peaks in the xylem spectrum allows us to identify the biomolecules that bind to the surface of CuO NPs, and determine the dynamics of binding.

Figure 4.9 shows FTIR spectra of xylem fluid interacting with CuO NPs for different time periods. To obtain these spectra we coated a Ge IRE with a thin, uniform layer of CuO NPs. We optimized the preparation conditions and used scanning electron microscopy to verify complete, uniform coverage with CuO NPs as demonstrated in the micrograph in Fig. S7. The CuO layer is

sufficiently thin that the electric field of the infrared light penetrates through the CuO layer. To collect *in situ* ATR-FTIR measurements a reference spectrum was first collected of the CuO film in nanopure water. After the background scan was collected, xylem fluid was flowed through the ATR cell and *in situ* FTIR spectra were collected continuously; after 52 spectra were obtained (3. h) the sample was rinsed with water and additional spectra were obtained. **Figure 4.9** shows a representative FTIR spectra acquired at different time points. Water gives rise to an asymmetric peak centered near 1638 cm^{-1} . The first spectrum measured after introducing xylem (“S0”, shown here magnified 10-fold) shows only a very weak water feature at 1638 cm^{-1} , that overlaps with the Amide I band peaking at 1636 cm^{-1} . Subsequent spectra S1, S5, S25, and S51 show new peaks with maxima near 1073 cm^{-1} , 1398 cm^{-1} , and 1562 cm^{-1} . Based on the analysis of pure xylem fluid and spectra collected of model molecules (**Figure 4.8, Figure A4.8**), we attribute these peaks to carbohydrates or phosphate containing molecules (1073 cm^{-1}), small molecules with carboxylate moieties (1398 cm^{-1}), and proteins (1562 cm^{-1}). The absorbance of these three features increases with exposure duration. To test the degree that molecular layer adsorption is reversible, we rinsed the sample for 3 h with ultrapure water to remove weakly adsorbed molecules. Spectra S67 (approximately 1 h rinsing) and S76 (approximately 1.5 h rinsing) show that the carbohydrate or phosphate containing molecule feature at 1073.2 cm^{-1} is removed during rinsing while the vibrational features at 1398 cm^{-1} and 1562 cm^{-1} persist. These results indicate that protein interacts more strongly with the CuO surface than do carbohydrates, and protein binding is largely irreversible on the time-scale of our experiments; this implies that the XPS data and BCA assays (which require a brief rinsing of the sample) are good probes of the protein component of the

coronas, but that more weakly adsorbed carbohydrates or phospholipids were rinsed away during XPS and phenol assay sample preparation.

4.4 Conclusion

Our results demonstrate that CuO NPs acquire a biomolecular corona from native pumpkin xylem fluid that contains both proteins and carbohydrates. Results from LDS-PAGE, ATR-FTIR, and XPS experiments show that the corona is composed primarily from proteins, despite the mass concentration of carbohydrates in xylem fluid being substantially higher than that of proteins. Furthermore, our results show that protein adsorption is *selective*; a subset of proteins having a molecular mass of approximately 6 kDa appear to be enriched in the corona relative to other xylem fluid proteins. Selectivity of protein binding is evidenced by the distribution of proteins extracted from the CuO NP surfaces differing from that of the starting xylem fluid (Fig. 7) and by the decrease in average corona thickness from ~5.1 nm to ~1.9 nm as the CuO NP concentration increases from 50 to 1000 mg L⁻¹, even though the total concentration of protein in solution is reduced by only approximately 15% (Fig. 3b).

While quasi-irreversible binding of proteins to metal oxides has been reported previously, most studies have primarily focused on isolated proteins or on corona formation in mammalian vasculature.^{68, 69} In comparison, xylem fluid has a higher ratio of carbohydrates to proteins,^{31, 32} suggesting that carbohydrates could play a more important role in corona formation in xylem fluid than in matrices investigated previously. The high carbohydrate concentration in xylem is demonstrated in our own measurements (Fig. 3) showing that the total mass concentration of carbohydrates is more than three times that of proteins. The high concentration of carbohydrates in xylem fluid suggests that nanoparticle coronas formed in plants could differ significantly from

those formed in the mammalian serum and tissue. Carbohydrates in xylem are present predominantly as oligosaccharides, with polysaccharides constituting <5% of total carbohydrate mass.⁷⁰ While we have not investigated the distribution of carbohydrate masses in the xylem fluid used here, our data show that carbohydrates in xylem fluid interact with CuO nanoparticles, but that over time periods of minutes these carbohydrates are replaced by protein and can be readily removed by simple water rinse. These observations indicate that even though carbohydrates are present at higher concentrations than proteins in xylem, CuO nanoparticles exhibit a stronger affinity for the amino acid residue side chains of proteins than for the sugar residues making up carbohydrate. As a consequence, proteins dominate the corona composition formed in xylem. These observations are consistent with past studies demonstrating low abundance of carbohydrates in nanoparticle coronas even when abundant in the biological matrix.^{28, 71} We hypothesize that the higher affinity for amino acid side chains likely arises from the presence of negatively charged functional groups on the proteins, driving an electrostatic attraction to the positively charged CuO NP surface. Electrostatically driven binding is consistent with the strong reduction in CuO NP electrophoretic mobility when suspended in xylem fluid. Electrostatics has been previously reported to play an important role in corona acquisition from animal and bacterial systems.⁷² We note that most metal oxide nanoparticles commonly investigated, including TiO₂, Fe₂O₃, and SiO₂, often have negative zeta potential at circumneutral pH,⁷³ while that of the studied CuO is positive. The nature of the protein corona is expected to depend on the sign and magnitude of nanoparticle surface charge, such that coronas formed on CuO might be distinct from those formed on many other metal oxides.

The acquisition of a biomolecular corona from xylem fluid may influence dissolution and distribution of inorganic NPs in plants, as well as alter the available concentration of specific proteins within the xylem fluid. We expect protein coronas to form on the surface of NPs in xylem fluid from a wide range of plant species, as variation in xylem fluid composition is relatively small among plant species.^{33, 40} However, we anticipate that the identity of biomolecules in the corona to be influenced by nanoparticle surface charge, composition, and size.⁷⁴ In an agricultural or environmental setting, engineered nanoparticles may encounter other complex media before entering xylem fluid. For instance, nanoparticles that enter the plant *via* the root may have previously acquired corona that contains components from soil organic matter, the soil microbiome, or root exudates. Particles on the leaf surface interact with a hydrophobic interface populated by a diverse microbial community that may lead to corona formation. Future studies to understand the effect of soil, root, phloem, and other extracellular matrices on NP corona formation are needed to provide a more complete view of NP transformations after environmental exposure, enable more accurate assessment of NP risk, and guide the tunable synthesis of responsive materials as part of nano-enabled precision agriculture.

4.5 Figures

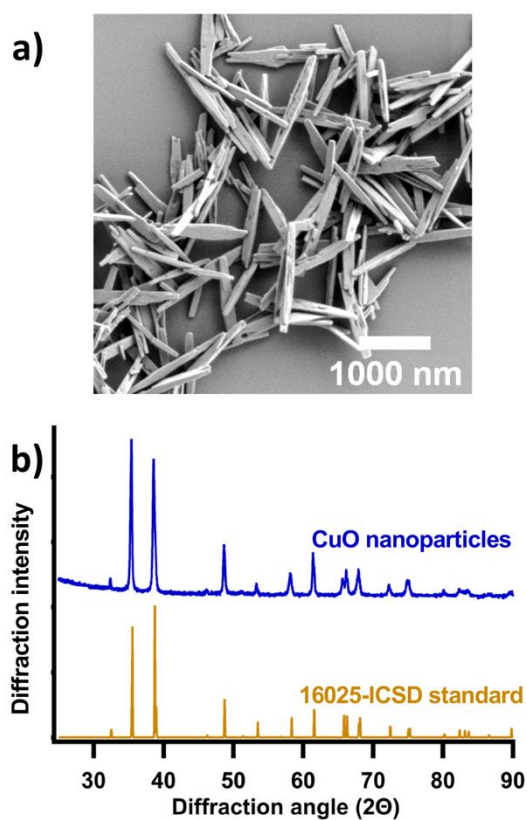


Figure 4.1 Properties of as-synthesized CuO NPs: (a) scanning electron micrograph showing the morphology of the CuO NPs; and (b) X-ray diffraction pattern indicating a crystal structure consistent with literature data from the International Crystal Structure Database (ICSD) entry 16025 for CuO (tenorite).³⁸

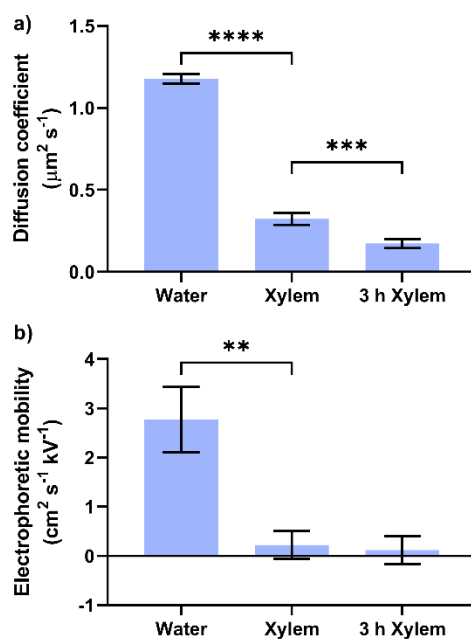


Figure 4.2 (a) Diffusion coefficients and (b) electrophoretic mobilities of CuO NPs are lower in xylem fluid than in water. Diffusion coefficients were determined from dynamic light scattering experiments; electrophoretic mobilities were determined by laser Doppler electrophoresis. Measurements are for $50 \text{ mg}\cdot\text{L}^{-1}$ suspensions of CuO NPs. Error bars denote one standard deviation. Notation for significance of differences: **, $p < 0.01$; ***, $p < 0.001$, ****, $p < 0.0001$.

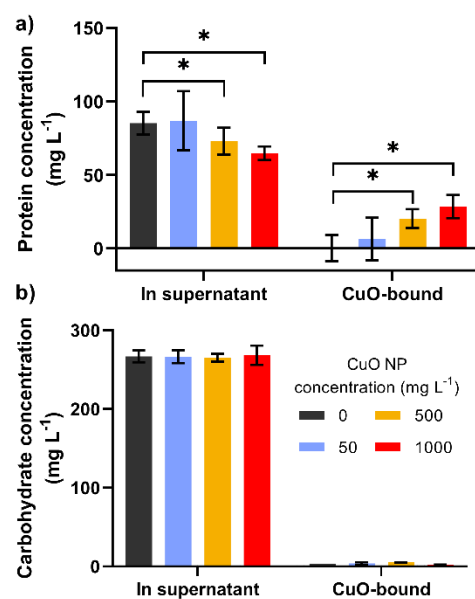


Figure 4.3 Concentrations of (a) carbohydrate or (b) protein remaining in supernatant and associated with CuO NPs after 3 h incubation and subsequent sedimentation at the indicated concentration of CuO NPs in xylem fluid. Carbohydrate concentration was measured by the phenol total carbohydrate assay; protein concentration was measured by the BCA assay. Error bars represent one standard deviation. Notation for significance of differences: *, $p < 0.05$.

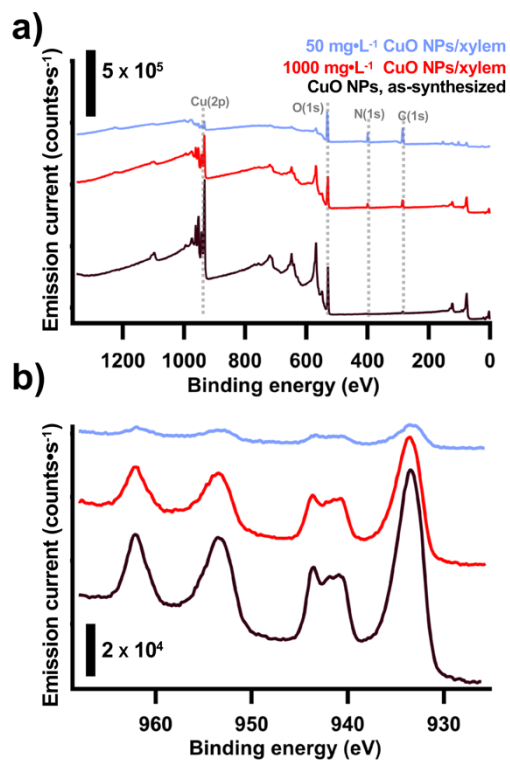


Figure 4.4 (a) Survey and (b) Cu(2p) X-ray photoelectron spectra from CuO NPs after interaction with xylem fluid.

