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Cerium oxide nanoparticles for the detection of antimicrobial resistance

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CERIUM OXIDE NANOPARTICLES FOR THE DETECTION OF ANTIMICROBIAL RESISTANCE

by

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ABSTRACT

The rise of antimicrobial resistance demands the development of more rapid screening methods for the detection of antimicrobial resistance in clinical samples to both give the patient the proper treatment and expedite the treatment of patients. Cerium oxide nanoparticles may serve a useful role in diagnostics due to their ability to exist in a mixed valence state and act as either oxidizing agents or reducing agents. Considering that cerium oxide nanoparticles have been shown to shift in absorbance upon oxidation, a useful method of antimicrobial resistance detection could be based on the oxidation of cerium oxide nanoparticles. Herein, an assay is described whereby cerium oxide nanoparticle oxidation is a function of glucose metabolism of bacterial samples in the presence of an antimicrobial agent. Cerium oxide nanoparticles were shown to have an absorbance in the range of 395nm upon oxidation by hydrogen peroxide whereas mixed valence cerium oxide nanoparticles lacked an absorbance around 395nm. In the presence the hydrogen peroxide-producing glucose oxidase and either increasing concentrations of glucose or bacterial medium supplemented with increasing concentrations of glucose, cerium oxide nanoparticles were shown to increase in absorbance at 395nm. This oxidation assay was capable of measuring differences in the absorbance of *E. coli* and *S. aureus* samples grown in the presence of inhibitory and non-inhibitory concentrations of ampicillin in as little as six hours. Therefore, this cerium oxide nanoparticle oxidation assay may be very useful for use in clinical laboratories for the detection of antimicrobial resistance due to the relatively low cost, no requirement for specialized equipment and, most importantly, the reduced incubation time of the assay to as little as six hours compared to current gold standard antimicrobial resistance detection methods that

require 24 hours. This assay may thus also help partially circumvent the issue of knowledge of antimicrobial resistance in infected patients before prescribing improper regimens.

ACKNOWLEDGEMENTS

I cannot thank Dr. J. Manuel Perez enough for giving me the opportunity to not only work in his research laboratory but also write this thesis. I also greatly thank Dr. Bambos Kaittanis for all his assistance in training me for conducting many of the lab experiments and helping me plan experiments. I would also like to thank Dr. Santimukul Santra and Oscar Santiesteban for all their help and suggestions for my project as well as their support and confidence in me. I greatly thank Dr. Saleh Naser for letting me work in his laboratory to grow the bacterial samples and conduct the M.I.C. tests and also Sammer Elwasila for assisting me with the bacterial growth and M.I.C. tests. I would also like to thank Dr. Saleh Naser and Dr. Diego J. Diaz for taking the time to offer their support as members of my thesis committee and offering suggestions for my research project. I would also like to thank all the members at the University of Central Florida's Microbiology Prep Room for the donation of bacterial media and allowing me to conduct my initial M.I.C. tests in the Prep Room.

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INTRODUCTION

Background

The use of antimicrobials has vastly reduced the morbidity and mortality associated with many infectious diseases (1). However, the rise of antimicrobial resistance in bacteria now poses a major threat to public health and has even threatened antibiotic efficacy in treating infectious diseases (2, 3). Many bacteria are becoming resistant to the most commonly used antimicrobial treatments, which not only increases the risk of serious infections in the human population but also the spread of these antimicrobial resistant bacteria throughout the human population (3). In addition to the development of novel antimicrobials, faster and more sensitive antimicrobial resistance screening tests are needed. These improved tests will not only expedite the diagnosis of antimicrobial susceptibility in infected patients but also allow the proper antimicrobial treatment to be administered rather than using a broad-spectrum antimicrobial based on disease symptoms or diagnosis without knowledge of antimicrobial susceptibility of the infectious agent. Currently, two of the gold standards for antimicrobial resistance detection are the disk diffusion (Kirby-Bauer) method and the minimum inhibitory concentration (M.I.C.) (4). Despite their effectiveness and, both of these tests require approximately 24 hours for a definitive result (4), and sometimes the patient may not have this amount of time to spare. Therefore, a novel method is needed that is much faster and does not compromise sensitivity or specificity.

Recently, new antimicrobial resistance detection methods have been developed that can give results in as little as three hours (5, 6). These methods use nanoparticles as diagnostic agents to detect antimicrobial resistance using methods such as shifts in surface resonance or magnetic relaxometry (5, 6). However, these methods require specialized, expensive materials and

equipment. In this study, we planned to develop an innovative type of nanoparticle-based detection test for antimicrobial resistance that uses a cheaper and more common method of detection that can be adapted for use in a clinical laboratory setting.

Cerium oxide nanoparticles may offer a useful role in molecular diagnostics. Cerium oxide nanoparticles exist in a mixed valence state between the 3+ cerium state (as Ce_2O_3) and 4+ cerium state (as CeO_2) (Figure 1), making cerium oxide nanoparticles useful as both reducing and oxidizing agents, depending on the ionic state (7, 8). Cerium oxide nanoparticles have also been shown to shift in absorbance in the ultraviolet region upon oxidation/reduction, which allows the change in valence state to be detected using a spectrophotometer (9). Therefore, a spectrophotometer-based diagnostic assay could be developed that detects changes in the redox state of the cerium oxide nanoparticles in order to identify the presence of antimicrobial resistance. These cerium oxide nanoparticles thus have the capability to be used in a range of molecular diagnostic assays, including antimicrobial resistance detection.

To detect the presence of antimicrobial resistance, an assay is needed that can either oxidize or reduce the cerium oxide nanoparticles, and then measure this change spectrophotometrically. In this study, we propose using mixed valence cerium oxide nanoparticles, which can be oxidized in the presence of an oxidizing agent such as hydrogen peroxide (H_2O_2) (7). The presence of H_2O_2 may thus cause the absorbance of the nanoparticles to change at a specific wavelength, which can be detected by a spectrophotometer. Rather than directly adding H_2O_2 to the cerium oxide nanoparticles, the oxidation of the nanoparticles can be coupled to bacterial metabolism in an assay using glucose oxidase. Glucose oxidase is an enzyme that catalyzes the conversion of glucose to D-glucono-1,5-lactone and H_2O_2 (10). This

H_2O_2 product can then oxidize the cerium oxide nanoparticles (Figure 2). We hypothesized that the amount of H_2O_2 produced would be dependent on the amount of glucose present in the medium; if the bacteria are resistant to the antimicrobial agent, the bacteria would continue to grow and metabolize the glucose, leaving little glucose behind for glucose oxidase to produce H_2O_2 and consequently oxidize the cerium oxide nanoparticles (Figure 3a). However, bacteria that are susceptible to the antimicrobial agent would stop growing and thus fail to metabolize the glucose. Therefore, more H_2O_2 will be produced, which would then oxidize a greater amount of the cerium oxide nanoparticles (Figure 3b). As a result, the absorbance change would be much greater for antimicrobial-susceptible bacteria than if the bacteria were resistant to the antimicrobial agent. This method was expected to be more rapid than the zone of clearing and turbidity-based methods of detection associated with the Kirby-Bauer and M.I.C. tests, respectively. Furthermore, the hypothesized assay would require no specialized equipment other than cerium oxide nanoparticles and a spectrophotometer. This method may thus offer a quicker detection method without requiring expensive materials or equipment while also being compatible for high-throughput screening methods.

Hypothesis

The hypothesis for this experiment was that cerium oxide nanoparticles can be used for the detection of antimicrobial resistance by measuring the absorbance change of the cerium oxide nanoparticles upon oxidation by the H_2O_2 produced in the glucose oxidase-catalyzed oxidation of glucose. This process would be dependent on the amount of glucose present in the medium and thus the metabolic activity of the bacteria.

Specific Aims

The specific aims for this experiment were:

1) Determine if the absorption spectrum of the cerium oxide nanoparticles changes upon the addition of H_2O_2, and then determine the wavelength at which the absorbance of the oxidized cerium oxide nanoparticles (CeO_2) is greater than the absorbance of the mixed valence state nanoparticles.

2) Determine if the absorbance change for the cerium oxide nanoparticles upon oxidation occurs in the presence of glucose oxidase in a glucose-containing medium, such as the bacterial medium Muller-Hinton (MH) broth supplemented with a defined concentration of glucose.

