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**The role of redox chemistry of disulfide bonds in cysteine residues of membrane proteins by cuprous and cupric ions in cell death of *E. coli***

by

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An undergraduate honors thesis submitted in partial fulfillment of the

requirements for the degree of

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## ABSTRACT

The antimicrobial properties of copper have been thoroughly researched, but is still unclear what the actual mechanism of cell death is. This study explores the theory that copper ions and other copper sources act as an antibiotic for *E. coli* by cleaving the disulfide bonds of membrane proteins through redox chemistry, disrupting the cell membrane and causing cell death. The focus of this study is Cu(I) and Cu(II) interactions with the thiol containing amino acid, cysteine, and how these interactions may be responsible for copper's toxicity. Cuprous ions have been found to be more toxic to *E. coli* than cupric ions. The difference between cysteine's reactions with Cu(I) and Cu(II) are explored.

## INTRODUCTION

Copper is a well known antimicrobial agent for a variety of organisms, including the bacteria *Escherichia coli*. It's toxicity has been shown in different forms of the metal, such as dry metallic surfaces<sup>1</sup>, nanostructures<sup>2,3</sup>, and ions<sup>4,5</sup>. Copper can be highly reactive in its ionic forms due to its ability to perform redox reactions. Because of this reactivity, there are many possible explanations for its toxicity. Previous theories suggest that an excess of reactive copper ions in the cell would create reactive oxidative species (ROS). ROS could cause damage to lipids in the cell membrane or damage genetic material, DNA and lead to mutations, which would lead to cell death. These proposals, however, have either been shown to be less likely or have less evidence to suggest they are the main cause of cell death<sup>6</sup>. Another theory, which involves the destruction of proteins, either membrane or intracellular, has received more attention in recent years, and is the one that will be explored in this study.

Humans rely on many microorganisms to fulfill a range of functions that we do not have the means to perform ourselves<sup>7</sup>. This relationship we have with such organisms depends on balance and control<sup>7</sup>. Among the bacteria humans host is *E. coli*<sup>7</sup>, however, in the wrong system, or the wrong amount, or even the wrong type, *E. coli* can be

dangerous<sup>7,8,9</sup>. Antibiotic agents have been studied for a long time due to their importance in returning control when the bacteria present become dangerous. As antibiotics were introduced to strains of bacteria, individual organisms which were genetically resistant to a particular kind of antibiotic would live on and reproduce, rendering the antibiotic ineffective, as the new strain of bacteria was now resistant to it<sup>10</sup>.

*E. coli* is a gram-negative bacteria<sup>11</sup>, meaning the cellular envelope of the bacteria is composed of an asymmetrical outer membrane layer in addition to the peptidoglycan inner membrane layer<sup>12</sup>. This asymmetry of the outer membrane is caused by the inclusion of both glycerophospholipids (a lipid containing a glycerol-3-phosphate backbone and positions 1 and 2 esterified with fatty acyl chains), and lipopolysaccharides (a glycolipid composed of lipid A, core oligosaccharide, and O-antigen domains)<sup>12</sup>. The purpose of the outer membrane composition's asymmetry is to act as protection for the organism. The outer membrane allows for restriction of both large polar molecules, which would normally be restricted by a phospholipid bilayer, and lipophilic molecules<sup>12</sup>. The organization of the cellular envelope of gram-negative bacteria allows it to be highly

selective about what can enter the cell, making the organism largely reliant on membrane proteins to bring nutrients and waste into and out of the cell respectively.

The proposed mechanism of denaturation of membrane proteins in *E. coli* involves the redox chemistry of the thiol groups of cysteine groups. Rigo et al. reports complexes formed cysteine and Cu(II), and the UV-Visible absorption spectrum associated with such complexes<sup>13</sup>. Often these complexes are utilized by the cell for catalytic function, or to bind copper for transport throughout the cell<sup>14</sup>. Cysteine residues are also known to be found in membrane proteins such as transport proteins<sup>15</sup>. Proposed here is the theory that these complexes are formed, not with catalytic cysteine residues, but with structural ones, specifically in membrane proteins.