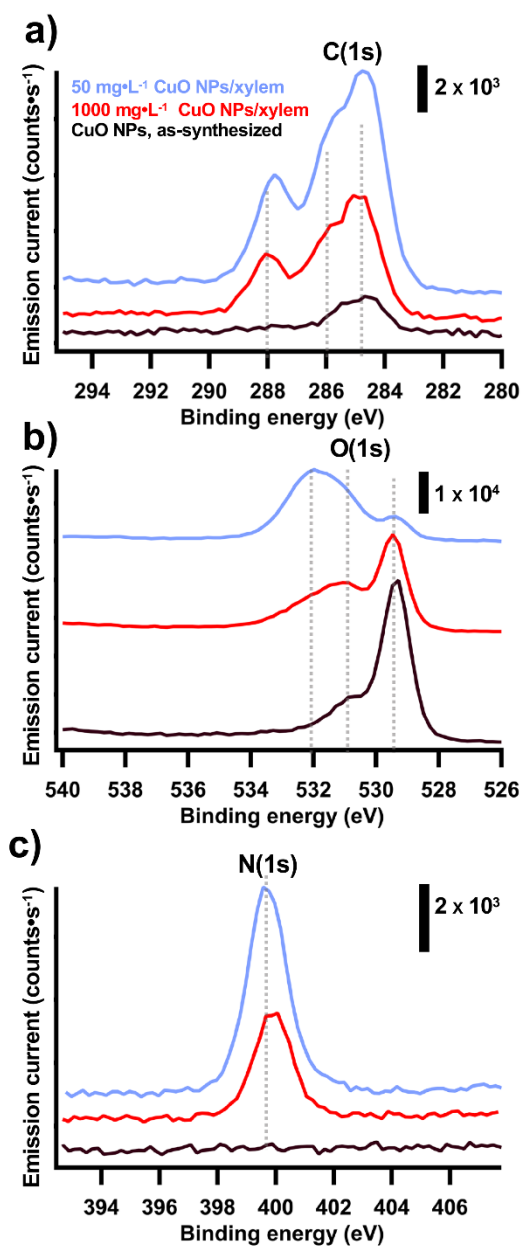


Figure 4.5 High resolution XPS spectra in the (a) C(1s), (b) O(1s), and N(1s) regions for CuO NPs that had been incubated with xylem fluid.

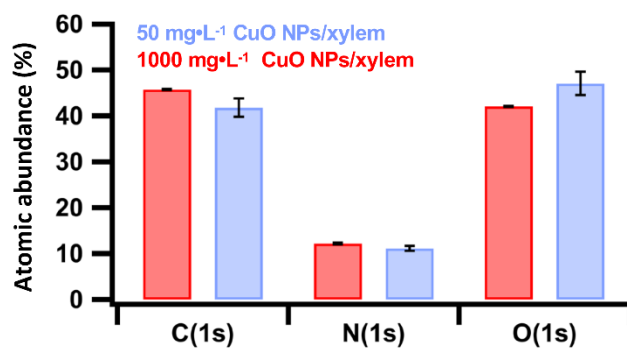


Figure 4.6 The atomic abundances of C, O, and N in the coronas on CuO NPs are consistent with the biomolecular corona being composed primarily of proteins. Atomic abundances are similar in elemental composition at both NP concentrations ($p > 0.05$).

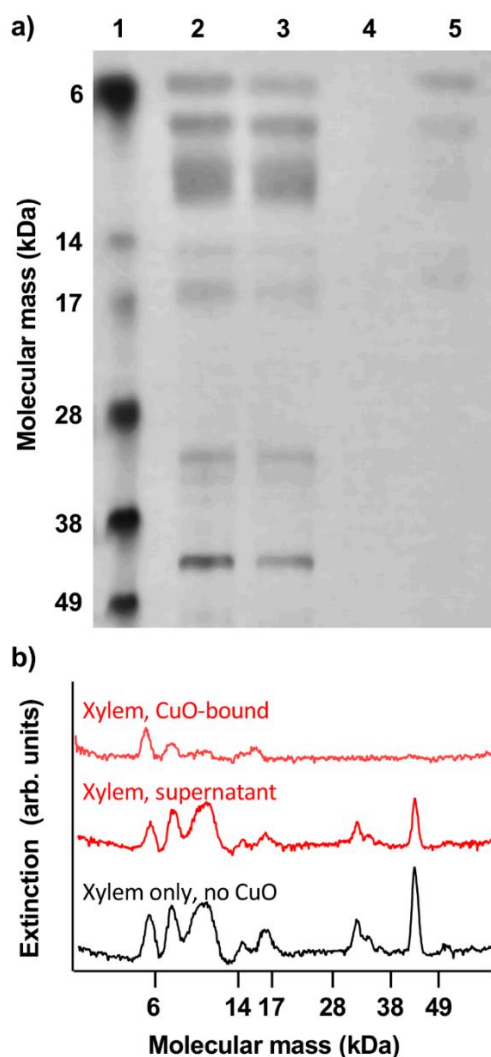


Figure 4.7 Protein binding to the CuO NPs was selective. (a) Proteins from xylem fluid before and after association with CuO NPs were fractionated by LDS-PAGE and visualized by silver staining: lane 1, molecular mass calibration standard; lanes 2, 3, supernatant after incubation with 0 and 1000 mg·L⁻¹ CuO NPs, respectively; lanes 4, 5, proteins extracted from NPs after incubation of xylem fluid with 0 and 1000 mg·L⁻¹ CuO NPs, respectively. (b) Extinction profiles showing intensity changes along lanes 2 and 3 (xylem fluid after incubation with 0 and 1000

mg·L⁻¹ CuO NPs, respectively) and lane 5 (proteins removed from 1000 mg·L⁻¹ CuO NP corona).

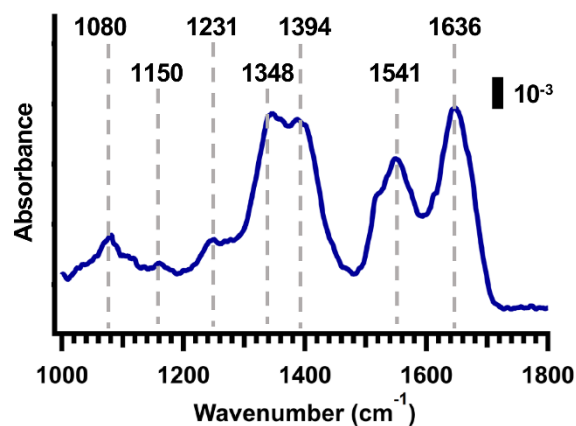


Figure 4.8 ATR-FTIR spectrum of xylem fluid. Peaks between 1700 and 1500 cm⁻¹ reflect protein amide bonds or the presence of carboxylates. The peaks between 1450 and 1250 cm⁻¹ indicate the presence of carboxylate moieties, that we attribute to small organic acids or the side chains of asparagine or glutamine residues in proteins or amino acids in xylem fluid. The peak between 1200 and 1000 cm⁻¹ is likely due to the presence of carbohydrates.

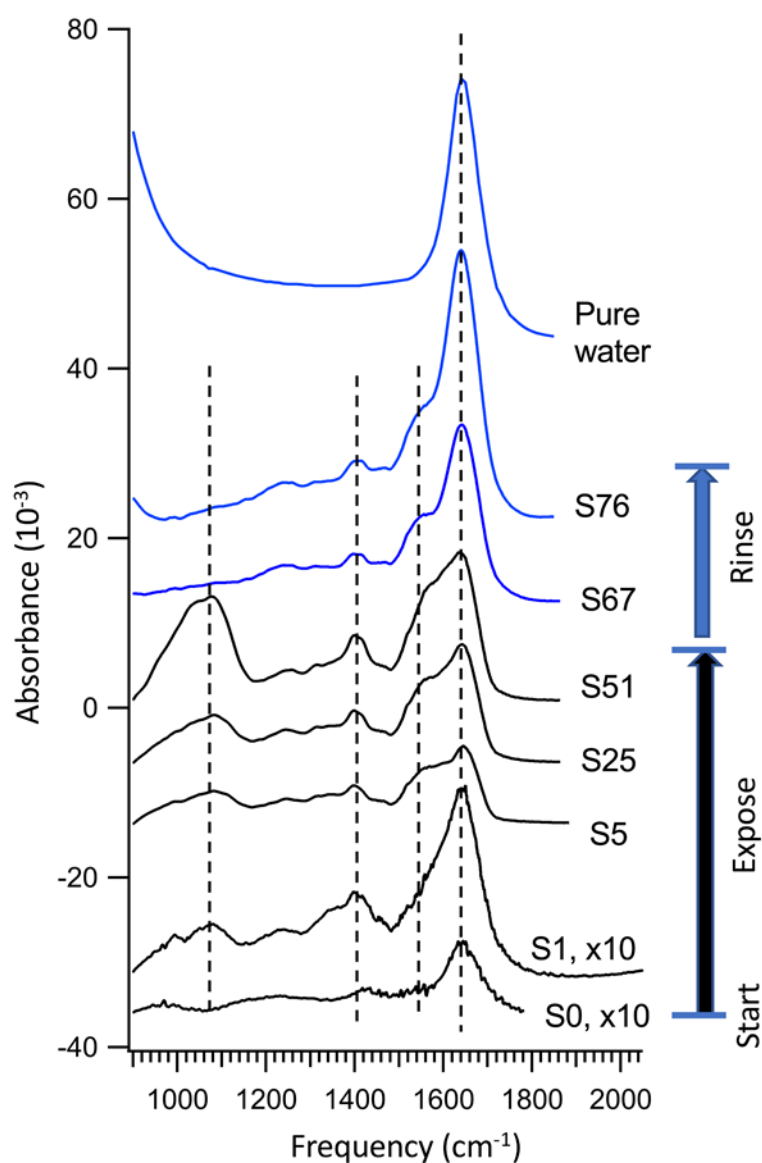


Figure 4.9 FTIR spectra of xylem fluid interacting with CuO-coated ZnSe as a function of exposure and rinsing time. The scans are identified by S#, where # is the number of scans that have been collected, with the label (S0, S1, etc.) representing the order in which the spectra were obtained; also shown is an absorption spectrum of pure water for reference.

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Chapter 5: Conclusions and future directions

5.1 Conclusions

In this thesis, we studied the impact of nanoparticle properties on disease suppression in agriculture. We studied the effect of nanoparticle composition and ion release on the suppression of *F. oxysporum* in watermelon using $\text{Cu}_3(\text{PO}_4)_2$ and CuO nanoparticles. We showed that in a variety of different matrices, $\text{Cu}_3(\text{PO}_4)_2$ nanosheets release Cu more rapidly than CuO nanoparticles; we believe that the ion release from CuO is slower because it requires conversion to $\text{Cu}(\text{OH})_2$ before dissolution. A simulated xylem matrix promoted ion release indicating that interactions with plants may increase Cu availability. In disease suppression studies, we showed that $\text{Cu}_3(\text{PO}_4)_2$ nanosheets suppress disease at 100 times lower concentrations than CuO nanoparticles, and that $\text{Cu}_3(\text{PO}_4)_2$ increases plant biomass. We believe composition impacted disease suppression, however, the CuO nanoparticles also differed by morphology and size. We adapted a synthesis method to make CuO nanosheets, and collaborated with the White group to show that CuO nanosheets have improved disease suppression efficacy when compared to $\text{Cu}_3(\text{PO}_4)_2$.¹ Overall, these studies indicate that shape and composition are important factors to consider when designing nanoparticles for nutrient delivery.