3) Determine if antimicrobial susceptible and resistant bacteria, when grown in a glucose-containing medium, produce different absorbance changes in the cerium oxide nanoparticles upon the addition of glucose oxidase.

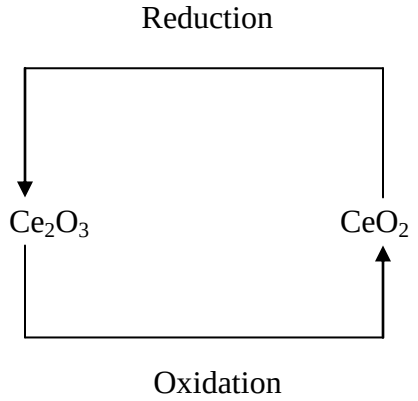


Figure 1: Mixed Valence State of Cerium Oxide Nanoparticles
Ionic cerium exists in a mixed-valence state between the 3+ (as Ce_2O_3) and 4+ (as CeO_2) states. Ionic cerium can thus change its ionic state in the presence of mild oxidizing or reducing agents, altering the spectral properties of the cerium oxide nanoparticles.

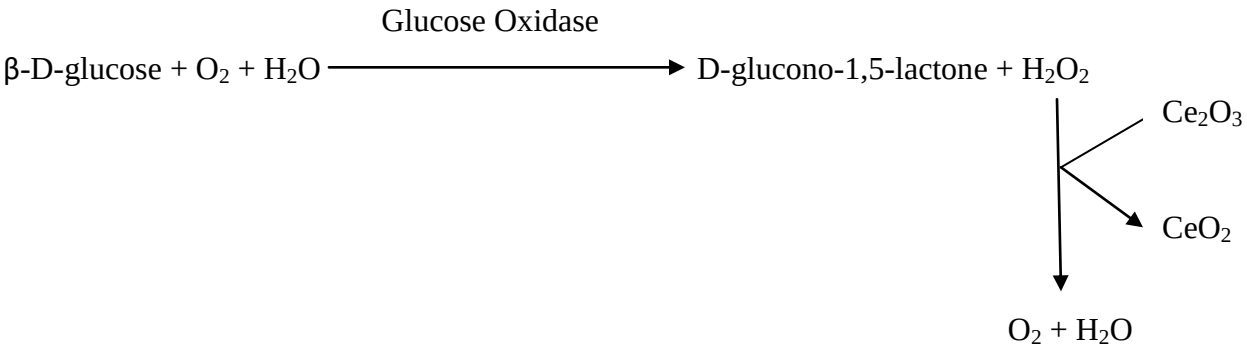
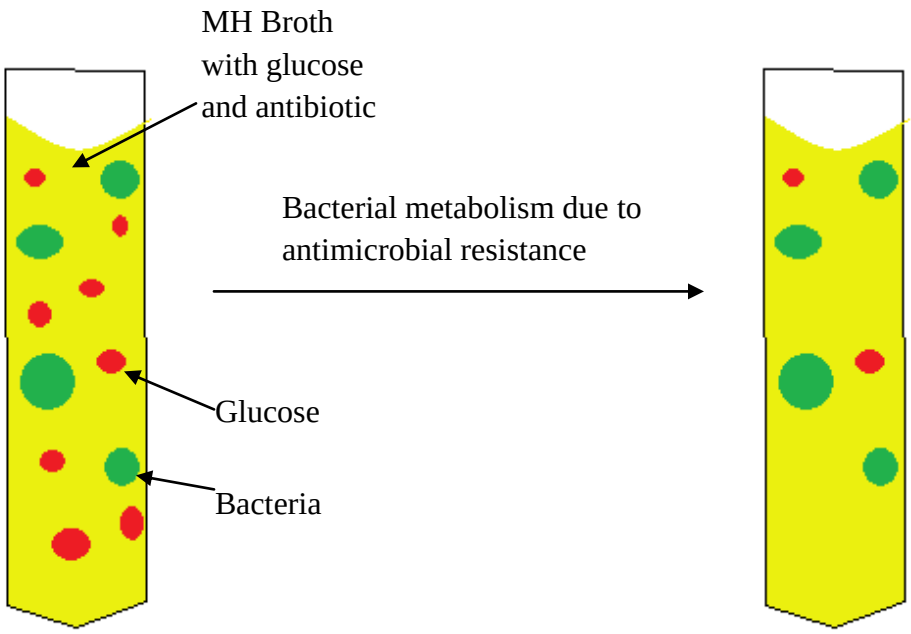


Figure 2: Chemical Reactions of the Hypothesized Oxidation Assay for Cerium Oxide Nanoparticles

Glucose oxidase is an enzyme that converts β-D-glucose to D-glucono-1,5-lactone and H_2O_2. The H_2O_2 product is then capable of oxidizing cerium oxide nanoparticles in the 3+ ionic state to the 4+ ionic state.

a)



- Very little glucose for oxidation
- Low CeO_2 produced
- Low Absorbance

b)

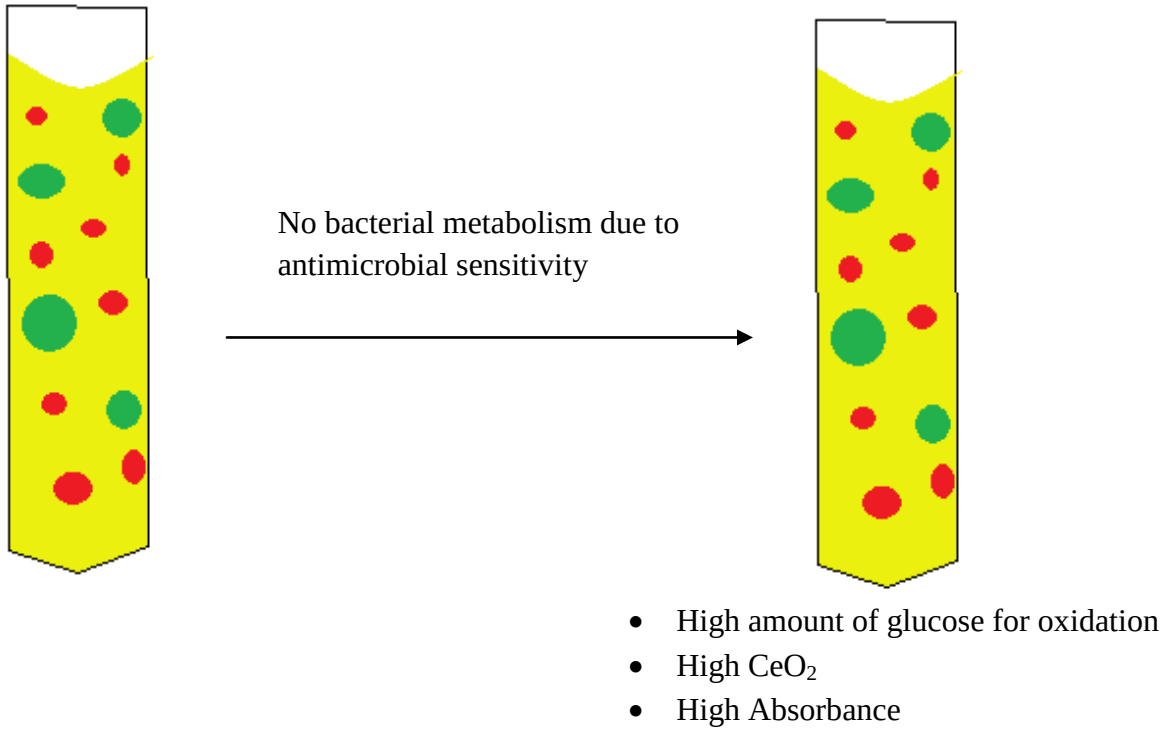


Figure 3: Proposed Model of Glucose Utilization by Sensitive versus Resistant Strains of Bacteria and the Effect on Cerium Oxide Nanoparticle Oxidation and Absorbance.

a) If the bacteria are resistant to the antimicrobial, the bacteria will continue to metabolize the glucose present in the medium. This will cause the amount of glucose to decrease over time, leaving less glucose substrate for glucose oxidase to produce H_2O_2. There will be little oxidation of Ce_2O_3 to CeO_2 and thus very little increase in absorbance.
b) If the bacteria are sensitive to the antimicrobial, bacterial metabolism will be halted. The amount of glucose in the medium will not markedly decrease, so there will be much glucose substrate for glucose oxidase to produce H_2O_2, which will in turn oxidize Ce_2O_3 to CeO_2.

