MECHANISMS OF ZINC OXIDE ANTIBACTERIAL ACTIVITY

IN STAPHYLOCOCCUS AUREUS

by

Iman Ali

Submitted in partial fulfillment of the

requirements for Departmental Honors in

the Department of Biology

Texas Christian University

Fort Worth, Texas

May 3, 2021

MECHANISMS OF ZINC OXIDE ANTIBACTERIAL ACTIVITY

IN STAPHYLOCOCCUS AUREUS

Project Approved:

Supervising Professor: Shauna McGillivray, Ph.D.

Department of Biology

Meredith Curtis, Ph.D.

Department of Biology

Yuri Strzhemechny, Ph.D.

Department of Physics & Astronomy

ABSTRACT

Antibiotic resistance has been increasing rapidly; however, the amount of new and effective antibiotics is declining. One area of growing interest is the use of metal oxide nanoparticles because they are relatively easy to make and can be synthesized into different shapes, sizes, and with various chemical properties. In particular, zinc oxide nanoparticles have shown to be effective against various bacterial strains; however, the mechanisms of zinc oxide antimicrobial activity is still unknown. It is also not clear what properties of zinc oxide, such as size or proximity to bacterial cells, are critical for its antimicrobial activity. In order to gain a better understanding of the mechanism behind zinc oxide's antimicrobial activity, we tested *Staphylococcus aureus* with various zinc oxide particles under different conditions. Specifically, we looked at whether particle size, contact with bacterial cells, and media type influenced antimicrobial activity. Our results suggest that particle size does not influence zinc oxide activity, but media type significantly impacts antimicrobial activity. Physical contact, although more effective, is not absolutely required to see inhibition of bacterial growth. Understanding the mechanisms that zinc oxide utilizes may guide design for future particles that will improve their effectiveness.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

INTRODUCTION

Antibiotics have played a crucial role in treating infectious diseases. Since their introduction, they have revolutionized healthcare. Traditional antibiotics elicit their effects by targeting specific structures on microbes or interfering in their microbial processes in order to limit or stop their growth. For instance, penicillin targets the enzyme in the bacterial cell that is responsible for forming the cell wall. Lack of a cell wall causes osmotic pressure to build up resulting in lysis of the bacterium[1]. Other antibiotics target protein production, DNA/RNA synthesis, or metabolic pathways. However, with all the benefits antibiotics provide, the ability to treat infectious diseases is becoming increasingly challenging with the rise in antibiotic resistance. Antibiotic resistance occurs when microbes such as bacteria gain the ability to overcome the effects of antibiotics that are made to kill them. For every antibiotic that is produced, bacterial resistance develops shortly after. These resistance genes can be passed on to future bacterial generations as the bacteria multiply therefore increasing the spread of antibiotic resistance[2]. Antibiotic overuse, misuse, and lack of drug development also contribute to the increase in resistance[3]. This public health crisis is a global issue as people around the world are dying from infections that have become untreatable. Since 2013, deaths by antibiotic resistance has decreased by 18 percent; however, there are more than 2.8 million cases of antibiotic resistant infections each year in the United States and more than 35,000 people die from these afflictions[4]. It is projected that by 2050, more people will die from antibiotic resistance related infections than from cancer[2]. It is imperative to address the issue of antibiotic resistance as we are reaching the point where we could lose the ability to treat infectious diseases.

Previously, most antibiotics were found in the natural environment; however, because of the antibiotic resistance crisis, scientists are broadening their scope and seeking novel ways to