Nanoparticle charge has been shown to impact nanoparticle uptake and translocation. We studied the influence of nanoparticle charge on disease suppression of *F. oxysporum* in tomato using positively charged nanospikes, negatively charged nanospikes, and negatively charged nanosheets. The nanoparticles were terminated with reactive (001) crystal faces, and the zeta potential was correlated to the relative amount of O and Cu on the nanoparticle surface. We found that the negatively charged nanospikes and nanosheets significantly repressed disease and

increased biomass when compared to the positively charged nanoparticles and CuSO₄ control. The positively charged nanospikes and CuSO₄ control showed the most Cu on the leaf surface at the end of the experiment, which is consistent with previous studies showing increased adsorption of positively charged particles to the root surface.²⁻³ The negatively charged CuO nanosheets showed an increase in the root Cu concentration, indicating that negatively charged nanoparticles may correlate to increased translocation. We used self-assembled monolayers to show that charge-based interactions and hydrogen bonding influence nanoparticle adsorption processes. This study indicates that negatively charged particles interact differently with plants and facilitate disease suppression despite the lower concentration of Cu that is retained at the leaf surface. Understanding the influence of charge on uptake and molecular level interactions in diseased systems would be an interesting future direction.

Corona formation influences nanoparticle surface chemistry and environmental fate. We studied the interaction between pumpkin xylem fluid and CuO nanospikes. We used *in situ* ATR-FTIR to show that molecules with peaks consistent with carbohydrates adsorb weakly and are removed by rinsing, while proteins bind irreversibly. The isolated corona consisted predominantly of irreversibly bound proteins, despite the abundance of carbohydrates, and small organic acids present in xylem fluid. We also showed that the protein adsorption is selective for specific proteins. The adsorption of the protein corona decreases the nanoparticle diffusion coefficient and electrophoretic mobility, indicating that the corona may alter how the nanoparticles interact with the plant system.

We showed that nanoparticle charge, composition and shape influence the disease suppression efficacy of nanomaterials in plant systems. Our research suggests that negatively charged sheet-like nanoparticles have the most significant impact on disease suppression, this finding may be used to guide future nanomaterial design for agricultural applications. Furthermore, the composition and ion release profile of the nanomaterials may also play an important role, however, the study that we performed compared nanoparticles that had different shapes and compositions.

5.2 Future directions

Cu-based nanoparticles and disease suppression

To understand the mechanism of nanoparticle charge on disease suppression future studies could be designed to measure the transcriptomic response and the polyphenol oxidase and peroxidase activity.¹ Studies measuring the distribution of Cu in the plant tissues with respect to time could be performed to determine whether uptake and translocation influence the disease suppression efficacy of the negatively charged nanoparticles.⁴ Some of these studies are already underway at the Connecticut Agricultural Experiment Station. There is also the opportunity to study how the timing of the nanoparticle application impacts disease suppression. Could the nanoparticles be used as a seed treatment? How does the age of the plant influence plant defense pathways and disease prevention?

The comparison of positively and negatively charged CuO nanoparticles could be extended to other plant systems and pathogens. For instance, the research reported here was performed on the dicot tomato, future studies could use the monocot maize which is known to

have fungal pathogens.⁵ In addition, the antifungal activity of the nanomaterials could be measured directly on fungal cultures.

Understanding the influence of nanoparticle charge and shape on corona formation

To probe the effect of nanoparticle charge on corona formation in xylem fluid the negatively charged CuO nanospikes could be compared to the positively charged nanospikes. The corona composition could be analyzed with the same combination of techniques reported in chapter 4. Furthermore, the effect of the (001) crystal face on corona formation could be studied through a comparison of the negatively charged CuO nanospikes to nanosheets, and the crystal face effect could be validated using single crystal studies.

Xylem fluid is a complex matrix, the effect of protein charge could be studied using a series of model proteins. The Pedersen and Hamers labs collaborated to select small proteins with variable isoelectric points. The proteins include lima bean trypsin inhibitor (LBTI, negative), bovine pancreatic trypsin inhibitor (BPTI, positive), and ubiquitin (neutral).⁶ **Figure A5.1a** shows that charge-based adsorption trends were followed for the positively charged CuO with adsorption of LBTI > ubiquitin > BPTI. However, for the negatively charged CuO nanoparticles the trend was LBTI > BPTI > ubiquitin, indicating that other structural factors of the protein influenced adsorption. Using pymol software to map the electrostatics of the protein, in **Figure A5.1b,c** that LBTI has positively charged tail but presents as negative charge overall. This preliminary study generates a lot of interesting research questions that could be probed further. Including how does the location of charge on a protein influence charge-based interaction, if it is buried, distributed across the protein, on mobile loops or tails? How does

adsorption of LBTI affect the protein secondary structure? How does hydrogen bonding influence adsorption processes? These studies could be performed using a combination of circular dichroism, *in situ* ATR-FTIR, NMR techniques, and methylation of the lysine residues to determine the impact of the charged protein tail.⁷ Furthermore, our preliminary findings motivate the investigation of other proteins that have isoelectric points that may not capture the nuance of the charge distribution across the protein.

Understanding the role of the Si impurity in CuO nanoparticle surface chemistry

We developed a synthesis method to prepare positively charged CuO nanopikes, negatively charged nanopikes, and negatively charged nanosheets. The negatively charged nanoparticles had a small surface Si impurity, that likely came from the glass vessels during synthesis. The major face of the nanoparticles was the (001) crystal face that has been reported as the most reactive and unstable face.⁸ In addition, the crystal structure at the (001) face appears to be polar. When a surface is polar it can be terminated with Cu or with O. The Zn and O terminated polar faces of ZnO have been extensively studied while polar termination of CuO has not been reported.⁹⁻¹¹ Preliminary studies performed using Teflon liners for synthesis and hydrothermal synthesis methods were able to produce CuO nanoparticles with positive and negatively charged surfaces, without the Si impurity. Understanding the surface chemistry of these nanoparticles, determining whether there is a polar termination, and understanding the implications for catalytic activity could all be interesting future studies.

5.3 References

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Appendix

A.2 Supporting information for chapter 2: Copper Based Nanomaterials Suppress Root Fungal Disease in Watermelon (*Citrullus lanatus*): Role of Particle Morphology, Composition and Dissolution Behavior.

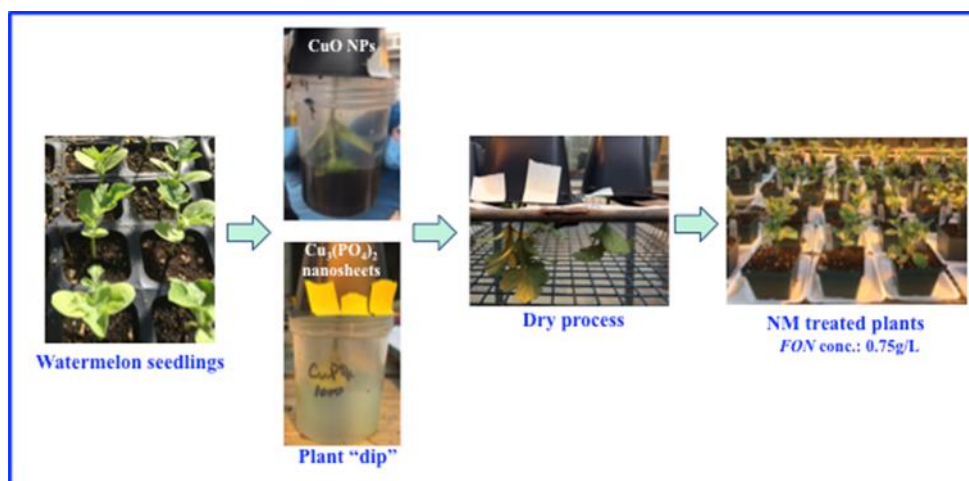


Figure A2.1 The dip method for nanomaterial foliar application.

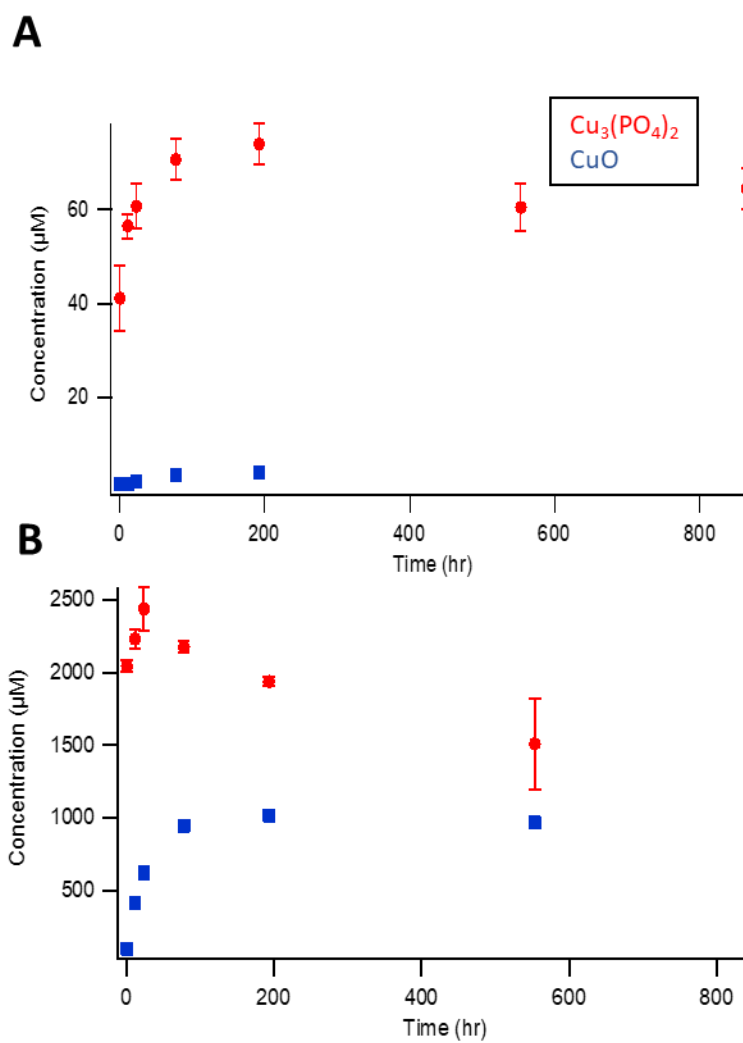


Figure A2.2 Dissolution at 1000 mg/L in A) deionized water and B) malic and citric acid matrix.