MATERIALS AND METHODS

Preparation of Polyacrylic Acid-Coated Cerium Oxide Nanoparticles

Polyacrylic acid-coated cerium oxide nanoparticles (PAA-CONP, Batch#AA-111809) were prepared as described (8).

Absorption Spectrum of PAA-CONP in the Presence and Absence of H_2O_2

The absorption spectrum of the PAA-CONP was measured in a Cary 300 Bio UV-Visible Spectrophotometer using Cary WinUV software. A 1:1000 dilution of the PAA-CONP was prepared and transferred to a quartz cuvette. A quartz cuvette containing 1ml of H_2O was used as a blank. Another quartz cuvette containing 1ml of H_2O was used to establish a baseline absorption spectrum. The absorption spectrum of the PAA-CONP sample was then measured. Next, hydrogen peroxide (H_2O_2, Acros) was added to a final concentration of 9.71mM (0.003%) in another quartz cuvette containing a 1:1000 dilution of the PAA-CONP, and the absorption spectrum of this sample was immediately measured and also six minutes after the addition of H_2O_2 to ensure no significant changes in the absorption spectrum of the H_2O_2-containing PAA-CONP sample occurred. It should be noted that, for this experiment only, a different batch of PAA-CONP was used (not Batch #111809), although similar trends were seen between the two batches in future experiments.

PAA-CONP Absorption after the Addition of H_2O_2 Dilutions

A 1:40 dilution of PAA-CONP was prepared. 200µl of the 1:40 dilution of PAA-CONP was mixed with 1µl of a range of H_2O_2 concentrations (0.0483mM, 0.0644mM, 0.0966mM, 0.483mM, 0.644mM, 0.966mM, 1.93mM, 4.83mM, 9.66mM, and 48.3mM). 1µl of dH_2O was added to 200µl of the 1:40 dilution of PAA-CONP to serve as the blank. Samples were then

transferred to a clear 96-well plate (CellStar) and placed in a BioTek µQuant Plate Reader (using Gen5 version 1.05 as the software). The absorption of the PAA-CONP was measured at 395nm, 400nm, and 410nm. The absorbance value was calculated by subtracting the absorbance of each sample containing H_2O_2 from the absorbance of the sample without H_2O_2.

PAA-CONP Absorption in the Presence of Glucose and MH Broth Supplemented with Glucose

PAA-CONP absorption was tested in a range of glucose concentrations (0, 0.0265, 0.06625, 0.1325, 0.199, 0.265, 0.6625, 1.325, 1.99, and 2.65mM) dissolved in either H_2O or Muller-Hinton (MH) broth (donated by the University of Central Florida's Microbiology Prep Room). The samples were prepared by adding 100µl of a 1:20 dilution of PAA-CONP to 100µl of a respective glucose solution, and by adding 100µl of a 1:10 dilution of PAA-CONP to 100µl of MH Broth supplemented with a defined concentration of glucose. Note that a 1:10 initial dilution of PAA-CONP was used for the MH broth samples supplemented with glucose instead of a 1:20 dilution in order to improve sensitivity by increasing the final absorbance values. The samples were then transferred to a clear 96-well plate, and the absorbance of the PAA-CONP was tested at 395nm, 400nm, and 410nm. Next, 10µl of 1mg/ml glucose oxidase (Type X, MP Biomedicals) was then added to the samples to bring the final volume to 210µl. 10µl of H_2O was added to one sample containing PAA-CONP and 2.65mM glucose to serve as the blank. The 96-well plate was then placed in a BioTek µQuant Plate Reader and the absorption of the PAA-CONP was measured at 395nm, 400nm, and 410nm.

Bacterial Culture Preparation and Determination of PAA-CONP Absorbance in the Presence of Bacterial Cultures Supplemented with or without an Inhibitory Concentration of Antibiotic

A stock culture of *Escherichia coli* strain MM 294 (ATCC 33625) was grown in Nutrient Broth supplemented with a defined concentration of glucose to log phase growth at 37^{o}C with shaking (C24KC refrigerated incubator shaker, New Brunswick Scientific) as measured by OD_{600} measurements in a Bio-Rad SmartSpec 3000 spectrophotometer (log phase defined as no more than 5.0×10^{8} cells/ml or an absorbance of 1.0). A set of M.I.C. samples containing an inhibitory concentration of ampicillin, no ampicillin, and a sterile control were prepared for various incubation times. During log phase growth, 1ml of 1×10^{6} *E. coli* cells was added to 1ml of Nutrient Broth supplemented with a defined concentration of glucose. 50µl of a 2mg/ml solution of ampicillin (Sigma) was added to one of the *E. coli* samples (50µg/ml ampicillin) and 50µl of H_2O was added to a second *E. coli* sample (no ampicillin). The sterile control contained 2ml of Nutrient Broth and 50µl of 2mg/ml ampicillin (50µg/ml ampicillin) was also prepared for each time point. An M.I.C. sample was specifically prepared for turbidity determination (and confirmed by OD_{600} measurements) at 24 hours to both determine and ensure the sensitivity/resistance profiles of the M.I.C. test. 50µg/ml ampicillin was found to be inhibitory towards the growth of *E. coli* strain MM 294 after 24 hours as determined by turbidity measurements. The M.I.C. samples were then incubated at 37^{o}C with shaking in a C24KC refrigerated incubator shaker (New Brunswick Scientific) for the time period of interest. M.I.C. samples were then autoclaved after the time point of interest to inhibit further metabolism, and then samples were centrifuged at 13,200rpm (16,100rcf) in an Eppendorf 5415D centrifuge for two minutes to remove the bacteria from the samples. 100µl of the M.I.C. samples were then added to 100µl of a 1:10 dilution of PAA-CONP and transferred to a clear 96-well plate. 10µl of

1mg/ml glucose oxidase was then added to the wells. A duplicate of each sample was prepared, but these samples had 10 µl H_2O added instead of glucose oxidase to serve as a blank.

PAA-CONP Absorbance Comparison to the Minimum Inhibitory Concentration (M.I.C.) Experiment

Stock cultures of *Escherichia coli* strain MM 294 (ATCC) and *Staphylococcus aureus* strain 25178 (ATCC) were grown in Nutrient Broth supplemented with 0.25mg/ml glucose. Cultures were allowed to incubate at 37°C on a C24KC refrigerated incubator shaker (New Brunswick Scientific) overnight. For *E. coli*, the bacteria was allowed to reach logarithmic phase growth, but the OD_{600} of the *S. aureus* culture was determined to be greater than 1.0, so this culture may not have been in logarithmic phase growth when used. 1ml of 1×10^6 of *E. coli* or *S. aureus* cells/ml (in Nutrient Broth supplemented with 0.25mg/ml glucose) was then added to 1ml of Nutrient Broth supplemented with 0.25mg/ml glucose. Ampicillin solutions (dissolved in Nutrient Broth supplemented with 0.25mg/ml glucose) were then added to each tube to the following concentrations: 0, 15, 30, 45, 60, and 75µg/ml for *E. coli* and 0, 0.25, 0.5, 0.75, 1, and 60µg/ml for *S. aureus*. Sterile controls that contained 2ml of Nutrient Broth supplemented with 0.25mg/ml glucose and either 75µg/ml ampicillin (for the *E. coli* sterile controls) or 60µg/ml (for the *S. aureus* sterile controls) were also prepared.

Three sets of each *E. coli* and *S. aureus* samples were prepared for 4 hours, 6 hours, and 24 hours of incubation. The 4 hour and 6 hour samples were autoclaved and centrifuged at 13,200rpm (16,100rcf) in an Eppendorf 5415D centrifuge for two minutes to remove the bacteria from the samples. 100µl of the supernatant was added to 100µl of a 1:10 dilution of PAA-CONP and then transferred to a clear 96-well plate. The absorbance was then measured at 395nm,

400nm, and 410nm. 10µl of 1mg/ml glucose oxidase was then added to each sample. A sample containing 100µl of the 4 hour or 6 hour sterile control, 100µl of PAA-CONP, and 10µl of H_2O was used as a blank. The absorbance of the samples was measured immediately after the addition of glucose oxidase as well as 15 and 30 minutes after the addition of glucose oxidase at 395nm, 400nm, and 410nm. Absorbance measurements were calculated by subtracting the absorbance of the sample of interest by the absorbance of the same sample before the addition of glucose oxidase and the absorbance of the blank sample.