Previous experiments conducted in Dr. Shankar Rananavare's lab at Portland State University found that in a comparison between copper nanowires, nanoparticles, and both the cupric and cuprous ionic forms, that Cu(I) had the highest antimicrobial effect when exposed to *E. coli* (Appendix A). Similar results are reported by Park et al, finding that Cu(I) has a higher antimicrobial effect on *E. coli* than Cu(II)<sup>16</sup>. The purpose of this study is to explain the discrepancy between the different forms of copper and their toxicity levels, and explore the mechanism by which copper achieves cell death in *E. coli*.

Antimicrobial properties of copper lead to the question: how does copper cause cell death? The mechanism of copper induced cell death remains unclear. Many theories have been proposed to explain this phenomenon, including damage to DNA, proteins, or lipids or some combination thereof. Some studies have observed the

rupturing of the cellular envelope with exposure to copper, while other studies have explained how copper disrupts protein structure and catalytic ability. This study aims to gain insight into one mechanism, which has shown a lot of promise as the leading cause. Specifically, we will examine why the mechanism proposed, disruption of membrane proteins through oxidation or reduction of disulfide bonds of cysteine residues, is more likely than others. Additionally, this study will examine the differences between Cu(I) and Cu(II) interactions with cysteine and attempt to explain why one would be more effective than the other.

## LITERATURE REVIEW

The effects of exposing *E. coli* to copper in various forms such as dry metallic surfaces, nanostructures, and ionic solutions, has been studied previously in literature. The results of such experiments will be reviewed and discussed here. As previously stated, one proposed theory, the damage of DNA, has been ruled out, in favor of the theory of protein damage. A discussion of why this is will be included. Because the mechanism theorized in this study as the cause of cell death suggests the oxidation of the disulfide bonds of thiol groups of membrane proteins, both the role of cysteine residues in proteins known in *E. coli* will also be discussed. In addition, previous studies which focus on the effect of copper on disulfide bonds will be reviewed and discussed. Lastly, previous studies of copper ion interactions with cysteine have been conducted with cupric ions, this study aims to gain insight into how Cu(I) ions would behave in place of Cu(II) ions in the same mechanisms, and why a difference was observed in the two in preliminary experiments.

## Copper's Antibacterial Effects

There have been many studies on the effectiveness of different forms of copper (dry metallic surfaces, nanomaterials, and ions) as an antibacterial agent, which have all reported similar findings, that copper's ability to kill bacteria is rapid, and effective<sup>1,2,4,14,17,18,19,20,21</sup>. Dry metallic copper has been used by humans for the purpose of sterilization from as early as 2000 BC<sup>22</sup>. More recently, copper is a popular choice for biomedical applications due to copper alloy surfaces' ability to kill up to 99% of bacteria within two hours<sup>22</sup>. Some antimicrobial agents cause the organism affected to become "non-viable", meaning they are not destroyed, but only unable to reproduce. Copper has the ability to completely inactivate bacteria<sup>22</sup>. Because of this, copper has been registered by the Environmental Protection Agency (EPA) as one of the only antimicrobial solid surfaces that can do so<sup>22</sup>. Additionally, it has been reported that copper has the fastest inactivation rate for human coronavirus, with the longest reported lifetime for the virus on copper surfaces being 4 hours<sup>23</sup>.

The search for more efficient antibacterial technology is being pushed forward as many strains of organisms become resistant to previously used antibiotics. The success of copper surfaces' ability to act as an antimicrobial agent has led to the development and use of copper nanostructures for the same purpose, with hopes of increasing surface area, and increasing effectiveness. Nanostructures are a classification of materials in the nanoscale, including nanoparticles, nanowires, and many more<sup>24</sup>. Recent studies of copper oxide nanomaterials' toxicity to bacteria have shown that against *Staphylococcus epidermidis*, CuO nanoparticles above 100 µg/mL render the bacteria inviable<sup>2</sup>.