Table A2.1 Content of macronutrients in Cu-based NM treated watermelon

NMs	Route	Conc. (mg/L)	Ca (mg/kg)				P (mg/kg)				S (mg/kg)				K (mg/kg)				Mg (mg/kg)			
			Non-infested		Infested		Non-infested		Infested		Non-infested		Infested		Non-infested		Infested		Non-infested		Infested	
			Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Cu ₃ (PO ₄) ₂ nanosheets	Foliar	0	4000±400	9400±600	28000±2000	14000±1000	5600±200	11000±800	7400±200	14000±800	2800±100	6500±900	3300±100	6800±800	33000±2000	30000±4000	38000±2000	33000±4000	6500±200	5972±265	5100±300	3515±378
		10	43000±2000	9500±600	30000±1000	12000±1000	6200±600	11000±800	7600±200	12000±600	2800±100	7500±900	3100±100	6400±500	34000±2000	34000±4000	37000±2000	29000±3000	6900±300	4900±200	5300±200	4532±17
		50	37000±6000	14000±3000	33000±2000	12000±600	6900±500	8300±200	7600±300	12000±700	3500±200	5600±1000	3300±100	7400±700	34000±3000	21000±10000	35000±1000	33000±4000	6500±900	3500±1000	5600±200	4062±307
		100	37000±2000	9200±500	31000±2000	12000±400	6700±1000	12000±1000	7500±200	12000±700	3300±400	8400±200	3400±200	5900±600	34000±2000	36000±3000	36000±2000	27000±3000	6200±200	4600±200	5400±300	4551±328
		250	42000±3000	9700±700	31000±2000	16000±2000	6100±1000	11000±600	7000±300	13000±600	2900±100	6900±900	3000±100	5300±600	34000±1000	33000±3000	33000±1000	23000±4000	6900±400	4900±200	5300±300	4154±175
		500	40000±1000	11000±1000	32000±2000	12000±900	6600±400	11000±500	7500±100	12000±300	3200±300	6400±1000	3400±900	5600±500	35000±2000	33000±4000	37000±1000	27000±3000	6500±300	4600±300	5500±300	4557±221
		1000	36000±5000	11000±700	29000±1000	19000±5000	6400±900	11000±1000	7300±200	17000±5000	3100±300	6600±1000	3200±200	8000±2000	35000±3000	32000±6000	38000±2000	39000±10000	5800±700	4300±500	5000±100	5654±609
		20×2	40000±3000	10000±300	33000±2000	14000±1000	6500±300	12000±1000	7700±400	12000±500	3100±300	7200±1000	3300±300	6200±600	34000±3000	33000±6000	33000±2000	27000±4000	6500±500	5000±200	5500±300	4502±366
		200×2	40000±800	8900±700	33000±2000	14000±1000	6300±200	11000±200	7800±300	12000±500	3000±400	7500±300	3300±200	5300±900	34000±300	34000±2000	35000±1000	25000±5000	6300±100	6000±300	5500±300	4309±286
CuO NPs	Foliar	0	40000±400	9400±600	28000±2000	14000±1000	5600±200	11000±800	7400±200	14000±800	2800±100	6500±900	3300±100	6800±800	33000±2000	30000±4000	38000±2000	33000±4000	6500±200	5972±265	5100±300	3515±378
		10	38000±1000	9200±1000	33000±2000	16000±900	6200±400	12000±700	8700±300	13000±600	2900±200	7800±700	3800±100	6100±600	34000±3000	36000±3000	38000±2000	23000±4000	6100±200	5482±435	5900±400	4549±506
		50	43000±2000	8900±600	34000±2000	17000±2000	5800±200	9200±400	8100±300	12000±500	2600±200	6000±1000	3500±100	4900±500	31000±2000	28000±6000	35000±1000	19000±4000	6400±200	5012±204	5600±200	4237±337
		100	46000±100	9600±500	31000±3000	15000±2000	6100±1000	10000±1000	8200±300	12000±700	3000±200	5700±500	3500±200	4800±600	34000±2000	25000±4000	34000±1000	22000±5000	7100±900	4642±460	5500±400	3829±328
		250	41000±2000	9000±600	29000±1000	16000±1000	5800±300	13000±2000	7100±200	13000±400	2900±200	7000±200	3100±100	5800±500	33000±600	33000±10000	35000±1000	27000±3000	6800±300	5467±422	5000±200	4819±332
		500	39000±2000	9300±300	32000±3000	13000±1000	5500±1000	10000±500	7500±300	11000±300	2500±200	7300±400	3100±200	5100±400	31000±1000	34000±3000	36000±1000	25000±3000	6300±400	4598±700	5400±400	4407±225
		1000	39000±2000	9000±1000	30000±1000	13000±600	5100±300	11000±400	7400±300	12000±300	2700±100	6600±1000	3100±200	6000±400	29000±3000	31000±6000	34000±1000	32000±2000	6100±300	5499±700	5100±300	4476±292
		20×2	41000±4000	12000±2000	33000±1000	14000±1000	6900±1000	11000±700	7700±300	12000±300	3300±700	4900±800	3000±100	5200±300	32000±4000	21000±6000	33000±800	25000±2000	6500±400	4220±607	5500±200	4833±313
		200×2	40000±2000	9800±1000	34000±2000	13000±1000	5300±400	12000±1000	7600±400	12000±700	2300±100	7900±300	3000±200	5900±300	27000±1000	35000±2000	32000±2000	28000±2000	6000±400	5404±185	5800±400	5000±487

Note: A student t-test was used to calculate the *p* value. Values in blue in Cu treated groups represent a significant difference at

p<0.05 as compared to the control; values in red in Cu treated groups represent a highly significant difference at *p*<0.01 as compared to the control.

Table A2.2 Content of micronutrients in Cu-based NM treated watermelon

NMs	Route	Conc. (mg/ L)	Cu (mg/kg)				Fe (mg/kg)				Zn (mg/kg)				Mn (mg/kg)				Si (mg/kg)			
			Non-infested		Infested		Non-infested		Infested		Non-infested		Infested		Non-infested		Infested		Non-infested		Infested	
			Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Cu ₃ (PO ₄) ₂ nanosheets	Foliar	0	17±4	37±3	10±0.6	210±50	49±5	78±20	60±3	140±50	94±7	320±30	84±4	280±30	280±10	110±20	160±20	160±20	520±30	290±30	370±30	52±100
		10	13±1	34±2	13±0.5	100±50	46±4	72±6	52±2	73±7	93±4	350±30	85±2	260±30	290±40	150±30	180±10	140±10	370±200	130±20	340±40	94±20
		50	37±9	130±80	29±2	86±40	62±8	130±40	61±3	71±5	94±9	340±30	81±3	250±30	270±60	170±20	210±20	180±30	470±60	120±10 0	410±30	140±30
		100	47±8	33±2	48±3	35±7	59±7	59±7	71±7	76±5	100±7	350±30	89±5	220±30	240±2	290±20	210±20	140±9	370±90	340±70	380±40	37±40
		250	95±8	33±2	100±3	30±5	46±5	78±30	58±3	62±5	100±10	330±10	87±9	190±10	270±5	200±40	180±10	150±20	500±50	170±40	400±30	ND
		500	160±20	38±2	220±20	34±9	60±7	68±5	67±5	74±7	94±2	300±50	82±5	200±20	280±7	160±20	180±30	120±10	830±300	220±40	400±30	ND
		1000	420±90	34±3	360±30	150±80	57±8	58±10	63±4	110±30	97±4	320±60	83±3	290±70	230±40	130±9	140±9	200±50	1200±10 0	260±10 0	340±10	ND
		20×2	35±2	33±4	42±4	63±30	47±3	50±8	71±6	67±5	93±6	350±70	90±6	230±30	290±40	180±20	210±20	160±20	1000±30 0	160±30	380±30	ND
		200×2	240±50	55±30	230±10	52±30	50±4	49±10	67±4	75±8	96±7	340±50	84±4	210±30	260±6	97±10	160±20	140±20	1400±70	130±9	350±30	ND
		0	17±4	37±3	10±0.6	210±50	49±5	78±20	60±3	140±50	94±7	320±30	84±4	280±30	280±10	110±20	160±20	160±20	520±30	290±30	370±30	52±100
CuO NPs	Foliar	10	20±1	29±2	26±4	80±30	51±2	41±4	69±3	110±10	87±9	280±7	97±5	280±20	270±10	160±40	240±20	210±20	490±30	130±20	310±30	ND
		50	45±20	29±3	64±10	63±20	44±1	39±0.7	69±3	84±20	100±4	270±30	95±7	210±10	310±20	110±6	220±20	170±20	350±200	230±60	490±70	ND
		100	73±7	35±2	120±30	79±10	52±3	61±7	67±4	99±20	110±5	300±40	90±6	260±30	320±2	120±9	210±20	160±20	450±60	87±20	620±20	ND
		250	160±20	31±5	260±20	53±10	48±3	51±5	62±3	84±5	100±6	320±80	74±3	260±30	270±30	140±7	180±10	170±10	360±80	200±60	510±30	ND
		500	400±40	30±2	350±60	48±10	39±5	60±4	63±5	82±8	92±7	340±20	77±5	230±20	270±20	120±8	180±30	120±7	500±50	170±80	490±90	100±200
		1000	590±100	26±3	1200±100	60±20	43±3	45±9	65±5	78±6	92±7	300±30	77±5	240±20	270±20	130±20	170±20	130±5	800±300	110±20	480±30	28±30
		20×2	75±9	29±4	79±8	38±20	51±10	72±20	56±3	62±4	110±10	260±20	75±3	200±10	270±40	120±20	190±30	130±10	1100±10 0	410±20 0	530±40	16±30
		200×2	360±60	27±4	630±70	47±20	46±6	53±9	61±5	69±9	90±5	360±40	84±6	220±20	280±10	100±8	230±10	120±10	980±300	160±20	520±70	ND
		0	17±4	37±3	10±0.6	210±50	49±5	78±20	60±3	140±50	94±7	320±30	84±4	280±30	280±10	110±20	160±20	160±20	520±30	290±30	370±30	52±100
		10	20±1	29±2	26±4	80±30	51±2	41±4	69±3	110±10	87±9	280±7	97±5	280±20	270±10	160±40	240±20	210±20	490±30	130±20	310±30	ND
		50	45±20	29±3	64±10	63±20	44±1	39±0.7	69±3	84±20	100±4	270±30	95±7	210±10	310±20	110±6	220±20	170±20	350±200	230±60	490±70	ND
		100	73±7	35±2	120±30	79±10	52±3	61±7	67±4	99±20	110±5	300±40	90±6	260±30	320±2	120±9	210±20	160±20	450±60	87±20	620±20	ND
		250	160±20	31±5	260±20	53±10	48±3	51±5	62±3	84±5	100±6	320±80	74±3	260±30	270±30	140±7	180±10	170±10	360±80	200±60	510±30	ND
		500	400±40	30±2	350±60	48±10	39±5	60±4	63±5	82±8	92±7	340±20	77±5	230±20	270±20	120±8	180±30	120±7	500±50	170±80	490±90	100±200
		1000	590±100	26±3	1200±100	60±20	43±3	45±9	65±5	78±6	92±7	300±30	77±5	240±20	270±20	130±20	170±20	130±5	800±300	110±20	480±30	28±30
		20×2	75±9	29±4	79±8	38±20	51±10	72±20	56±3	62±4	110±10	260±20	75±3	200±10	270±40	120±20	190±30	130±10	1100±10 0	410±20 0	530±40	16±30
		200×2	360±60	27±4	630±70	47±20	46±6	53±9	61±5	69±9	90±5	360±40	84±6	220±20	280±10	100±8	230±10	120±10	980±300	160±20	520±70	ND

Note: A student t-test was used to calculate the p value. Values in blue in Cu treated groups represent a significant difference at

$p < 0.05$ as compared to the control; values in red in Cu treated groups represent a highly significant difference at $p < 0.01$ as compared

to the control. ND= not detected (detection limit is 0.1mg/L)

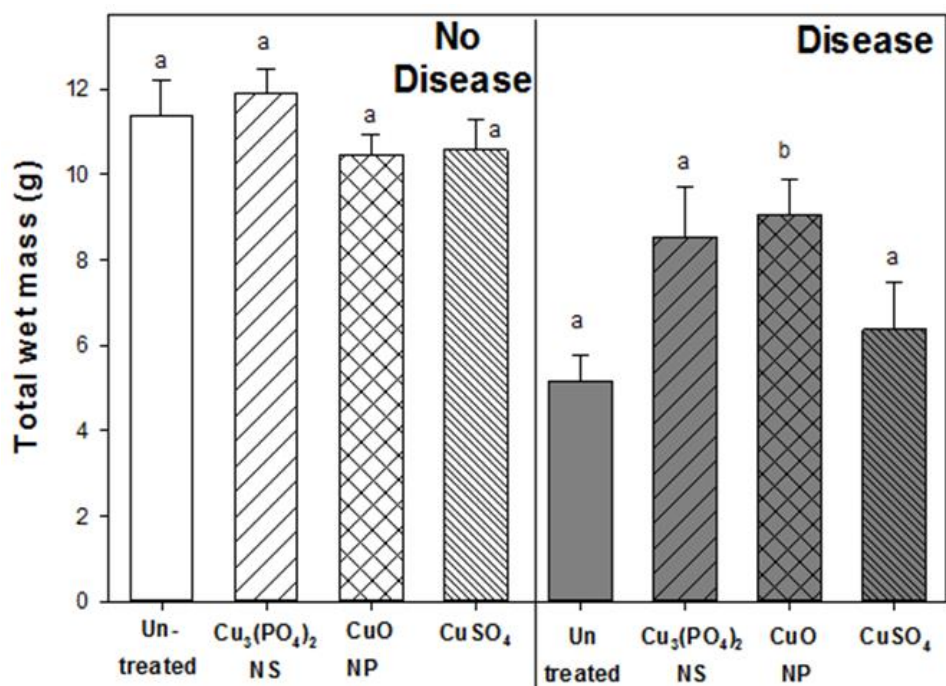


Figure A2.3 Influence of Cu₃(PO₄)₂ nanosheets, CuO nanoparticles, and CuSO₄ salt treatment on the wet biomass of non-diseased and diseased watermelon. Statistical significance (one-way ANOVA with SNK multiple comparison test) is shown above the bars.