The 24 hour M.I.C. samples were used for both turbidity determination and OD_{600} measurements and thus as a comparison to the results obtained for the 4 hour and 6 hour samples. The OD_{600} for the 24 hour samples was determined by transferring 1ml of each M.I.C. sample to a plastic cuvette. The OD_{600} was measured in a Bio-Rad SmartSpec 3000 spectrophotometer, using the sterile control as the blank.

RESULTS

First, the absorption spectrum of mixed valence polyacrylic acid-coated cerium oxide nanoparticles (PAA-CONP) was obtained. The PAA-CONP were then oxidized via the addition of H_2O_2, and the absorption spectrum of the oxidized PAA-CONP was then measured and compared to the absorption spectrum of the PAA-CONP without H_2O_2 oxidation.

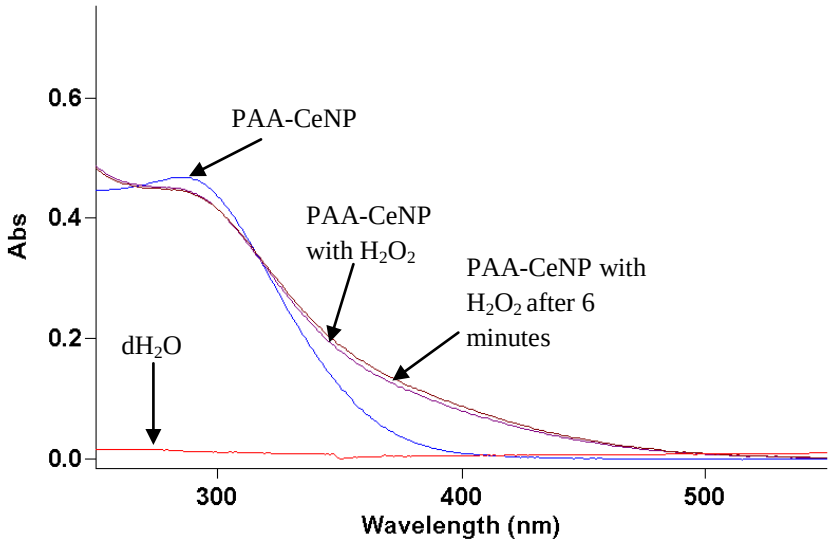


Figure 4: Absorption Spectra of PAA-CONP before and after the addition of H_2O_2. The absorption spectrum of PAA-CONP was before (blue line) and after (purple line) the addition of H_2O_2. The absorption spectrum was also measured six minutes after the addition of H_2O_2 (maroon line) to ensure no other changes in the absorption spectrum occurred over time. The absorption spectrum of dH_2O (red line) served as the baseline. Note that the absorption spectra were measured between 200nm and 800nm, but the graph shown here only represents the absorption spectra between 250-550nm, since this was the only range of wavelengths where differences in the absorption spectrum of PAA-CONP with and without H_2O_2 could be seen. Note that the oxidized PAA-CONP has a measurable absorbance around 400nm whereas mixed valence PAA-CONP (no H_2O_2 added) does not have an absorbance in this region.

The results of the absorption spectra showed that oxidized PAA-CONP has a measurable absorbance around 400nm whereas mixed valence PAA-CONP did not show a significant absorbance. The slight increase in absorbance for the oxidized PAA-CONP after six minutes

indicated that oxidation of the PAA-CONP was still occurring. Note that there was no observable difference in the absorption spectra of the PAA-CONP with and without H_2O_2 before 250nm and between 550nm and 800nm (data not shown).

Next, the change in absorbance of the PAA-CONP around 400nm was tested by first comparing the absorbance of the PAA-CONP at 395nm, 400nm, and 410nm incubated with different concentrations of H_2O_2 to the PAA-CONP without H_2O_2 present.

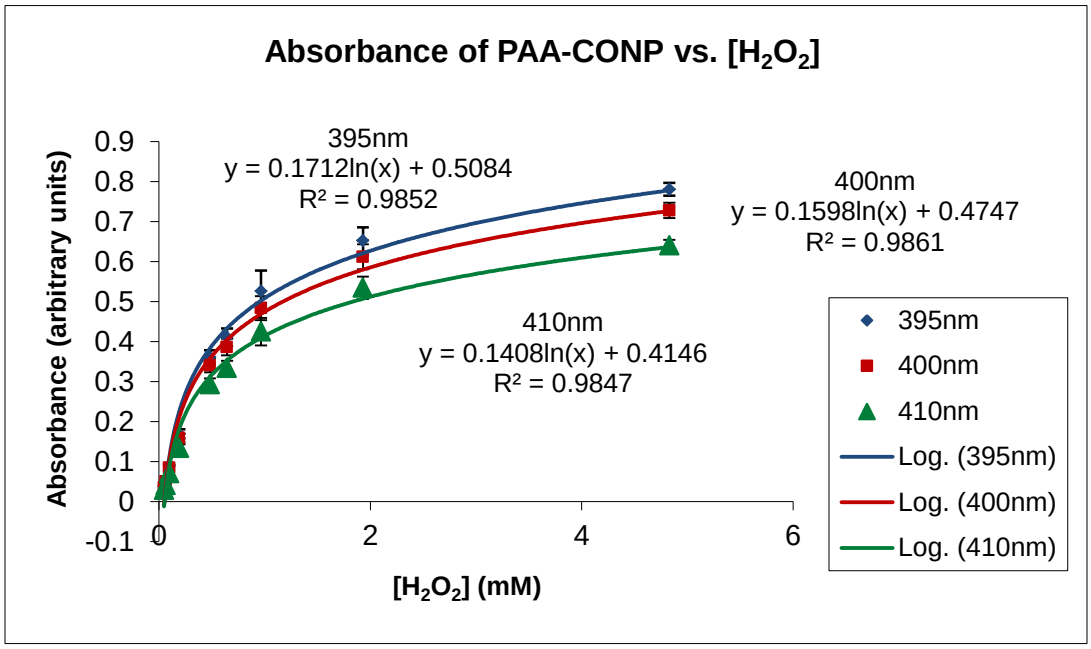


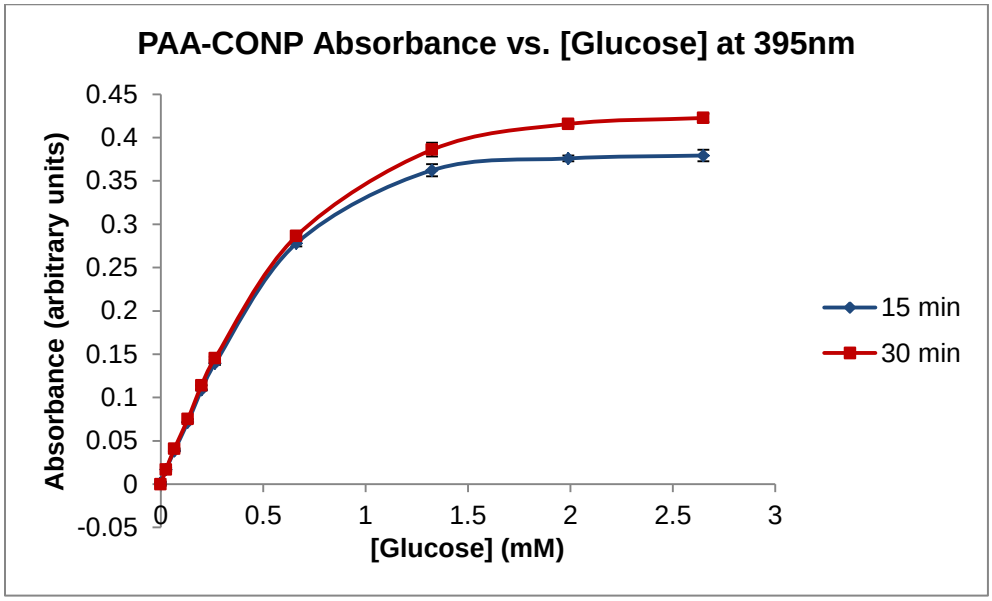
Figure 5: Absorbance Values of PAA-CONP upon the Addition of Various Concentrations of H_2O_2 at 395nm, 400nm, and 410nm.
The absorbance values were calculated by subtracting the absorbance of the cerium oxide nanoparticles in the presence of no H_2O_2 from the absorbance of the cerium oxide nanoparticles in the presence of H_2O_2. Note that the greatest absorbance values occurred at 395nm. Three independent experiments were performed, and the average values were plotted. Error bars represent the standard deviation for each value. The absorbance values for 9.66mM and 48.3mM H_2O_2 are not shown but were still a part of the logarithmic trend seen in this figure.