inhibit bacterial growth[5]. One area of growing interest is the use of nanoparticles as potential antibiotics. Nanoparticles are generally less than 100 nm in size, relatively easy to make, and are unique in their chemical and physical features as they can be synthesized into different shapes, sizes, and with various chemical properties[6]. They are able to effectively elicit their antimicrobial activity at low concentrations because of their high surface area to volume ratio[6]. Research on the use of zinc oxide nanoparticles specifically has shown to be effective against various bacterial species such as *Escherichia coli* and *Staphylococcus aureus*[6]. The additional benefit of low toxicity to humans makes this antimicrobial agent an even more appealing potential antibiotic for combating the rise in antibiotic resistance[7]. Changing the properties of zinc oxide particles can alter its antimicrobial activities. The mechanism that zinc oxide utilizes to exhibit its antimicrobial activity is still unknown. Currently, there are several theories suggesting the way zinc oxide inhibits bacterial growth such as electrostatic interactions, reactive oxygen species formation, bacterial internalization of zinc oxide nanoparticles, and Zn^{2+} solubility in the media[7]. As of now, the most accepted theory is that smaller particle sizes are more effective at inhibiting bacterial growth because the bacterium internalizes the particles where they interfere with biochemical processes and cause cell death[6].

The bacterial strain we will use to investigate these possible mechanisms for this project is *Staphylococcus aureus*, a gram-positive, round-shaped, cluster-forming bacterium. It is part of the normal human microbiota, commonly found on the skin. The majority of *S. aureus* infections are superficial soft tissue infections but can become fatal if the bacterium invades into deeper tissues or enters the bloodstream, heart, or lungs[8]. Staph infections can be treated with antibiotics; however, strains such as methicillin-resistant *S. aureus* (MRSA) are very challenging to treat as they can become resistant to multiple antibiotics. *S. aureus* is one of the most common

bacterial pathogens and its notorious ability to develop antibiotic resistance makes it crucial to find new effective antibiotics against it[9].

In this study, we will address some of the proposed mechanisms of action in order to better understand the antibacterial activity of zinc oxide. Specifically, we will investigate the importance of media type, particle size, and physical contact for the antimicrobial properties of zinc oxide against *S. aureus*. It is important to gain a better understanding of zinc oxide's antimicrobial activity as changes to its properties can result in significantly different results. Understanding the mechanisms that zinc oxide utilizes may guide design for future particles that will improve their effectiveness.

<p style="text-align: center;">EXPERIMENTAL PROCEDURES</p>

Bacterial strains and media

S. aureus Newman, a methicillin-susceptible *S. aureus* strain, was used in all assays. Unless otherwise noted, bacteria were grown in Mueller Hinton Broth (MHB; Fisher Scientific) at all stages.

Zinc Oxide Synthesis

Zinc oxide was either obtained commercially (Sigma-Aldrich) or synthesized by John Reeks in Dr. Yuri Strzhemechny's lab at TCU. Using a teflon-lined stainless steel autoclave reactor, chemical precursors underwent heat treatments under constant temperatures and pressures for designated amounts of time. These reactions used hexamethylenetetramine (HMT) as a surfactant and hydroxide supplier with zinc suppliers being 99.999% pure Zn foil and zinc acetate dihydrate ($Zn(O_2CCH_3)_2(H_2O)_2$). Varying equimolar concentrations of HMT and zinc acetate dihydrate were stirred at medium speed for 30 minutes to 2 hours. Once the mixing was complete, the solution was transferred to a Teflon-lined stainless steel autoclave reaction chamber along with a sheet of cleaned Zn foil. The lid on the Teflon liner was immediately closed and placed inside the steel reactor (shown below).
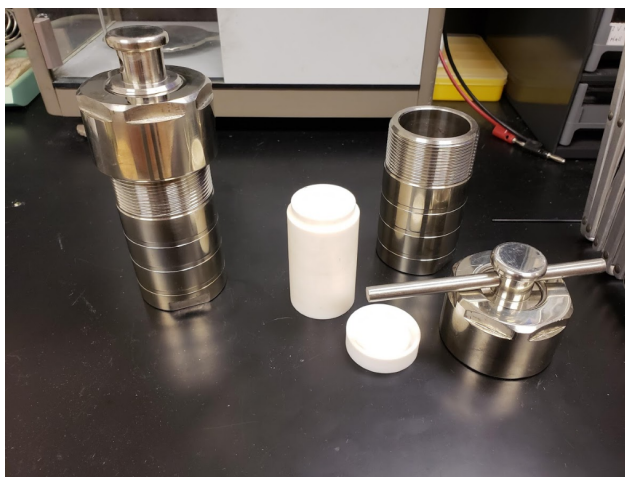
The reactor was then heated using a programmable oven (Across international STABLETEMP series forced air oven) was employed, shown below, to provide consistent heat treatment conditions. Samples were treated at temperatures ranging from 90 °C to 110 °C for times ranging from 1 to 20 hours (methods description courtesy of J. Reeks).