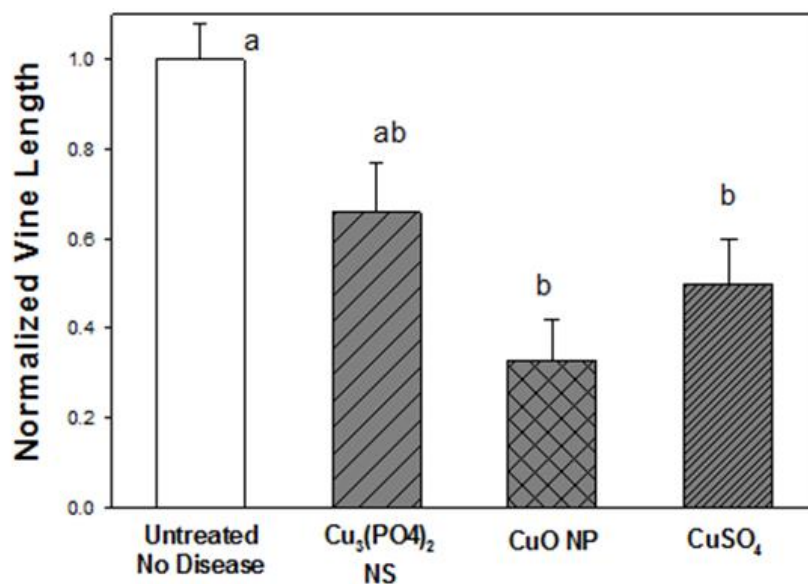


Figure A2.4 Influence of $\text{Cu}_3(\text{PO}_4)_2$ nanosheets, CuO nanoparticles, and CuSO_4 salt treatment on the vine length growth of watermelon. The vine length was measured in cm but then all treatments were normalized to the untreated no disease controls. Statistical significance (one-way ANOVA with SNK multiple comparison test) is shown above the bars.

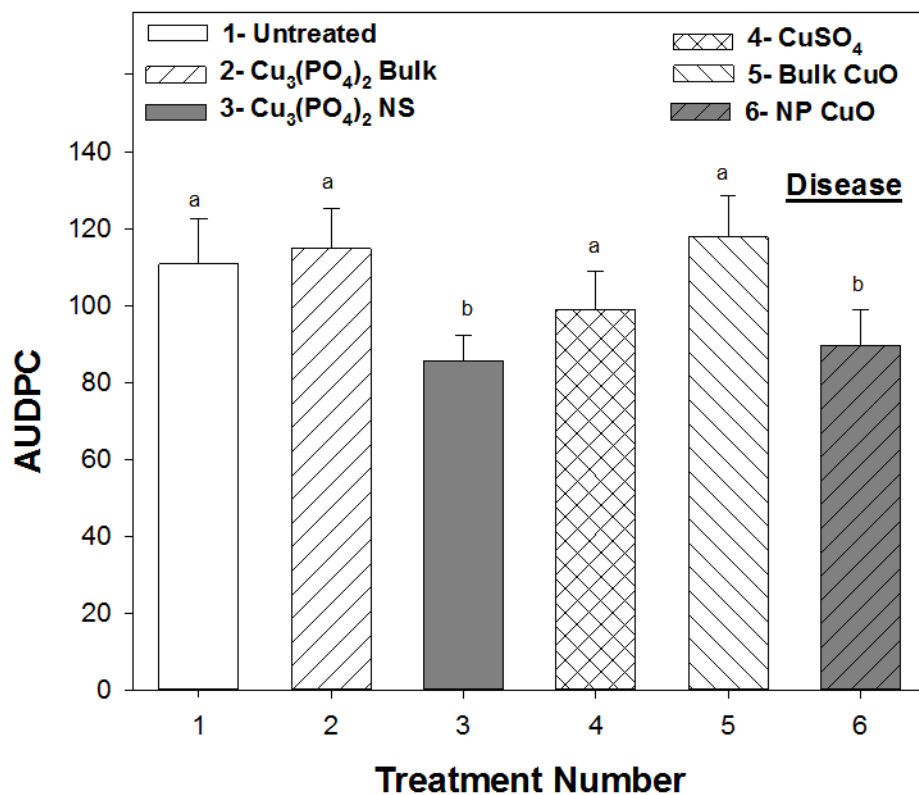


Figure A2.5 Disease progression using foliar spray methods to apply $\text{Cu}_3(\text{PO}_4)_2$ nanosheets, bulk $\text{Cu}_3(\text{PO}_4)_2$, CuO nanoparticles, bulk CuO, and CuSO_4 salt. Statistical significance (one-way ANOVA with SNK multiple comparison test) is shown above the bars.

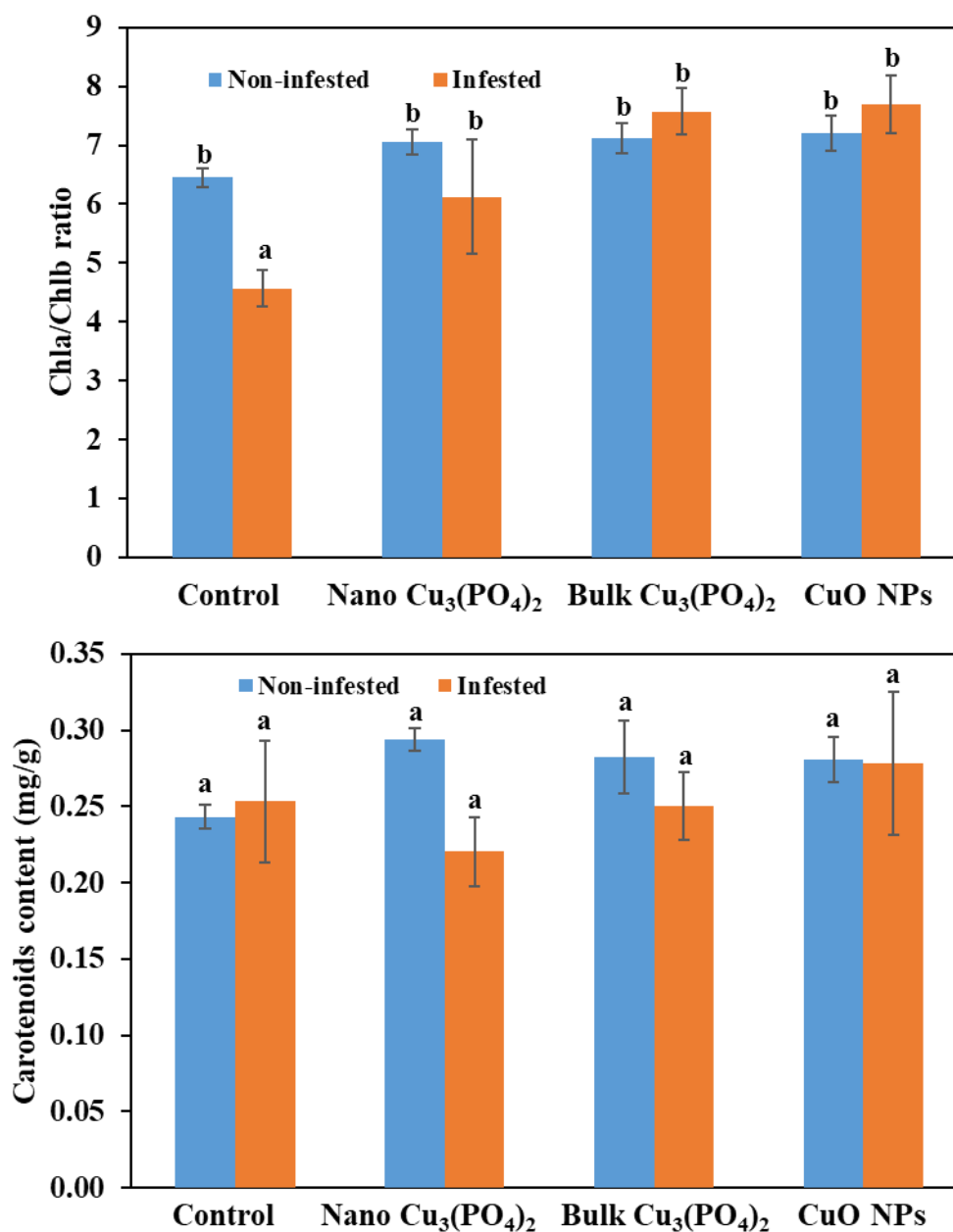


Figure A2.6 Pigment analysis in Cu-based material treated watermelon leaves. (A) and (B) represents Chla/Chlb ratio and carotenoids content, respectively. Statistical significance (one-way ANOVA with SNK multiple comparison test) is shown above the bars.

Table A2.3 The content of macro- and micronutrients in watermelon at Lockwood farm.

	Element	Treatment	Non-infested watermelon		Infested watermelon	
			Shoot	Root	Shoot	Root
Macronutrients (mg/kg)	Ca	Control	10000±400	5800±400	7400±700	8100±700
		Nano Cu ₃ (PO ₄) ₂	10000±1000	6000±600	7800±1000	8300±600
		Bulk Cu ₃ (PO ₄) ₂	11000±900	8400±400	8200±1000	6900±500
		CuO NPs	9400±1000	5500±400	11000±1000	7600±700
	P	Control	2800±200	2300±200	2200±200	2500±100
		Nano Cu ₃ (PO ₄) ₂	2400±300	2200±200	2300±200	2400±200
		Bulk Cu ₃ (PO ₄) ₂	2400±300	2400±80	2400±200	2500±200
		CuO NPs	2400±400	2500±200	2300±200	2300±100
	S	Control	1900±80	1800±70	1400±60	1700±100
		Nano Cu ₃ (PO ₄) ₂	1700±100	1800±100	1600±100	1900±80
		Bulk Cu ₃ (PO ₄) ₂	1700±100	2100±200	1600±80	2200±100
		CuO NPs	1800±100	2100±80	1700±80	1700±20
	K	Control	32000±2000	17000±800	24000±2000	16000±2000
		Nano Cu ₃ (PO ₄) ₂	27000±900	15000±1000	23000±2000	15000±2000
		Bulk Cu ₃ (PO ₄) ₂	26000±1000	21000±2000	26000±1000	19000±1000
		CuO NPs	30000±2000	18000±800	29000±2000	18000±2000
	Mg	Control	4500±200	2400±200	3300±300	2000±100
		Nano Cu ₃ (PO ₄) ₂	4100±100	2200±200	3200±300	2000±100
		Bulk Cu ₃ (PO ₄) ₂	3600±200	2600±200	3400±300	2300±200
		CuO NPs	3300±400	2300±100	3600±400	2000±300
Micronutrients (mg/kg)	Cu	Control	10.2±0.1	10.2±0.5	6.5±0.2	12±0.9
		Nano Cu ₃ (PO ₄) ₂	7.8±0.2	11±0.6	7.3±0.7	12±0.6
		Bulk Cu ₃ (PO ₄) ₂	7.7±0.4	11±0.6	7.3±0.3	11±0.3
		CuO NPs	8.9±0.6	13±2	7.7±0.4	11±0.5
	Fe	Control	130±40	440±80	69±10	340±60
		Nano Cu ₃ (PO ₄) ₂	210±100	590±200	180±80	380±60
		Bulk Cu ₃ (PO ₄) ₂	86±10	490±50	70±6	310±70
		CuO NPs	160±60	420±90	130±20	310±70
	Zn	Control	54±6	35±4	50±4	42±5
		Nano Cu ₃ (PO ₄) ₂	48±7	39±7	51±6	41±4
		Bulk Cu ₃ (PO ₄) ₂	60±6	49±3	54±6	44±5
		CuO NPs	56±3	41±2	69±7	57±8
	Mn	Control	27±2	32±5	25±2	42±6
		Nano Cu ₃ (PO ₄) ₂	31±6	39±10	32±4	46±3
		Bulk Cu ₃ (PO ₄) ₂	29±3	39±3	27±4	38±5
		CuO NPs	29±2	31±4	35±5	38±2
	Si	Control	84±6	290±30	78±5	140±50
		Nano Cu ₃ (PO ₄) ₂	90±8	410±200	81±6	160±50
		Bulk Cu ₃ (PO ₄) ₂	83±7	350±40	80±3	130±40
		CuO NPs	86±7	320±70	90±2	130±20

Note: A student t-test was used to calculate the p value. Values in blue in Cu treated groups represent a significant difference at $p < 0.05$ as compared to the control; values in red in Cu treated groups represent a highly significant difference at $p < 0.01$ as compared to the control.