The results of this experiment showed that the absorbance of PAA-CONP around 400nm increased logarithmically as the concentration of H_2O_2 increased, which suggested that higher

concentrations of H_2O_2 would have a more significant impact on the absorbance until a point of saturation was reached. Although Beer's Law states that there is a linear relationship between absorbance and concentration, the reason a logarithmic trend was seen between the absorbance of the PAA-CONP and the concentration of H_2O_2 was that this method detects a product of a chemical reaction (oxidized PAA-CONP). Therefore, since the product of a chemical reaction is being detected, the kinetics should be similar to a chemical reaction, so a point of saturation is expected to be reached after a large amount of substrate (in this case, H_2O_2) is added. The highest absorbance values occurred at 395nm, so this wavelength was selected for future experiments since this wavelength was expected to give the most sensitive results.

Due to the fact that the absorbance increased as the [H_2O_2] increased, the absorbance should also increase if the [glucose] in the medium was increased because, in the presence of glucose oxidase, the increased [glucose] would result in increased H_2O_2 production. Therefore, the PAA-CONP was incubated with a range of glucose concentrations, and the A_{395} was measured (Figure 6). The A_{395} was calculated for each sample containing glucose oxidase by subtracting the A_{395} from both the absorbance the sample prior to the addition of glucose oxidase as well as the absorbance of a blank sample containing 2.65mM glucose and PAA-CONP but no glucose oxidase.

a)


PAA-CONP Absorbance vs. [Glucose] at 395nm
Absorbance (arbitrary units)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
0
0.5
1
1.5
2
2.5
3
[Glucose] (mM)
15 min
30 min

b)

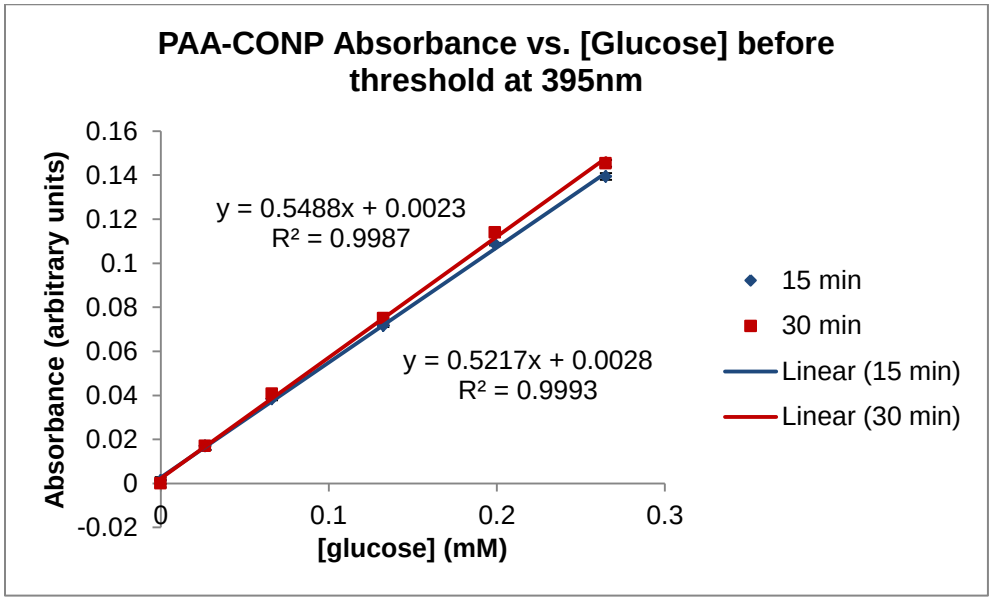
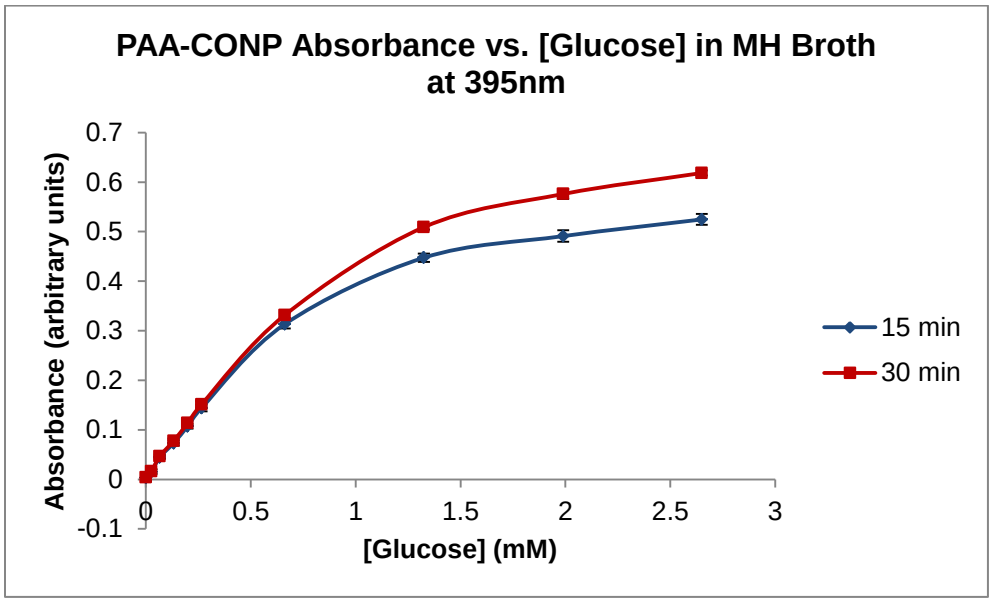


Figure 6: PAA-CONP Oxidation by Glucose Oxidase in the Presence of Glucose Dilutions.
a) The absorbance of the CONP was measured at 395nm over a range of glucose dilutions. Absorbance measurements were measured 15 minutes and 30 minutes after the addition of glucose oxidase.
b) The absorbance of the CONP at 395nm without the glucose concentrations seen in the threshold of part (a). The data shows a linear trend in absorbance versus the concentration of glucose.
The absorbance in these figures was calculated as the absorbance of the sample of interest after the addition of glucose oxidase minus both the absorbance of the sample before the addition of glucose oxidase and the absorbance of a blank sample containing 2.65mM glucose and no glucose oxidase. The CONP was diluted 42-fold in these experiments. The values shown are the average values from three independent experiments, and error bars represent the standard deviation for each value.

Figure 6 showed that the absorbance of the PAA-CONP at 395nm increased as the concentration of glucose in the sample increased, which would be useful for measuring differences in glucose levels in a bacterial medium. Furthermore, the absorbance was initially a linear trend (as shown in part b of Figure 6) but eventually reached a threshold (as seen in Figure 6a). Therefore, quantitation of glucose levels using PAA-CONP could be possible in this small

range of glucose concentrations (0-0.3mM). To confirm if the absorbance of the PAA-CONP also increased as the concentration of glucose was increased in a bacterial medium, a complex bacterial medium (Muller-Hinton broth) was supplemented with a range of glucose concentrations, and these samples were incubated with glucose oxidase and a 1:21 final dilution of the PAA-CONP. The absorbance of the PAA-CONP was then measured.

a)



b)