Minimum Inhibitory Concentration (MIC) Assays

Bacteria cultures were grown overnight in MHB and the next day were grown to early log phase with an optical density (OD) around 0.4 at wavelength 600 nm in MHB. This culture was diluted 1:100 in fresh MHB and 200 ul was transferred to 1.7 ml microcentrifuge tubes along with 200 ul of varying concentrations of zinc oxide suspended in MHB. This resulted in a final dilution of 1:200 of bacteria in a total volume of 400 ul with zinc oxide. Tubes without bacteria were also prepared in the exact same way to serve as the negative control. The tubes containing zinc oxide (with and without bacteria) were inverted in a nutator overnight (approximately 16 hrs) at 37 °C. After incubation, these overnight tubes were spun at 100 relative centrifugal force (rcf) for 30 seconds to pellet the zinc oxide particles before transferring 200 ul of the supernatant to a 96-well plate to read the OD. The absorbance readings from the

negative controls (zinc oxide, no bacteria) were subtracted from the experimental tubes (zinc oxide plus bacteria) in order to account for any background interference from any remaining zinc oxide particles that were not pelleted during centrifugation.

For assays under static conditions, the bacterial cultures were prepped the exact same way as described above except that the 1.7 ml microcentrifuge tubes containing zinc oxide (with and without bacteria) were placed in a microcentrifuge rack instead of a nutator and incubated under static conditions overnight at 37 ℃. After spinning tubes at 100 rcf for 30 seconds, 200 ul were transferred from the tubes to the 96-well plate to obtain absorbance readings.

For assays with RPMI + 5% LB, bacterial cultures were prepped the same way as described above; however, after *S. aureus* was grown to log phase, cultures were washed once in PBS before being diluted 1:100 in RPMI + 5% LB. The rest of the assay was completed in RPMI + 5% LB.

Calculating Percent Growth

Percent growth was calculated by setting the growth of 0 mg/ml of zinc oxide at 100% to normalize for differences in overall growth. The OD at each subsequent concentration of zinc oxide was divided by the OD at 0 mg/ml and then multiplied by 100 to obtain percent growth.

Conditioned Media Assay

Conditioned medium was prepared by making a stock of commercial zinc oxide in MHB at a particular concentration, and the zinc oxide particles were immersed in the MHB medium for the indicated amount of time in the figures. In order to keep the stock concentrations constant for each trial, the tube was mixed to ensure equal distribution of the zinc oxide particles and 1 ml of

the stock was removed, placed in 1.7 microcentrifuge tubes, and centrifuged at 16,000 rcf for 5

minutes to pellet the zinc oxide. 100 ul of the supernatant was then removed and this conditioned

medium was placed with 100 ul of the 1:100 diluted log-phase *S. aureus* culture (prepared as

described above) in a well of the 96-well plate. In another well, 100 ul of the 1:100 diluted log-

phase *S. aureus* was placed with 100 ul of non-conditioned MHB medium to serve as a control.

The designated zinc oxide concentrations refer to the stock concentration used to prepare the

conditioned medium although the zinc oxide was removed before adding it to the assay and

conditioned medium was diluted 1:2. For instance, a concentration of 5 mg/ml means the

conditioned medium was incubated with 5 mg/ml of zinc oxide before particles were removed,

and this conditioned medium was diluted 1:2 for incubation in the 96-well plate resulting in a

final concentration of 2.5 mg/ml of zinc oxide. The 96-well plate was placed in the incubator

overnight at 37 °C. An absorbance reading was taken directly from the 96-well plate the next

day.