Table A2.4 The content of macro- and micronutrients in watermelon in Griswold (Location 2)

	Element	Treatment	Non-infested watermelon		Infested watermelon	
			Shoot	Root	Shoot	Root
Macronutrients (mg/kg)	Ca	Control	10000±1000	4700±200	10000±2000	6100±500
		Nano Cu ₃ (PO ₄) ₂	9800±800	4700±200	8600±1000	5500±200
		Bulk Cu ₃ (PO ₄) ₂	11000±500	4700±90	7700±1000	5800±300
	P	Control	1700±30	1700±50	1500±200	2300±300
		Nano Cu ₃ (PO ₄) ₂	1900±80	1800±60	2200±600	2500±500
		Bulk Cu ₃ (PO ₄) ₂	1900±100	1800±90	1500±200	2000±100
	S	Control	2800±60	2900±200	1700±100	2300±300
		Nano Cu ₃ (PO ₄) ₂	3500±200	2900±300	1900±200	2500±200
		Bulk Cu ₃ (PO ₄) ₂	3200±300	2800±200	1800±200	2100±200
	K	Control	14000±1000	9400±600	15000±3000	12000±2000
		Nano Cu ₃ (PO ₄) ₂	15000±1000	8900±400	18000±3000	13000±2000
		Bulk Cu ₃ (PO ₄) ₂	16000±2000	9900±800	16000±3000	12000±1000
	Mg	Control	11000±500	5300±300	8500±2000	3800±400
		Nano Cu ₃ (PO ₄) ₂	12000±600	4900±200	8600±2000	4300±300
		Bulk Cu ₃ (PO ₄) ₂	12000±1000	4800±200	8000±400	4200±300
Micronutrients (mg/kg)	Cu	Control	10.2±0.3	10.2±0.8	6.3±0.2	13±0.4
		Nano Cu ₃ (PO ₄) ₂	7.8±0.4	14±0.4	6.4±0.2	11±0.3
		Bulk Cu ₃ (PO ₄) ₂	7±0.4	12±0.6	6.1±0.5	12±0.6
	Fe	Control	130±10	220±50	100±20	280±60
		Nano Cu ₃ (PO ₄) ₂	140±20	210±30	92±10	260±40
		Bulk Cu ₃ (PO ₄) ₂	140±20	280±40	110±30	310±40
	Zn	Control	94±7	54±5	98±8	86±9
		Nano Cu ₃ (PO ₄) ₂	110±6	60±5	100±4	84±9
		Bulk Cu ₃ (PO ₄) ₂	93±7	57±3	95±10	79±7
	Mn	Control	80±10	23±1	110±20	59±4
		Nano Cu ₃ (PO ₄) ₂	70±9	22±2	73±10	39±4
		Bulk Cu ₃ (PO ₄) ₂	75±9	23±2	77±10	49±7
	Si	Control	97±7	110±6	88±2	96±9
		Nano Cu ₃ (PO ₄) ₂	97±5	110±3	88±5	100±8
		Bulk Cu ₃ (PO ₄) ₂	100±2	110±7	80±6	110±10

Note: A student t-test was used to calculate the p value. Values in blue in Cu treated groups represent a significant difference at p<0.05 as compared to the control; values in red in Cu treated groups represent a highly significant difference at p<0.01 as compared to the control.

Table A2.5 The content of macro- and micronutrients in fruit from Lockwood (Location 1).

	Element	Treatment	Non-infested Fruit
Macronutrients (mg/kg)	Ca	Control	242.97
		Nano Cu ₃ (PO ₄) ₂	264.78
		Bulk Cu ₃ (PO ₄) ₂	315.08
		CuO NPs	332.87
	P	Control	438.66
		Nano Cu ₃ (PO ₄) ₂	303.38
		Bulk Cu ₃ (PO ₄) ₂	411.03
		CuO NPs	425.35
	S	Control	161.24
		Nano Cu ₃ (PO ₄) ₂	107.58
		Bulk Cu ₃ (PO ₄) ₂	153.76
		CuO NPs	125.45
	K	Control	4738.31
		Nano Cu ₃ (PO ₄) ₂	2824.78
		Bulk Cu ₃ (PO ₄) ₂	3604.10
		CuO NPs	3949.11
	Mg	Control	305.31
		Nano Cu ₃ (PO ₄) ₂	280.96
		Bulk Cu ₃ (PO ₄) ₂	336.83
		CuO NPs	392.04
Micronutrients (mg/kg)	Cu	Control	0.99
		Nano Cu ₃ (PO ₄) ₂	0.67
		Bulk Cu ₃ (PO ₄) ₂	0.95
		CuO NPs	1.24
	Fe	Control	5.20
		Nano Cu ₃ (PO ₄) ₂	4.69
		Bulk Cu ₃ (PO ₄) ₂	5.60
		CuO NPs	6.54
	Zn	Control	2.56
		Nano Cu ₃ (PO ₄) ₂	1.47
		Bulk Cu ₃ (PO ₄) ₂	1.66
		CuO NPs	2.38
	Mn	Control	0.65
		Nano Cu ₃ (PO ₄) ₂	0.58
		Bulk Cu ₃ (PO ₄) ₂	0.94
		CuO NPs	0.73
	Si	Control	14.13
		Nano Cu ₃ (PO ₄) ₂	8.31
		Bulk Cu ₃ (PO ₄) ₂	14.70
		CuO NPs	15.01

Table A2.6 The content of macro- and micronutrients in fruit from Griswold field (Location 2)

	Element	Treatment	Non-infested fruit	Infested Fruit
Macronutrients (mg/kg)	Ca	Control	1077.1	2669.1
		Nano Cu ₃ (PO ₄) ₂	850.5	939.8
		Bulk Cu ₃ (PO ₄) ₂	804.5	949.3
	P	Control	1348.4	1740.1
		Nano Cu ₃ (PO ₄) ₂	2516.8	1362.8
		Bulk Cu ₃ (PO ₄) ₂	2221.0	815.3
	S	Control	664.0	1155.6
		Nano Cu ₃ (PO ₄) ₂	839.9	496.2
		Bulk Cu ₃ (PO ₄) ₂	702.0	367.7
	K	Control	13804.2	18345.3
		Nano Cu ₃ (PO ₄) ₂	15985.5	13849.2
		Bulk Cu ₃ (PO ₄) ₂	12114.7	13132.9
	Mg	Control	1436.7	1981.1
		Nano Cu ₃ (PO ₄) ₂	1683.0	1404.5
		Bulk Cu ₃ (PO ₄) ₂	1363.6	1352.9
Micronutrients (mg/kg)	Cu	Control	4.80	2.81
		Nano Cu ₃ (PO ₄) ₂	8.05	4.035
		Bulk Cu ₃ (PO ₄) ₂	6.60	3.000
	Fe	Control	22.83	24.68
		Nano Cu ₃ (PO ₄) ₂	29.57	19.08
		Bulk Cu ₃ (PO ₄) ₂	30.94	19.08
	Zn	Control	8.55	11.33
		Nano Cu ₃ (PO ₄) ₂	17.33	6.98
		Bulk Cu ₃ (PO ₄) ₂	15.51	7.13
	Mn	Control	237.7	439.7
		Nano Cu ₃ (PO ₄) ₂	183.8	236.5
		Bulk Cu ₃ (PO ₄) ₂	234.3	318.3
	Si	Control	110.2	121.7
		Nano Cu ₃ (PO ₄) ₂	65.53	66.41
		Bulk Cu ₃ (PO ₄) ₂	72.41	78.19

A.4 Supporting information for chapter 4: Biomolecular Corona Formation on CuO Nanoparticles in Plant Xylem Fluid

A.4.1 Experimental

A.4.1.1 Calibration curve for total carbohydrate assay.

Calibration of total carbohydrate assay was performed with 0, 25, 50, 100, 150, and 300 mg·L⁻¹ glucose standards. The assay was performed by combining 200 µL of 5% w/v phenol in water solution with 250 µL of standard in a glass vial. A 1 mL volume of 98% sulfuric acid was rapidly added and the solution was vortexed briefly. The solution was left to cool for 1 hour and the absorbance was measured at 460 nm using a BioTek Synergy 2 plate reader. Three replicate measurements were performed for each concentration of glucose. A calibration curve was generated for each 96 well plate separately (Figure S3).

A.4.1.2 Calibration curve for total protein assay.

Calibration of total protein assay was performed with 0, 5, 25, 50, 125, and 250 mg·L⁻¹ bovine serum albumin (BSA) standards. Quantification of protein in solution was performed using a PierceTM BCA Assay Kit was purchased from Thermo Scientific (product number 23225). To use the kit 25 µL of sample was added to 200 µL working reagent in a 96-well plate, incubated for 30 min at 60 °C, and absorbance was measured at 562 nm using a BioTek Synergy 2 plate reader. Three replicate measurements were performed for each concentration of glucose. A calibration curve was generated for each 96-well plate separately (**Figure A4.4**).

A.4.1.3 In situ ATR-FTIR spectroscopy of model molecules interacting with CuO nanoparticles.

The nanoparticle films were prepared as described in the methods section. The internal reflection element with the nanoparticle film was then installed in the flow cell and allowed to equilibrate in ultrapure water (18 MΩ) for ~1.5 hours. The solution of interest was then flowed over the nanoparticle film and spectra were collected. The ubiquitin, dextran, and malic acid were purchased from Millipore Sigma and used without further purification. The 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was obtained from Avanti polar lipids (item number 850375).

A.4.2 Results and Discussion

A.4.2.1 Xylem fluid viscosity.

Estimated viscosity of xylem fluid is between 8.90×10^{-4} Pa·s (the viscosity of water at 25 °C) and 1.2×10^{-3} Pa·s.^{1, 2} The viscosity of xylem fluid may therefore be higher than that of water, leading to a smaller diffusion coefficient in xylem fluid than in water for the same sized particle according to the Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi\eta a}$$

where k_B is the Boltzmann constant, T is absolute temperature, η is dynamic viscosity, and a is particle radius. Using the maximum estimated value of xylem fluid viscosity (1.2×10^{-3} Pa·s), the Stokes-Einstein equation indicates that the diffusion coefficient of CuO NPs in xylem fluid could be lower than that in water by a factor of 0.74. The measured diffusion coefficients of CuO NPs

in water and xylem fluid was $1.18 \pm 0.03 \mu\text{m}^2\cdot\text{s}^{-1}$ and $0.32 \pm 0.04 \mu\text{m}^2\cdot\text{s}^{-1}$ respectively; that is, the diffusion coefficient in xylem fluid was lower than that in water by a factor of 0.27. This difference is larger than can attributable to the higher viscosity of xylem fluid than water.

A.4.2.2 CuO NP electrophoretic mobility.

The ionic strength of xylem fluid is $32 \pm 14 \text{ mM}$ (*Ricinus communis*)³ is significantly larger than ionic strength of water (estimated to be $1 \times 10^{-7} \text{ M}$). To estimate the effect of changing ionic strength from water to xylem fluid, we used Henry's equation:⁴

$$\mu_e = \frac{2\varepsilon\zeta F(\kappa a)}{3\eta}$$

where μ_e is the electrophoretic mobility, ε is the solvent dielectric permittivity, ζ is the zeta potential, $F(\kappa a)$ is Henry's function, and κ is the inverse Debye length. Henry's function was calculated for using Ohshima's relation⁵ for water (1.00) and xylem fluid (1.39). This was combined with the Debye-Hückel approximation:⁴

$$\sigma = \frac{\varepsilon\zeta(1 + \kappa a)}{a}$$

By solving for ζ in the Debye-Hückel equation and combining with Henry's equation, we estimated the impact of xylem fluid ionic strength on CuO NP electrophoretic mobility. Using the maximum estimated value of xylem fluid viscosity ($1.2 \times 10^{-3} \text{ Pa}\cdot\text{s}$) and the median value of xylem fluid ionic strength (32 mM), Henry's equation indicates that the electrophoretic mobility of CuO NPs in xylem fluid could be lower than that in water by a factor of 0.04. The measured electrophoretic mobility of CuO NPs in water and xylem fluid was $+2.7 \pm 0.6 \text{ cm}^2\cdot\text{mV}^{-1}\cdot\text{s}^{-1}$ and $+0.2 \pm 0.3 \text{ cm}^2\cdot\text{mV}^{-1}\cdot\text{s}^{-1}$ respectively.