Another recent study, which included the effects of copper nanoparticles on both gram-negative and gram-positive bacteria found that presence of copper nanowires greatly reduced growth of both types of organisms<sup>21</sup>.

Santo et al. hypothesized that the effects of copper on *E. coli* exposed to dry metallic surfaces are due to the ionization of surface copper molecules into either Cu(I) or Cu(II) ionic states and their effect on the bacteria<sup>1</sup>. Due to its high enthalpy of hydration, copper ions are highly unstable in an aqueous environment<sup>19</sup>, and, in the presence of oxygen, these ions can produce highly reactive oxidative species (ROS)<sup>13</sup>. These ROS can be damaging to DNA, which is the proposed mechanism for cell death through effects to DNA<sup>13</sup>. Copper's two most common ionic forms, Cu(I) and Cu(II), both have positive reduction potentials which makes them oxidizing agents. Previous studies on the effects of copper ions on *E. coli* have shown that exposure to copper acetate or copper nitrate destroys the cell wall and inactivates the cell in an hour or less<sup>4,19</sup> just as seen in experiments with dry metallic surfaces.

## Membrane vs. DNA Damage

Because copper can be highly reactive when in its ionic forms, there are many possibilities for the reason to its toxicity. Previously, one theory suggests that an excess of reactive copper ions in the cell would cause DNA mutations causing cell death. However, recent research has shown that this is not the case, and in fact, increase in copper ions in the cell affects copper permeability through the cell membrane wall<sup>6</sup>. When there is an excess of copper ions in the cell, the ComR gene, which is responsible for production of membrane protein ComC, is signaled to stop

production, thus reducing copper ion intake<sup>6</sup>, which would prevent interactions between copper and DNA. In addition to this, cells exposed to copper nanoparticles have been found to have cavities in their cell walls<sup>3</sup>, supporting the theory that copper is affecting the membrane. Additionally, in studies which observed *E. coli* when exposed to copper ions, damage to the cell membrane occurred rapidly<sup>18</sup>.

### **Cysteine's Role in Membrane Proteins**

Cysteine can serve many roles in a protein, depending on where it is located in the sequence, and where the protein is placed. Because of its polar nature and easily oxidized thiol group, cysteine can either coordinate metals, serve as a structural element, or participate in regulation of protein function<sup>13,25</sup>. There are two types of cysteine bonds in proteins<sup>26</sup>. Proteins with structural disulfide cysteine bonds are hardly found in cytoplasmic proteins<sup>26</sup>. If cell death is mainly caused by disruption of the membrane, and the denaturation of membrane proteins is the source of this disruption, it stands to reason that looking at how copper interacts with cysteine is a good place to start in an explanation of copper's role in cell death.

### **Copper's Effect on Proteins and Cysteine**

While copper can be toxic to many organisms, not just bacteria<sup>17,19</sup>, it is essential in certain levels for some biological functions<sup>13,15</sup>. Copper is often used as a cofactor in certain proteins, and is utilized for its ability to perform redox chemistry<sup>15</sup>. Because it is needed in the cell, *E. coli* has established pathways for dealing with it in the cell at biological concentrations<sup>27</sup>. It is proposed that at high concentrations, when the cell cannot handle

the amount of copper it is exposed to, that toxicity occurs.

There are several proposed theories which attempt to explain what exactly copper does to proteins to cause damage. One such theory suggests that copper ions oxidize active sites on catalytic reducing groups, such as cysteine residues, thus rendering them inactive<sup>2,28</sup>. Another focuses on the structural role of disulfide bonds in protein folding, and how the breaking of such integral structural components could cause cell death by "membrane fouling"<sup>29,30</sup>. Cu toxicity via damage to proteins may also be due to displacement of essential metals<sup>30</sup>. In much of the literature, these processes are dependent on the complexing or oxidation reactions of copper and cysteine<sup>13,21,28,30,31,32,33</sup>.