Killing Curve

Bacterial cultures were grown to log-phase as described above. Cultures were washed

and diluted 1:100 in MHB or phosphate-buffered saline (PBS). Microfuge tubes were used to

hold 750 ul of the 1:100 bacterial dilution and 750 ul of the 5 mg/ml zinc oxide stock made from

either MHB or PBS for a total volume of 1.5 ml. In addition, there were two other tubes with 750

ul of the 1:100 bacterial dilution and 750 ul of media (MHB or PBS) alone. Culture tubes were

inverted in the incubator at 37 °C for the duration of assay. At 0 hr, 1 hr, 2 hr, and 4 hr time

points, tubes were centrifuged for 2 minutes at 100 rcf, to pellet the zinc oxide particles and 25 ul

of culture from each tube was removed from the top and plated using serial dilutions to enumerate surviving CFU/ml.

Scanning Electron Microscopy (SEM) Imaging

Specific concentrations from the MIC assays were combined for SEM imaging. Samples were resuspended in PBS once then fixed in 2.5% glutaraldehyde for one hour. Next, there were a series of alcohol washes to dehydrate the bacteria. Samples were incubated for 10 minutes at each of the following ethanol concentrations: 30%, 50%, 70%, 85%, 90%, and 100% twice. Hexamethyldisilzane was then added to the samples with the lid left open overnight for the liquid to evaporate. The resulting pellet was crushed, placed on a pedestal, and sputter coated with 8nm of gold to image.
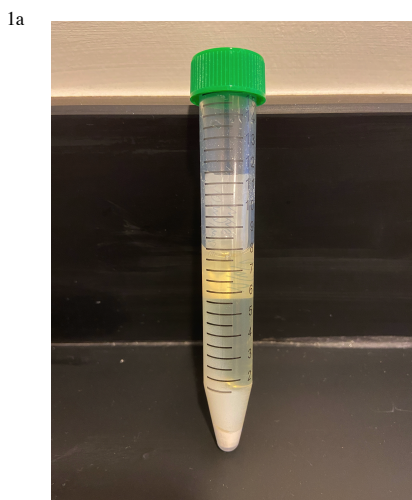
RESULTS

Figure 1a ZnO nanoparticles sitting in media for two weeks. These particles have settled out of the solution.
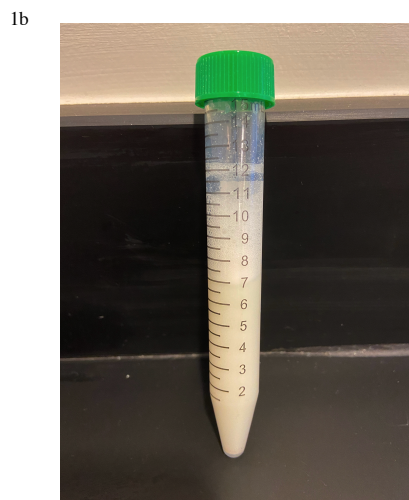

Figure 2a ZnO nanoparticles after shaking. These particles have now been suspended in the media.

Our first objective for this project was to determine the conditions for our assays. One challenge when working with zinc oxide particles is that they do not dissolve in the media and over time, due to their size, they will settle out of the media (Fig 1a). This is also a challenge because zinc oxide particles create background issues. As the concentration of zinc oxide increases, there are more particles and therefore there is a higher chance of interference. For instance, in one assay with commercial Sigma-Aldrich zinc oxide particles, the OD at 5 mg/ml without bacteria was 1.437; however, at 0.625 mg/ml, the OD was 0.195. In order to alleviate these discrepancies, after incubation with bacteria we briefly centrifuged the tubes with minimal force in order to pull down the zinc oxide particles into a pellet without also pelleting the bacteria. We then checked to make sure that this centrifugation step did not significantly decrease bacterial number in the supernatant (data not shown). Furthermore, for the larger zinc oxide particles that were synthesized by John Reeks, we used a sonicator in order to break the

zinc oxide particle agglomerations into finer particles using sound energy so that it would be better suspended in the media. This sonication process was done on the stock sample of the zinc oxide before assays were run. Additionally, before pipetting the desired volumes from the tubes with zinc oxide particles, the tubes were shaken to disperse the particles (Fig 1b).