$1\cdot s^{-1}$ respectively; that is, the electrophoretic mobility in xylem fluid was lower than that in water by a factor of 0.07. This difference is within the range attributable to ionic strength change.

A.4.2.3 Calculating organic layer thickness.

The approximate thickness of an overlayer on a substrate was determined using measurements of the N and Cu peak areas. ⁶ The detection of an electron from a specific element is influenced by the number density of the material (ρ), the sensitivity factor of the XPS instrument for that element (SF), the inelastic mean free path of an electron traveling through that material (λ), the angle of the detector with respect to the surface normal (θ), and the thickness of the material (z). The overall effects of electron scattering within the bulk CuO substrate (sub) and the overlayer can be described as:

$$\frac{(1 - e^{-z/\lambda_{overlayer}\cos\theta})}{e^{-z/\lambda_{sub\ through\ overlayer}\cos\theta}} = \frac{A_{overlayer} \rho_{sub} SF_{sub} \lambda_{sub}}{A_{sub} \rho_{overlayer} SF_{overlayer} \lambda_{overlayer}}$$

(S1)

The unknown side is then plotted as a function of thickness creating plots like the one displayed in **Figure A4.5**. The number density of CuO was calculated to be $47.84 \text{ molecules}\cdot\text{nm}^{-2}$. The number density of adsorbed N from protein was determined using the following expression:

$$\text{Number density } N = \frac{\rho_{protein} \times N_A \times \text{Number of N atoms in the amino acid backbone}}{\text{MolarMass}_{amino\ acid}}$$

where the molar mass of amino acids is $110 \text{ g}\cdot\text{mol}^{-1}$ (the average molecular mass of all the amino acids), $\rho_{protein}$ is the density of protein is $1.35 \text{ g}\cdot\text{cm}^{-3}$, N_A is the Avogadro constant, and the number of N atoms in the amino acid back bone is 1. The NIST standard reference database 71 was used to determine the inelastic mean free path (IMFP). ⁷ Since the identity of the proteins bound to the

CuO is not known, BSA was used as a reference protein.⁸ To perform the IMFP calculations the density of protein was assumed to be $1.35 \text{ g}\cdot\text{cm}^{-3}$ and a density of CuO was $6.31 \text{ g}\cdot\text{cm}^{-3}$ was used for CuO. To determine the energy-dependent IMFP, we used electron kinetic energies of 553 eV for Cu, 1202 eV for C(1s), and 1087 eV for N(1s). The inelastic mean free path for photoelectrons were estimated to be 1.2 nm for Cu(2P) in CuO, and 3.269 nm for N(1s) electrons in the protein layer. We used the following relationship to calculate the 2.07 nm inelastic mean free path of a Cu electron through the protein layer.

$$\frac{\lambda_1}{\lambda_2} = \left(\frac{KE_1}{KE_2} \right)^{0.67}$$

(S2)

The right side of equation S1 was then solved for using the experimental parameters and the value was then plotted to determine the layer thickness of protein.

A.4.2.4 Estimating protein removal from corona thickness.

Corona thickness, as determined by XPS, was used to estimate protein removal from solution.

The estimated protein removal was compared to experimental protein removal to show agreement between the BCA and XPS approaches. Particles were approximated as rectangular prisms of 900 nm × 120 nm × 50 nm for surface area and concentration calculations.

$$\begin{aligned} \text{Surface Area} &= (900 \text{ nm} \times 120 \text{ nm} + 900 \text{ nm} \times 50 \text{ nm} + 120 \text{ nm} \times 50 \text{ nm}) \times 2 \\ &= 3.18 \times 10^5 \frac{\text{nm}^2}{\text{particle}} \end{aligned}$$

$$\text{Volume} = 900 \text{ nm} \times 120 \text{ nm} \times 50 \text{ nm} = 5.4 \times 10^6 \frac{\text{nm}^3}{\text{particle}}$$

For the 50 and 1000 mg·L⁻¹ CuO concentration the corona thickness from XPS was estimated to be 5.1 and 1.9 nm respectively. Here we outline the approximation of protein depletion that would create a uniform corona of 1.9 nm thickness on 1000 mg·L⁻¹ CuO NPs. The same calculation was performed for a corona of 5.1 nm thickness on 50 mg·L⁻¹ CuO NPs. The density of CuO is 6.31 g·cm⁻³. The exposed surface area was calculated:

$$1000 \frac{\text{mg}}{\text{L}} \text{CuO NPs} \times \left(\frac{1 \text{ cm}^3}{6.31 \text{ g}} \right) \times \left(\frac{1 \text{ particle}}{5.4 \times 10^6 \text{ nm}^3} \right) \times \left(\frac{3.18 \times 10^5 \text{ nm}^2}{1 \text{ particle}} \right) = 9.2 \times 10^{18} \frac{\text{nm}^2}{\text{L}}$$

The density of protein corona was approximated as 1.35 g·cm⁻³. The protein required to form the corona was calculated:

$$9.2 \times 10^{18} \frac{\text{nm}^2}{\text{L}} \times 1.9 \text{ nm} \times \left(\frac{1.35 \text{ g}}{1 \text{ cm}^3} \right) = 24 \frac{\text{mg}}{\text{L}}$$

The approximated protein depletion for 50 and 1000 mg·L⁻¹ CuO NPs was 3.2 mg·L⁻¹ and 24 mg·L⁻¹ respectively. This estimation aligns very well with our experimentally determined values of 9 ± 18 and 28 ± 5 mg·L⁻¹ protein is depleted from xylem fluid.

A.4.2.5 In situ ATR-FTIR of model molecules interacting with CuO nanoparticles.

The spectra of xylem interacting with CuO has major peaks at 1542 cm⁻¹ and 1628 cm⁻¹, which are consistent with those in the ubiquitin spectrum that represent the Amide I and Amide II regions. The features between 1456 and 1250 cm⁻¹ are also consistent with the carboxylate moieties in the ubiquitin spectrum. The relative peak intensities between 1250 and 1628 cm⁻¹ in the xylem spectrum are similar to those in the ubiquitin spectrum. The carboxylate moieties could also be due to the presence of small organic acids. The malic acid sample also shows peaks within the region from 1420 to 1195 cm⁻¹ associated with C–O or CO₂⁻ modes. Small organic acids in the xylem fluid may contribute slightly to the corona that is formed. The peak at 1080 cm⁻¹ is in the same region where peaks associated with the C–O–C stretches seen in the dextran carbohydrate and the P–O–C stretches of the 1,2-dioleoyl-*sn*-glycero-3-phosphocholine DOPC lipid occur.

A.4.3 Results and Discussion



Figure A4.1 Collection of xylem fluid from a cut stem of a pumpkin plant.

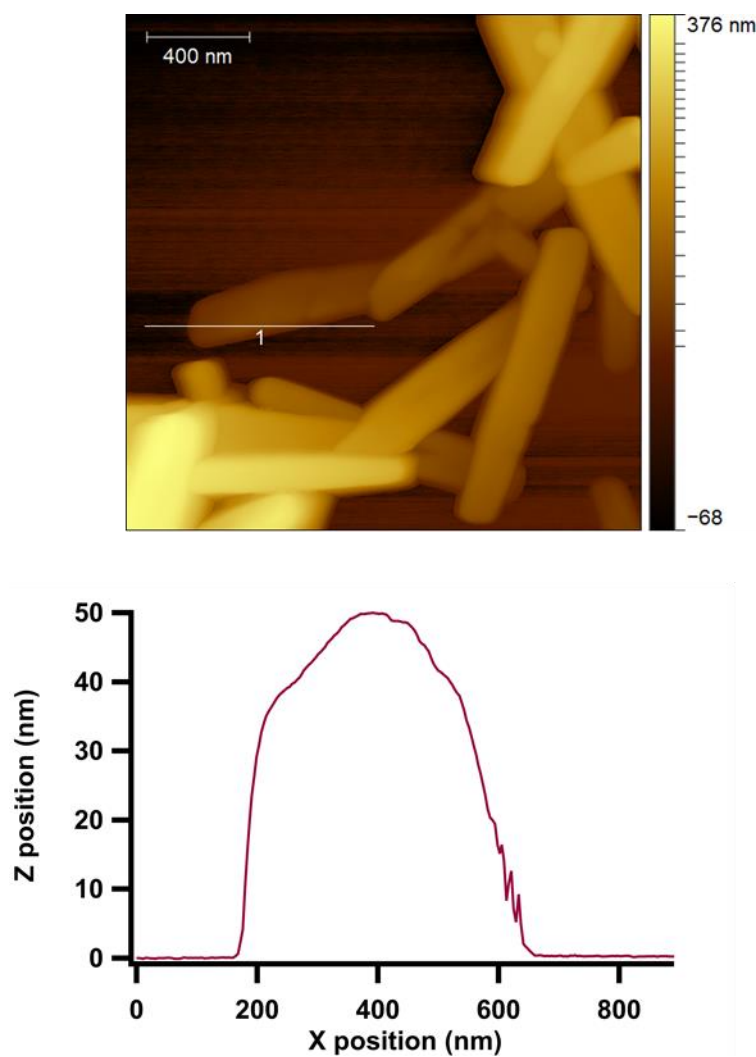


Figure A4.2 Atomic force microscope image of CuO nanoparticles on a mica substrate. The line labeled 1 corresponds to the line scan plot displayed below. The line scan indicates that the maximum nanoparticle height is approximately 50 nm.

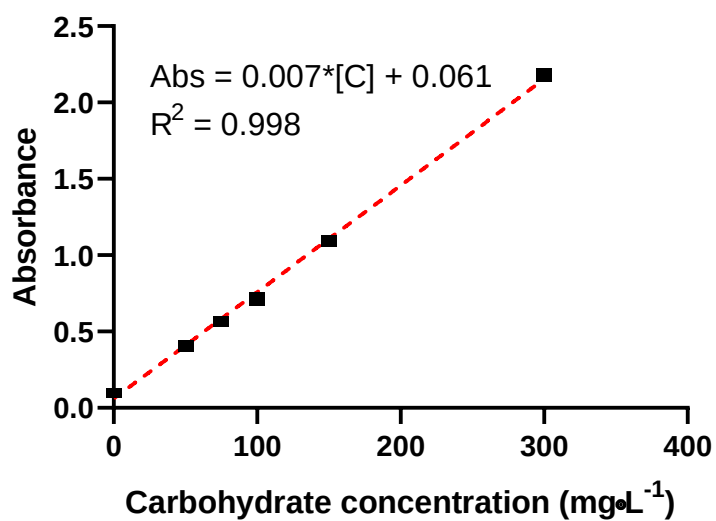


Figure A4.3 Absorbance measurements for 0, 25, 50, 100, 150, and 300 mg·L⁻¹ ppm glucose standards. Dotted red line is a linear fit of glucose concentration vs absorbance. Linear fit for glucose concentration vs absorbance was good with an R^2 of 0.998. Error bars represent one standard deviation of three replicate measurements.