### **Cupric vs. Cuprous Ions.**

The standard reduction potentials of Cu(I) and Cu(II) are 0.518 and 0.161, making Cu(I) a stronger oxidizing agent than Cu(II)<sup>34</sup>. Park et al. supports the finding that Cu(I) has a higher toxicity to *E. coli* than Cu(II), and proposes that the mode of inactivation of the cell is not due to reactive oxidative species formed by copper ions, but by the copper ions themselves<sup>16</sup>.

## **METHODS**

### **Absorption of copper-cysteine complexes**

Absorption spectra of cysteine's complexes with both Cu(I) and Cu(II) ions were collected through UV-Visible spectrophotometer. All trials were done in anaerobic conditions using nitrogen gas. Cuprous and cupric chloride salts were used as the source of copper ions. Due to cuprous

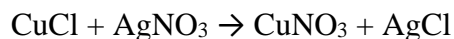
chloride's low solubility in water, a phosphate buffer of pH 6.1-6.4 was used as the mixing solution. The phosphate buffer consisted of 0.0607 M NaH<sub>2</sub>PO<sub>4</sub> and 0.0136 M Na<sub>2</sub>HPO<sub>4</sub>. The concentration of cysteine across all trials was maintained at 1.3 mM. Concentrations of copper ions were analyzed at 0.63 and 0.33 mM. The copper-cysteine solutions were prepared by first adding the cysteine aliquot to the buffer, bubbling N<sub>2</sub> gas through the solution, then adding the copper ion aliquot (to make either 0.63 or 0.33 mM), and bubbling with N<sub>2</sub> once more before spectrum was collected. Absorption spectra were collected using an Agilent 8453 UV-Visible Spectrophotometer.

### Absorption of cysteine at varying concentrations

In preliminary tests, a discrepancy between the absorption peak of cysteine was observed at different concentrations. This prompted a test of an analysis of concentration of uncomplexed cysteine at varying concentrations, in order to identify concentrations of uncomplexed cysteine in further trials. This analysis involved comparing cysteine's absorbance peak over a concentration range of 10-100 mM in both deionized water and in the phosphate buffer used in the UV-Visible spectra of copper-cysteine complexes.

### Preparation of CuNO<sub>3</sub>

Copper nitrate was prepared by combining solid copper chloride with aqueous silver nitrate through the reaction



The silver nitrate was filtered out and the copper nitrate was diluted to 0.63 mM and mixed with 1.13 mM cysteine in phosphate buffer.

## RESULTS AND DISCUSSION

The first comparison of Cu(I) and Cu(II) interactions with cysteine, used cuprous and cupric chloride. Their spectra show a strong peak at around 200 nm (Figure 1). Pecci et al. reports the absorbance spectrum of Cu(II)-cysteine complexes to have a peak at 260 nm and a shoulder at 300 nm<sup>33</sup>. These two characteristics are present in t Figure 1 for the solution prepared to be a Cu(I)-cysteine complex. This indicates that in the sampling process, some of the Cu(I) was oxidized to Cu(II). These findings suggested a different method of preparation of Cu(I) ions was needed. Additionally, neither of these peaks are present in the solution prepared to be a Cu(II)-cysteine complex. The cause of this is unknown.

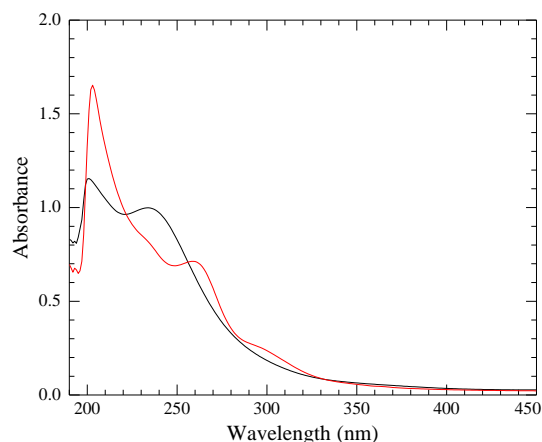


Figure 1: UV-Visible absorbance of 1.13 mM cysteine with 0.63 mM CuCl (red), and 0.63 CuCl<sub>2</sub> (black) in deoxygenated phosphate buffer.