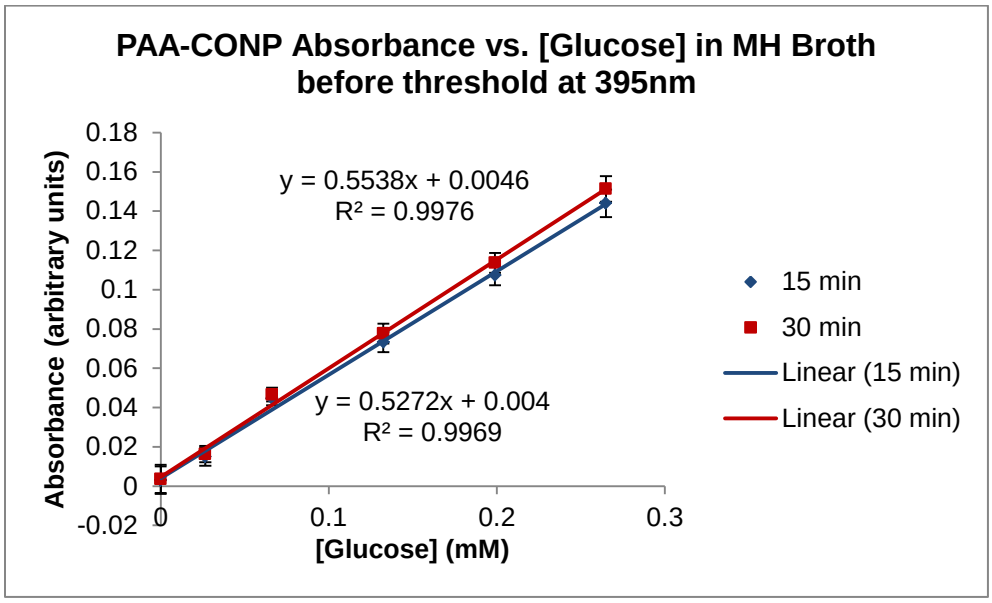


Figure 7: A_{395} of PAA-CONP in the Presence of MH Broth Supplemented with Glucose and Glucose Oxidase.

a) A_{395} for PAA-CONP in the presence of a range of glucose concentrations 15 minutes and 30 minutes after the addition of glucose oxidase.
b) A_{395} for PAA-CONP in the presence of a range of glucose concentrations 15 minutes and 30 minutes after the addition of glucose oxidase at glucose concentrations prior to the threshold seen in part (a). Note the linear trend in absorbance as the [glucose] increases.
The A_{395} for both part (a) and (b) was calculated by subtracting the absorbance of the sample of interest from both the absorbance of the sample of interest before the addition of glucose oxidase and the absorbance of a blank sample containing PAA-CONP and MH Broth supplemented with 2.65mM glucose but no glucose oxidase. The values shown are the average values from three independent experiments, and error bars represent the standard deviation for each value. A similar trend in absorbance values (although the actual absorbance values were lower) were obtained when the absorbance of the PAA-CONP was measured at 400nm and 410 nm (not shown).

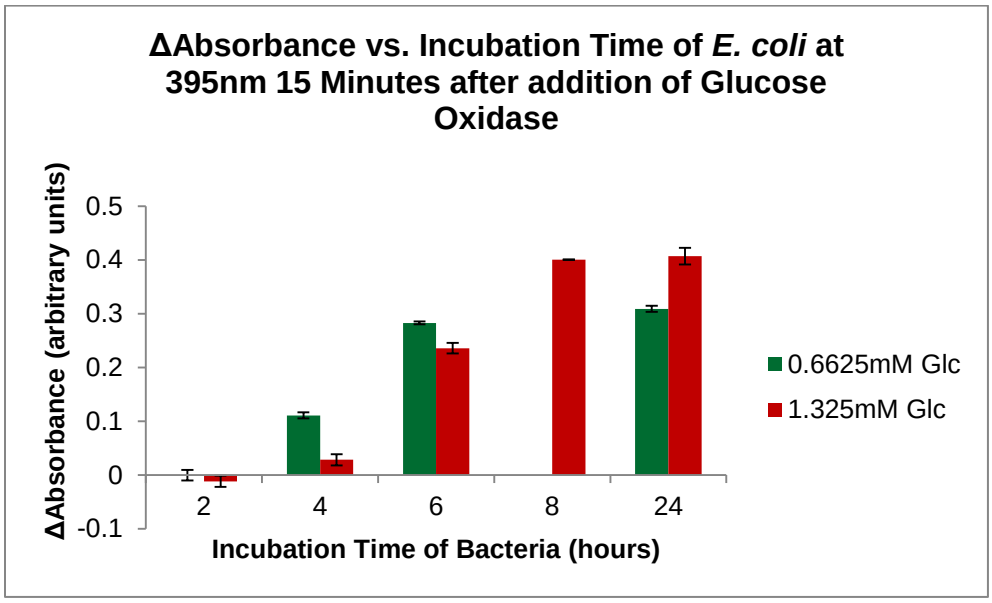
In the presence of PAA-CONP, a measurable A_{395} was seen, even as early as 15 minutes after the addition of glucose oxidase. As expected, the A_{395} increased as the [glucose] increased in the MH Broth. Furthermore, a point of saturation was reached, similarly to the data obtained when PAA-CONP was oxidized by a range of H_2O_2 concentrations (Figure 5). Based on the

obtained data, the glucose concentrations 0.6625mM and 1.325mM glucose were found to give absorbance readings that were greater than 0.2, which indicated that these glucose concentrations may produce significant differences in the A_{395} between bacterial samples in the presence of inhibitory versus non-inhibitory concentrations of an antimicrobial. However, the absorbance values for these glucose concentrations values did not fall on the linear portion seen in Figures 6 and 7, so these glucose concentrations would most likely not produce the greatest change in absorbance between bacterial samples grown in the presence of inhibitory versus non-inhibitory concentrations of an antimicrobial. These glucose concentrations were selected and used in future studies because these glucose concentrations produced PAA-CONP absorbance values greater than 0.2 and were expected to still produce an absorbance difference, even though this difference may not occur as readily as a concentration of glucose that fell on the linear portion of the graph in Figures 6 and 7.

Based on the current results of this study, the A_{395} was expected to decrease as the concentration of glucose also decreased. Therefore, an anticipated result would be that the A_{395} of PAA-CONP incubated with glucose oxidase and a bacterial sample containing an inhibitory concentration of the antimicrobial agent should be higher than the A_{395} of PAA-CONP incubated with glucose oxidase and a bacterial sample containing a non-inhibitory concentration of the antimicrobial agent since the bacterial sample with the non-inhibitory concentration of antimicrobial agent should have less glucose present in the medium, so less H_2O_2 would be produced by this sample relative to the bacterial sample containing an inhibitory concentration of the antimicrobial agent to oxidize the PAA-CONP. To test this hypothesis, *E. coli* strain MM 294 was grown in nutrient broth (NB) supplemented with 0.6625mM or 1.325mM glucose in the

presence or absence of an inhibitory concentration of the antibiotic ampicillin. Comparison of the A_{395} of MH Broth samples containing ampicillin showed no significant difference to samples lacking ampicillin (data not shown), so ampicillin would not interfere with the PAA-CONP absorbance measurements at 395nm. The *E. coli* samples were incubated for 2, 4, 6, 8, and 24 hours in the presence or absence of ampicillin (50µg/ml). A second set of 24 hour *E. coli* samples that contained or lacked ampicillin was also prepared to determine the turbidity of each tube to confirm the validity of this experiment. 50µg/ml of ampicillin was determined to be inhibitory to this *E. coli* strain based on the lack of turbidity at 24 hours, whereas the sample lacking ampicillin showed turbidity compared to a sterile NB sample with 50µg/ml ampicillin (data not shown). The presence/absence of bacterial growth in these 24 hour samples was confirmed by OD_{600} measurements (data not shown). The other bacterial samples were then autoclaved after the time of interest, centrifuged to remove the bacteria from the sample, and incubated with glucose oxidase and a final dilution of 1:21 of PAA-CONP, and the results were shown in Figure 8.

a)



b)