We next had to determine whether the zinc oxide particles needed to be in constant contact with the bacterial cells for the duration of the assay. Because the zinc oxide particles naturally settle, static incubation would reduce the potential for physical contact between the bacteria and the particles, and from the literature, it was unclear whether constant physical contact was a necessity. To answer this question, we conducted side-by-side assays to determine the minimum inhibitory concentration (MIC), which is defined as the concentration at which growth of the bacteria is completely inhibited. In one assay, the zinc oxide particles and *S. aureus* in 1.7 ml microcentrifuge tubes were placed in a tube rotator called a nutator that inverts the tubes so that there was continuous contact between the particles and bacteria. In the other assay, the zinc oxide particles and *S. aureus* in 1.7 ml microcentrifuge tubes were placed in a microcentrifuge tube rack with no movement. In this condition, the zinc oxide particles settle out of the media overtime and there is less mixing and contact between the particles and bacteria.

When the tubes were inverted, *S. aureus* growth is clearly inhibited in a dose-dependent manner (Fig 2b). However, even in the static conditions, the zinc oxide still exhibited inhibitory effects (Fig 2a). Under static conditions, the MIC is around 1.25 mg/ml and it is similar for the rotating conditions. One thing to note is that the S. *aureus* growth in the static conditions is a lot lower than the rotating conditions. For instance, at 0 mg/ml the OD is around 0.1 for static conditions and 1.5 for rotating conditions. This means there are fewer bacteria in the MHB solution with the zinc oxide particles in static conditions. The higher OD seen at 2.5 mg/ml in

rotating conditions may be due to simply higher levels of bacterial growth. To normalize for differences in growth in the two conditions and make it easier to compare them, Figure 2c and 2d depict percentage growth which sets the amount of growth in 0 mg/ml of zinc oxide to 100%. However, even with normalizing the data, it is difficult to compare zinc oxide efficacy between the two conditions (static vs rotating) since the starting bacterial count is so low for the static conditions. Therefore, we concluded that rotating conditions were better than static conditions since the *S. aureus* exhibited better growth in the rotating conditions.

2a

S. aureus growth, static conditions
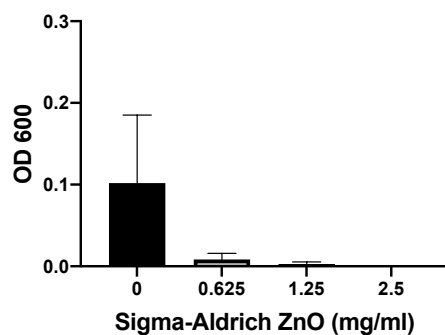
Figure 2a Growth of *S. aureus* at the indicated commercial-grade ZnO concentrations under static conditions. Zinc oxide stock freshly made about 24 hours before assay was run. Error bars represent mean +/- SD of three independent trials.
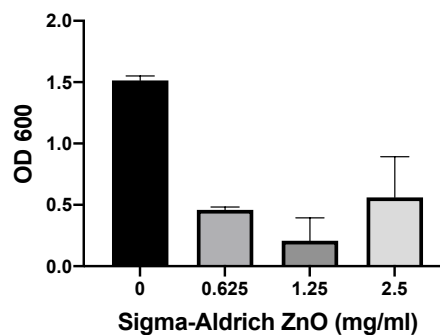
2b