Observed in the Cu(II)-cysteine complex in addition to the peak at 200, is a peak over the range 230-250 nm. A possible explanation for this peak is cysteine which did not bind into a complex with copper. Figure 2 shows the absorbance peak of cysteine falls between 235 and 245, depending on the concentration. Figure 2 also shows the effects of the phosphate

buffer on cysteine's UV-Visible spectrum. When compared to a cysteine dilution done in deionized water, several unidentified peaks are observed. This led to the conclusion that a different buffer be used when observing cysteine-containing samples. It is unclear what causes cysteine to have a shift in absorbance as the concentration increases. An analysis of the relationship between the major peak wavelength and the concentration of cysteine yields a linear trendline in both the phosphate buffer and water (Figure 3).

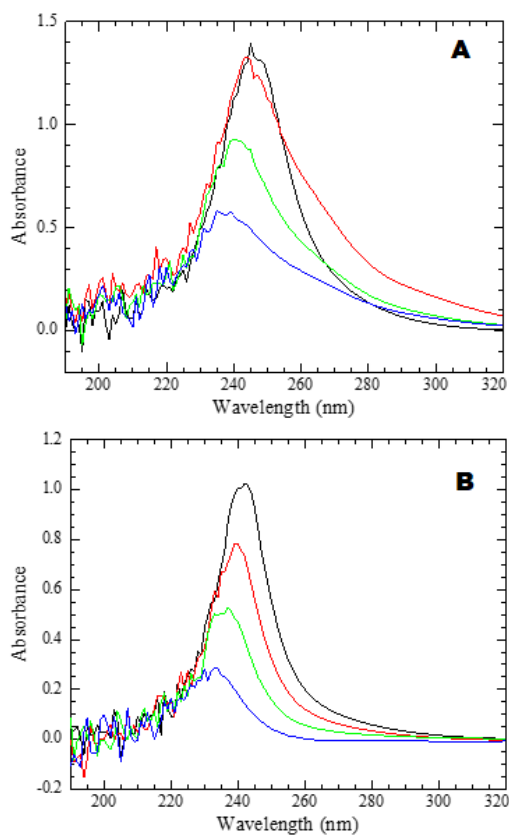


Figure 2: UV-Visible absorption spectra of cysteine dilutions in phosphate buffer (A) and deionized H<sub>2</sub>O (B). Black: 100 mM, Red: 50 mM, Green: 25 mM, Blue: 10 mM.

The slope of the curve for cysteine in the phosphate buffer is  $10 \pm 2$ , and the y-intercept is  $225 \pm 3$ . The slope for cysteine in water is  $9 \pm 0.6$  and the y-intercept is  $224.1 \pm 0.9$ . There is not significant difference between these results, which

indicates that a change in peak wavelength is not caused by matrix effects in the buffer, but is instead a property of cysteine itself.