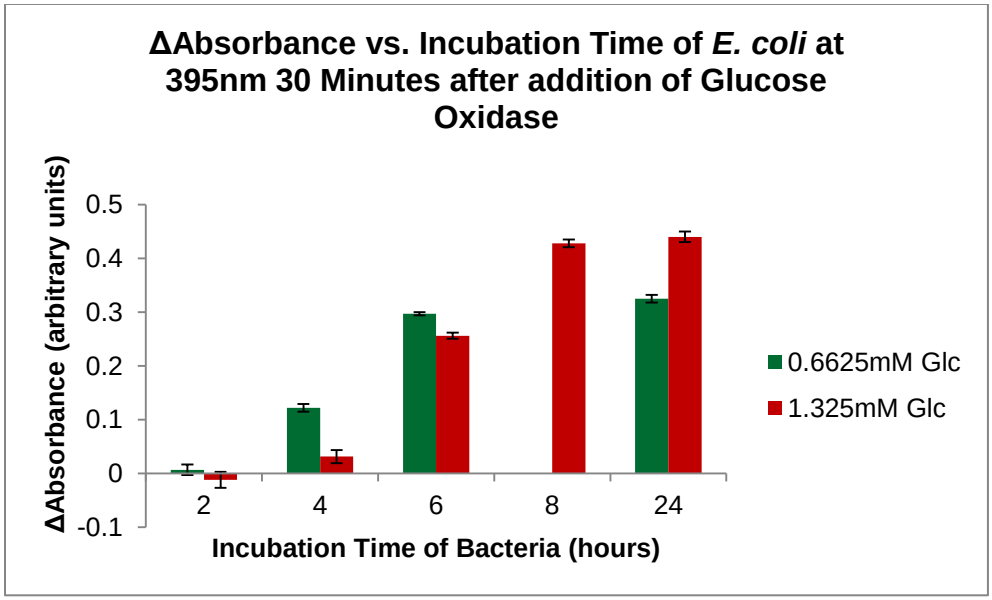


Figure 8: Comparison of the ΔA_{395} Values of *E. coli* in the Presence and Absence of Inhibitory Concentrations of Ampicillin.

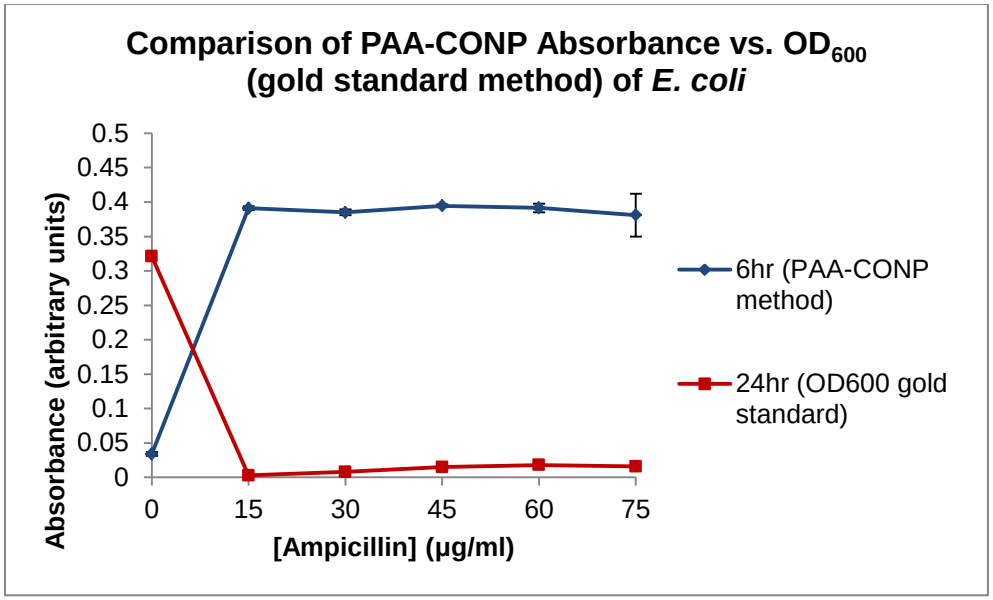
a) ΔA_{395} 15 minutes after the addition of glucose oxidase.
b) ΔA_{395} 30 minutes after the addition of glucose oxidase. The absorbance values at 395nm were measured and calculated by subtracting the absorbance of each sample from both the absorbance

of the sample before the addition of glucose oxidase and a blank sample containing the same ampicillin-treated sample in the absence of glucose oxidase. The ΔA_{395} was then calculated by subtracting the absorbance of the sample without antibiotic from the sample containing antibiotic. Note that no ΔA_{395} sample was prepared for the 8 hour time point for *E. coli* in the presence of 0.6625mM glucose. The ΔA_{395} values shown are the average of the ΔA_{395} values and error bars represent the standard deviation of two independent experiments of the same bacterial culture medium.

The results shown in Figure 8 showed that the difference in absorbance between the *E. coli* sample treated with 50µg/ml ampicillin (an inhibitory concentration of ampicillin on *E. coli*) and the *E. coli* sample treated with no ampicillin was indeed detectable. The A_{395} of the *E. coli* sample treated with 50µg/ml ampicillin was similar to the A_{395} of a sterile medium (nutrient broth supplemented with 10mg/ml glucose and 50 µg/ml ampicillin, data not shown), indicating that essentially no glucose metabolism was occurring in the ampicillin-treated *E. coli* sample, which was indicative of inhibition of growth by the ampicillin. Detectable ΔA_{395} occurred earlier for the 0.6625mM glucose samples (4 hours) compared to the samples containing 1.325mM glucose (6 hours). Although the samples grown in 0.6625mM glucose could produce a useful ΔA_{395}, the six hour sample in the presence of 0.6625mM glucose and no ampicillin did not show a detectable absorbance after the addition of glucose oxidase (data not shown), indicating that the six hour time point could differentiate between inhibitory and non-inhibitory concentrations of antibiotic based on if there is or is not a detectable A_{395}. Therefore, future bacterial samples would be incubated for both four hours and six hours in the presence of 0.6625mM glucose. It should be noted that turbidity was seen as early as four hours in the bacterial samples with no ampicillin present, and a bacterial pellet could also be detected upon centrifugation of the four hour sample.

To compare the results of the current study to the results for one of the gold standard methods of determining antimicrobial resistance, the Minimum Inhibitory Concentration (M.I.C.) method, samples of both *E. coli* and *S. aureus* were prepared similarly to the protocol for the M.I.C. method. The bacterial samples were incubated in Nutrient Broth supplemented with 0.25mg/ml glucose and also supplemented with a range of ampicillin concentrations (as described in Materials and Methods). Samples were then incubated for either four hours and six hours, and the results of the absorbance of the PAA-CONP incubated with the four hour and six hour samples was compared to the turbidity and OD_{600} of the samples incubated at 24 hours (the standard incubation time and method of assaying antimicrobial resistance for the M.I.C. method).

a)



b)

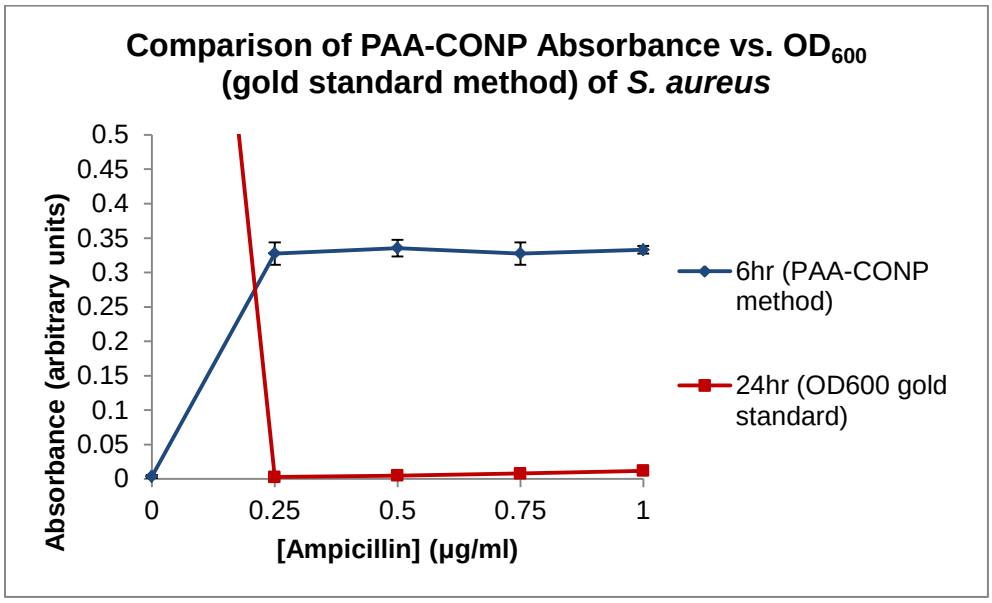


Figure 9: Comparison of PAA-CONP Absorbance with *E. coli* and *S. aureus* samples incubated with a range of ampicillin concentrations for 6hr versus OD_{600} of 24hr M.I.C. Samples