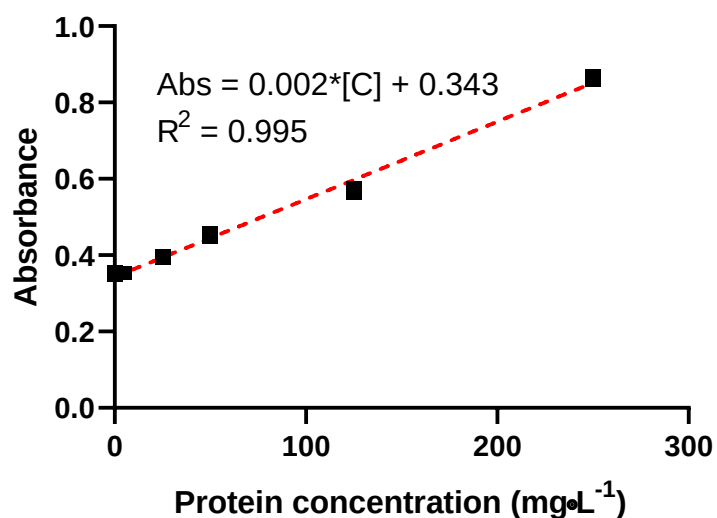


Figure A4.4 Absorbance measurements for 0, 5, 25, 50, 125, and 250 mg·L⁻¹ BSA standards after BCA treatment. Red dotted line represents a linear fit of protein concentrations vs absorbance. Linear fit for protein concentration vs absorbance was good with an R^2 of 0.995. Error bars represent one standard deviation of three replicate measurements. There was no significant difference between 0 and 5 mg·L⁻¹ standards ($p > 0.05$).

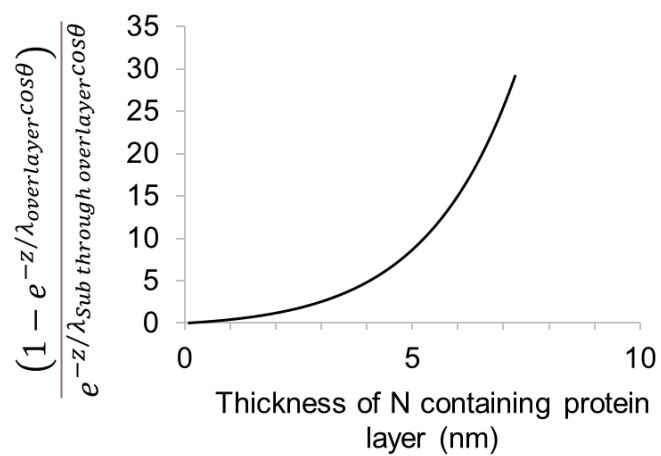


Figure A4.5 Plot of attenuation as a function of N containing protein layer thickness as calculated using equation S1. Attenuation factor refers to the right-side of equation S1.

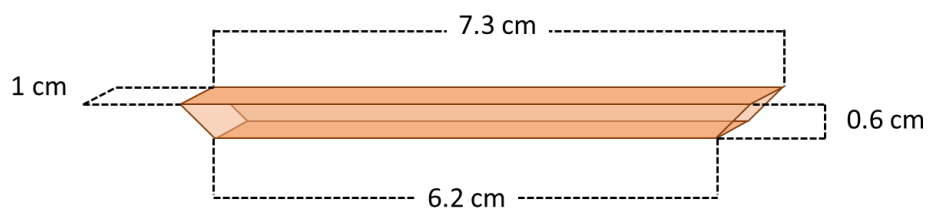


Figure A4.6 The dimensions of the ZnSe and Ge internal reflectance elements used for *in situ* attenuated total reflectance Fourier-transform infrared spectroscopy experiments.

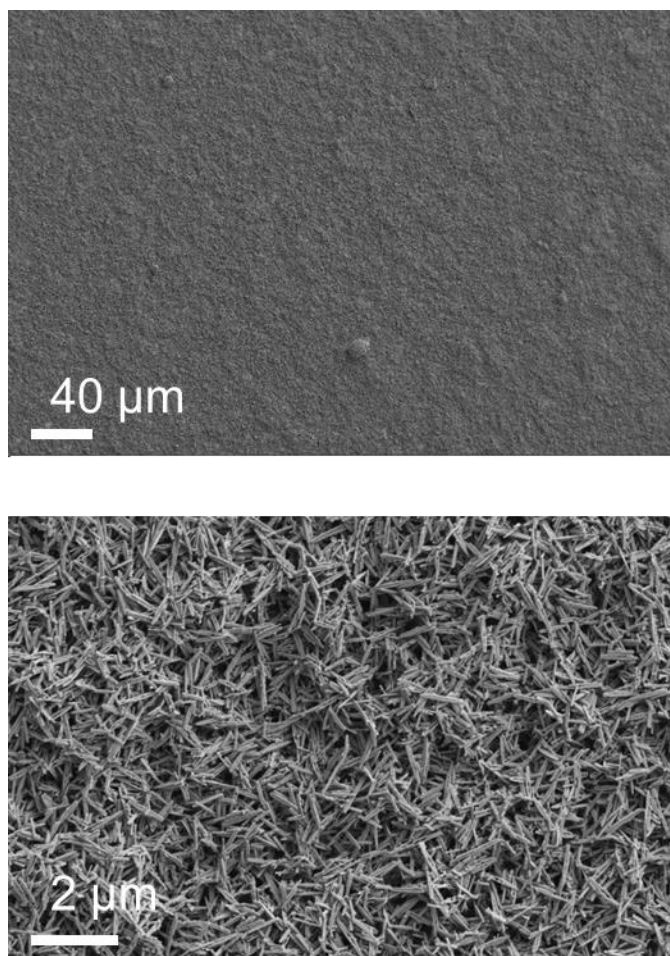


Figure A4.7 CuO nanoparticles deposited on Ge element showing a uniform, at low magnifications (above) and high magnifications (below).

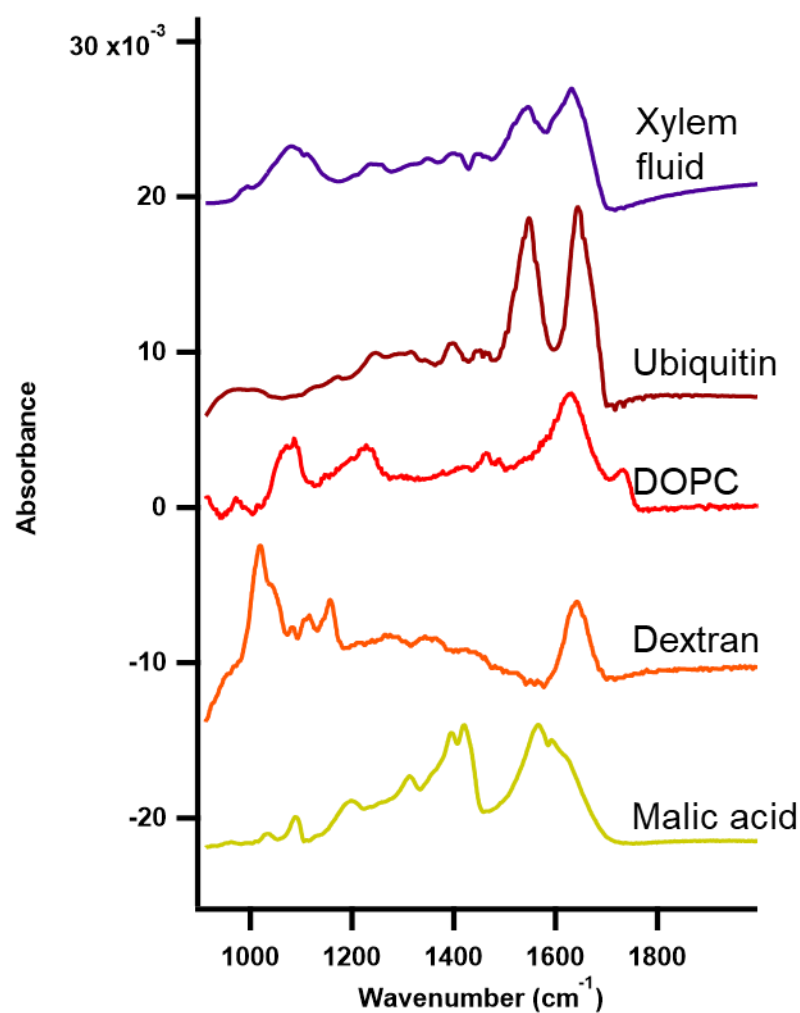


Figure A4.8 *In situ* ATR-FTIR spectra of model biomolecules interacting with the surface of CuO nanoparticles. The model biomolecules were malic acid to represent small carboxylic acids, dextran to represent carbohydrates, DOPC to represent lipids, ubiquitin to represent proteins, and xylem fluid for reference.

Table A4.1 Peaks present in the ATR-FTIR spectrum of xylem fluid, showing the presence of carbohydrate, protein, and carboxylate moieties.

Peak position (cm ⁻¹)	Identity	Associated molecule
1080	C–O , P–O, or P–O–C stretch	Carbohydrate, phospholipid
1150.4	C–O stretch	Carbohydrate
1231.3	Amide III	Protein
1243.9	Amide III	Protein
1348	Carboxylic acid O–H deformation vibration	Carboxylic acid moiety
1394.3	Symmetric CO ₂ ⁻ stretch	Carboxylic acid moiety
1524.5	Amide II	Protein
1541.8	Amide II	Protein
1636.3	Amide I	Protein
2337.3	CO ₂ peak	CO ₂
2361.5	CO ₂ peak	CO ₂
2962.2	C–H stretches for methylene and methyl groups	sp ² hybridized carbon
3266.9	O–H, amide N–H stretch	Protein, carbohydrate, carboxylic acid, phospholipid

A.4.4 References

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A.5 Supporting information for chapter 5: future directions

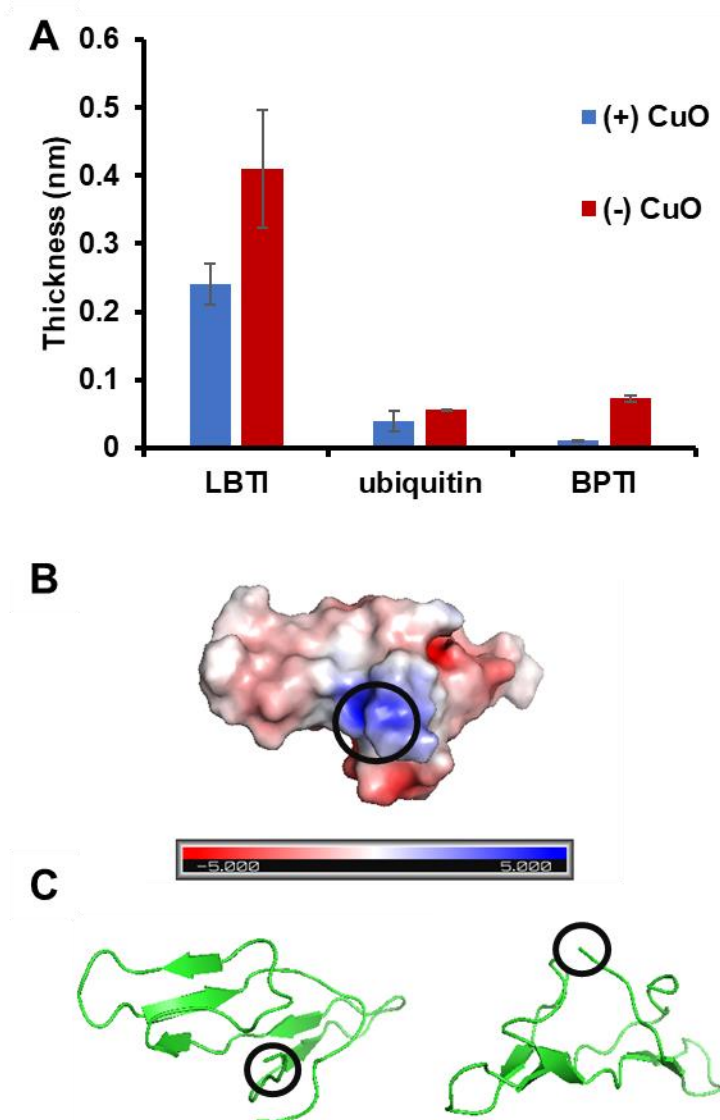


Figure A5.1 A) Protein layer thickness on the surface of positively and negatively charged CuO nanospikes as calculated using XPS and the thickogram approximation B) electrostatic map of the surface of LBTI as calculated using pymol, where red represents the negative domains and blue represents the positive domains C) protein structure showing that the positively charged region is at the tail of the protein.