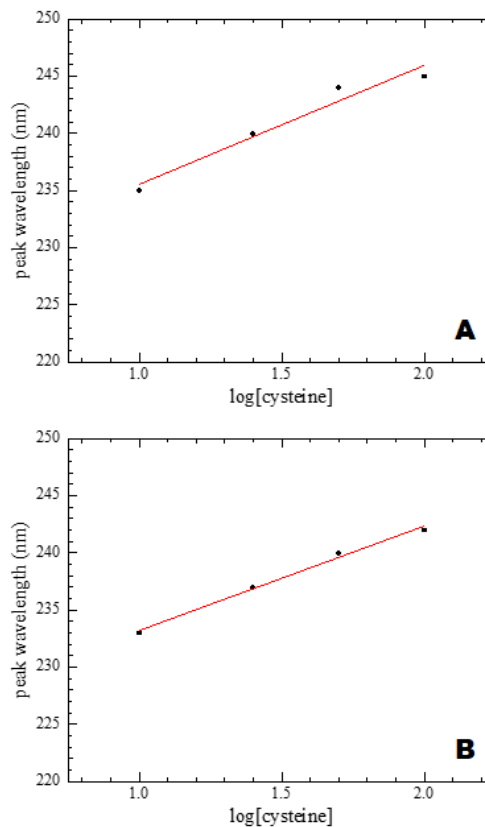


Figure 3: Plot of logarithm of concentration of cysteine (mM) versus absorption peak wavelength (nm) in pH 6.2 phosphate buffer (A) and in deionized water (B).

In addition to the Cu(II)-cysteine peaks observed in the red curve of Figure 1, there is also a shoulder present around 230 nm and a soft peak around 260 nm. The UV-Visible spectrum of cystine, the dimer form of cysteine, has been reported to have such characteristics<sup>35</sup>. The redox potential of the coupling of two cystine molecules into one cystine is  $-0.22V$ <sup>36</sup>. Taking into consideration the reduction potentials of both Cu(I) and Cu(II), the formation of cystine is possible, and is the most likely cause of the peaks observed in the red curve of Figure 1.



The unclear results shown in Figure 1 were theorized to be due to the low solubility of CuCl. It is possible that the absorbance spectra appearing closer to the expected spectra of a Cu(II) complex with cysteine was observed because Cu(I) was being oxidized to Cu(II), which would allow it to enter solution, and complex with cysteine. A new source of Cu(I) ions, CuNO<sub>3</sub> was chosen for a second test for complexed formed between cysteine and Cu(I). The result of CuNO<sub>3</sub> with cysteine is shown in Figure 4. This spectrum shows a rough peak around 245 nm. Because cysteine has been shown to have an absorbance peak at 245 nm at the concentration used in this trial, and no other clear peaks are seen, it can be concluded that copper nitrate did not interact with cysteine. During the filtration process, the copper nitrate solution was exposed to air, which may have caused it to become oxidized, however, the characteristic peak and shoulder associated with a Cu(II)-cysteine complex was not observed.

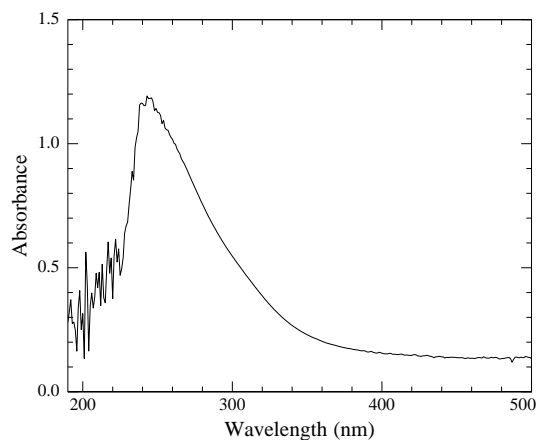


Figure 4: UV-Visible spectrum of 0.63 mM CuNO<sub>3</sub> with 1.13 mM cysteine in phosphate buffer .

In conclusion, results from previous studies which indicate a characteristic absorption peak for Cu(II)-cysteine complexes was confirmed in this study. Additionally, the literature review provides evidence for theory that copper acts as an antibacterial

agent in *E. coli* due to damage caused to membrane proteins through redox chemistry with thiol groups. In order to further explore this hypothesis, the following procedures are proposed: a more thorough analysis of the interactions between Cu(I) and cysteine, and, using results obtained from such analysis, an investigation into the presence of such copper-cysteine complexes in *E. coli* exposed to copper ions. This may be implemented using methods such as the detection of cleaved disulfide bonds using Ellman's reagent.