a) Absorbance of PAA-CONP at 395nm with *E. coli* samples incubated for 6 hours compared to the OD_{600} of the *E. coli* samples after 24 hours of incubation.
b) Absorbance of PAA-CONP at 395nm with *S. aureus* samples incubated 6 hours compared to the OD_{600} of the *S. aureus* samples after 24 hours of incubation.
Note that the 6hr absorbance measurements were measured 15 minutes after glucose oxidase was added to each sample, and the absorbance measurements were recorded at 395nm. However, the absorbance measurements at 24 hours were used to assay cell growth and thus measured at 600nm. Note that the absorbance measurements for the 6hr samples were calculated by subtracting the absorbance of the sample of interest by the absorbance of the same sample before the addition of glucose oxidase and the absorbance of the blank (sterile control with no glucose oxidase), and the OD_{600} values were measured at 24 hours using the sterile control as the blank. The OD_{600} of the *S. aureus* sample with 0µg/ml at 24 hours was in part (b) was 1.704 (too high to be shown on the graph). 60µg/ml of ampicillin was also inhibitory towards *S. aureus* growth, and the PAA-CONP and OD_{600} measurements for *S. aureus* at 60µg/ml were similar to other inhibitory concentrations of ampicillin (data not shown). The results shown are the average absorbance measurements of the PAA-CONP, and error bars represent the standard deviation of the PAA-CONP absorbance values for two independent experiments with the same bacterial culture medium.

As shown in Figure 9, the absorbance of the PAA-CONP increased as the OD_{600} decreased. In other words, a higher absorbance of the PAA-CONP occurred when the concentration of ampicillin inhibited the growth of the bacteria. This trend was seen for both *E. coli* and *S. aureus*, indicating that this trend was not specific for a particular bacterial strain. The inhibition of bacterial growth was seen as both a lack of turbidity in the bacterial samples (data not shown) and an OD_{600} measurement that was about zero when compared to the sterile control. Note that turbidity was only seen in samples with a measurable OD_{600} (data not shown). Furthermore, the absorbance of the PAA-CONP for the concentrations of ampicillin that inhibited the growth of the bacteria was similar to the absorbance of the sterile control (data not shown).

However, unlike the data seen in Figure 8, the difference in absorbance measurements between inhibitory and non-inhibitory concentrations of ampicillin was only slight after four hours of incubation (data not shown). Furthermore, the absorbance measurements of the inhibitory concentrations of ampicillin after 6 hours of incubation was not exactly zero for *E. coli*. The reason for this variation may have been due to differential growth between bacterial samples or due to differences in the glucose concentration due to errors in the measurement of the mass of glucose used during the preparation of the stock glucose solution. Nonetheless, this data indicated that the results after four hours of incubation were not as reliable as previously thought. Therefore, six hours of bacterial incubation before testing with the PAA-CONP was determined to be the ideal incubation time to allow sensitive and reliable A_{395} measurements. This data also confirmed that the PAA-CONP absorbance measurements produced similar results in terms of differentiating between inhibitory and non-inhibitory concentrations of ampicillin

when compared to turbidity determination and OD_{600} measurements of the M.I.C. gold standard method of antimicrobial resistance detection. Therefore, this data indicated that using PAA-CONP to detect glucose metabolism was a sensitive and reliable method of detecting inhibitory and non-inhibitory concentrations of ampicillin and also produced results in one quarter of the amount of time required for the M.I.C. gold standard method of antimicrobial resistance detection.

DISCUSSION

The rise of antimicrobial resistance demands quicker, more sensitive methods of detection. A novel detection method will help to improve the lives of people with bacterial infections by ensuring the patient receives both the proper and prompter treatment. The method described herein involving the oxidation of cerium oxide nanoparticles may offer a much more rapid assay relative to current gold standard methods of antimicrobial resistance detection, which may aid in quicker treatments for patients. In this study, the use of cerium oxide nanoparticles to detect the presence of glucose concentrations was shown to be capable of detecting an inhibitory versus non-inhibitory concentration the antibiotic ampicillin on both *E. coli* and *S. aureus* in as little as six hours of incubation, compared to the 24 hours needed in the M.I.C. and disk diffusion assays (4). This method only requires a spectrophotometer rather than more expensive machinery and is also well-suited for high-throughput screening, which would allow the detection of the M.I.C. of multiple antimicrobials at once. This combination of a reduced incubation time and the ability of this method to be adapted for high-throughput screening will both save time for the clinical microbiologist and decrease the time a patient must wait for the most proper therapy. The total time of sample preparation, incubation (as little as six hours), and absorbance measurements (15-30 minutes after the addition of glucose oxidase) would be quick enough to receive results by the end of the day rather than waiting until the next day. This method may also be useful for testing the efficacy of multiple concentrations of multiple candidate antimicrobials in the inhibition of growth of bacteria in high-throughput screening methods. Furthermore, this assay does not require the use of extreme conditions such as acidic pH or the use of highly reactive chemical reagents.

Despite the utility of this cerium oxide nanoparticle based method of antimicrobial resistance detection, this method has some limitation that should be noted. First, this method would most likely only be suitable for microorganisms that use glucose as the primary carbon source, since other carbon sources will be present in the complex growth medium. Furthermore, like the turbidity method, this method is also dependent on the growth rate of the microbe of interest, so slower growing microbes may require a longer incubation time to detect differences in glucose levels. In addition, similarly to the M.I.C. and disk diffusion methods, this assay requires a pure culture of the pathogenic agent of interest and can only determine if the antimicrobial agent inhibits bacterial growth, but does not determine the mode of action of the antimicrobial agent. In other words, this method cannot detect if the antimicrobial agent is bactericidal (kills the microbe) or bacteriostatic (inhibits the growth of the microbe but does not kill the microbe). Furthermore, cerium oxide nanoparticles, due to the different preparations present and lack of a reliable method for quantitation of the amount of cerium oxide nanoparticles present, would also need to be tested and calibrated to standardize the comparison of sensitive versus resistant antimicrobial concentrations. For example, absorbance spectra, differences in the valence state of the preparation, absorbance changes, and the kinetics of oxidation may vary among different preparations, and all these differences would need to be accounted for in each nanoparticle preparation. In addition, a large amount of nanoparticles (only a 1:10 dilution of the stock preparation) was used, which may be an issue in terms of cost and availability of the nanoparticles. This method also requires at least 6 hours of incubation for sensitive and reliable absorbance measurements, whereas previously described methods can give results in as little as three hours (5, 6). Of note, turbidity was actually seen in the non-inhibitory

concentrations of ampicillin as early as 4 hours, so the early presence of turbidity actually appeared earlier than expected. Another limitation may be the cost and/or availability of the reagents to clinical laboratories. Although this method would be less expensive than previously described novel methods of antimicrobial resistance detection (5, 6), this method still requires the availability of cerium oxide nanoparticles and glucose oxidase to clinical laboratories. These reagents, while not exceedingly expensive, can still increase the cost of antimicrobial testing. However, the increased cost comes with a reduced time to detect antimicrobial resistance, so the cost-benefit ratio of this method may be determined based on saving money versus the necessity in receiving the antimicrobial resistance profile for patients.

Despite the limitations posed by this cerium oxide nanoparticle based mode of detection of antimicrobial resistance, the many benefits offered by this method should make this method highly considered for not only clinical use but also use for other methods such as drug screening. For example, this method may also be well-suited in assessing both drug toxicity and the efficacy of chemotherapeutic agents against cancer cells due to the high metabolic rate of cancer cells relative to noncancerous cells. Furthermore, data in Figures 6 and 7 show that cerium oxide nanoparticles may be useful for the quantification of glucose levels, which may have a profound impact on assessing cell metabolism (although the range of glucose concentrations that result in a linear trend was not very large according to Figures 6 and 7), or for the quantification of the concentration of cerium oxide present in the nanoparticle preparation. Most importantly, this method can reduce antimicrobial resistance testing to 1/6 of the current time needed. Therefore, this method can allow the rapid screening of antimicrobials to determine the most effective regimen for patients. This will not only allow the patients to receive proper and prompter

treatment but also avoid the potential of giving a patient a broad spectrum antibiotic that the infecting pathogen is unknowingly resistant to while antimicrobial resistance testing is being performed, which may help prevent unwanted side effects in the patient.

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