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## APPENDIX A



### Antibacterial properties of copper nanowires and the role of copper ion/cysteine interactions in bacterial membrane proteins

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#### Abstract

Antibacterial properties of copper nanostructures on *Escherichia coli* are presented. This study focuses on the effectiveness of antibacterial properties of copper nanoparticles or nanowires and explores cytotoxic mechanisms of copper ions. Previously observed *E. coli* cell wall disruption can be understood in terms of membrane protein denaturation as seen through ultraviolet-visible spectroscopic studies of solutions of Cu(II)/cysteine complexes. The results of this study indicate that the Cu(I) ions were more effective than the Cu(II) ions and that there was a difference in the effectiveness of copper nanowires versus nanoparticles.

#### Methods

Copper nanowires were synthesized using a solution-based process. Data collection employed two disk diffusion assay procedures. The first method used five wells made in agar plates with *E. coli*, each with varying concentrations of nanowires or nanoparticles followed by measuring the zone of inhibition. The second disc method consisted of distributing the desired concentration of nanowires and nanoparticles on to sterile disks of filter paper, which are placed into plated *E. coli* cultures followed by measuring the zone of inhibition. Cu(II) cysteine solutions are prepared in phosphate buffer solution at pH 7 and deaerated by nitrogen gas bubbling before Ultraviolet visible spectra were collected.

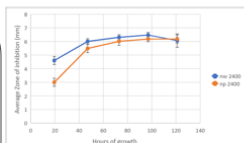


Figure 1- The Zone of inhibition in millimeters produced by a dose of 2400 µg of copper nanowires (blue) or copper nanoparticles (orange) over five days.

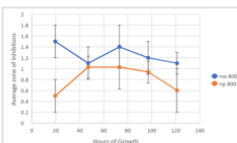


Figure 2- The zone of inhibition in millimeters produced by a dose of 800 µg of copper nanowires (blue) and copper nanoparticles (orange) over five days.

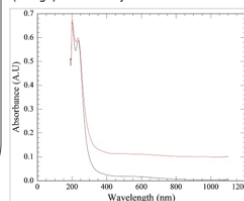


Figure 3- UV-Visible spectra of copper-cysteine (1.13 mM) complexes. At 0.33 mM (black) and 0.66 mM (red) of Cu(II) ions.

#### Results

The copper nanoparticles and nanowires were somewhat effective against *E. coli* XL blue.

- The average zone of inhibition for copper nanoparticles at 800 µg was less than 2 mm and at 2400 µg it was 5.5 mm after 48 hours; the nanowires showed similar results with the 800 µg showing an average zone of inhibition of 1 mm with the average zone for 2400 µg being 6 mm.
- Over the course of five days the highest zone of inhibition shown at the 800 µg dose of nanoparticles was in the first 24 hours.
- The largest zone of inhibition shown for 2400 dose was at 97 hours and was 6.5 and 6.2 for the nanowires and nanoparticles, respectively.
- The average zone of inhibition for the ampicillin was 11 mm after 24 hours.
- The average zone of inhibition of 1200 µg of copper nanoparticles at 24 hours was 2.4 mm, and the zone of inhibition of copper nanowires at 24 hours was 3.3 mm.
- The zone of inhibition of Cu(II) was 3.1 mm and the zone of inhibition of Cu(I) was 5.0 mm.

#### Discussion

- Significant differences between the copper nanoparticles and nanowires toxicity could be due to the nature of their surface structures.
- The Cu(I) ions were more effective than the Cu(II) ions.

#### References

- <sup>1</sup>Rigo, Adelio et al. "Interaction of copper with cysteine: stability of cuprous complexes and catalytic role of cupric ions in anaerobic thiol oxidation" *Journal of Inorganic Biochemistry* 98 (2004): 1495-1501
- <sup>2</sup>Espirito Santo, Christophe et al. "Bacterial Killing by Dry Metallic Copper Surfaces" *Applied and Environmental Microbiology* 77.3 (2011): 794-802