S. aureus growth, rotating conditions

Figure 2b Growth of *S. aureus* at the indicated commercial-grade ZnO concentrations under rotating conditions. Zinc oxide stock freshly made about 24 hours before assay was run. Error bars represent mean +/- SD of three independent trials.
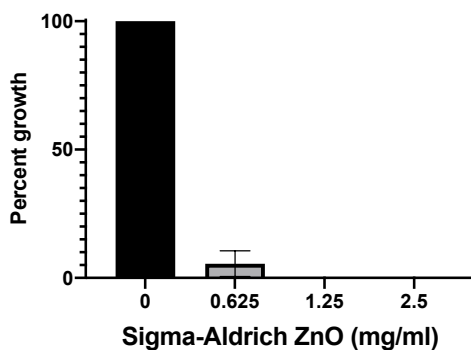
2c

S. aureus percent growth, static conditions

Figure 2c Percent growth of *S. aureus* at indicated commercial-grade ZnO concentrations under static conditions. Percent growth in 2a was calculated by setting growth in 0 mg/ml of ZnO at 100% to normalize for differences in overall growth.
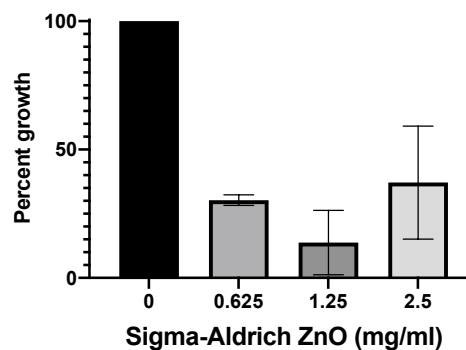
2d

S. aureus percent growth, rotating conditions

Figure 2d Percent growth of *S. aureus* at indicated commercial-grade ZnO concentrations under rotating conditions. Percent growth in 2b was calculated by setting growth in 0 mg/ml ZnO at 100% to normalize for differences in overall growth.

Next, we examined whether the efficacy of the zinc oxide particles is influenced by the time it is immersed in MHB media in order to determine how stable the stock solution remains over time. This determines if we need to make fresh zinc oxide stocks before running every assay or if we can use stocks that have been sitting out at room temperature over extended periods of time. The two zinc oxide stocks that were used in the assays we ran were made at least 6 months apart. The zinc oxide stock made 6 months prior to running the assay is denoted as old stock and the freshly made stock is denoted as new stock. The MIC for the old zinc oxide stock is around 2.5 mg/ml (Fig 3b) and the MIC for the new zinc oxide stock is 1.25 mg/ml (Fig 3a). Given that this is a very small difference in MIC, particularly because growth is nearly completely inhibited at 1.25 mg/ml in the old stock, we concluded that significant degradation of the zinc oxide particles is not occurring even after 6 months in the media. Therefore, we do not have to prepare a fresh stock of commercial zinc oxide before running every assay as it remains relatively stable over time (at least 6 months).
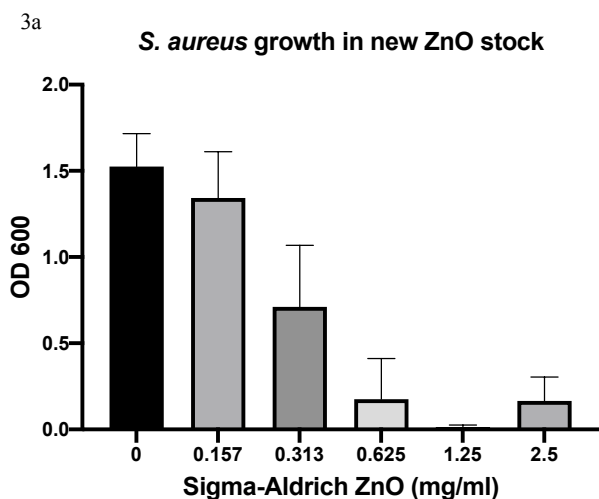
3a

S. aureus growth in new ZnO stock

3b

S. aureus growth in old ZnO stock

Figure 3a *S. aureus* **growth in freshly made commercial-grade ZnO stock at the indicated concentrations.** Zinc oxide stock made within 2 days of completing the assay. Error bars represent mean +/- SD of three independent trials.
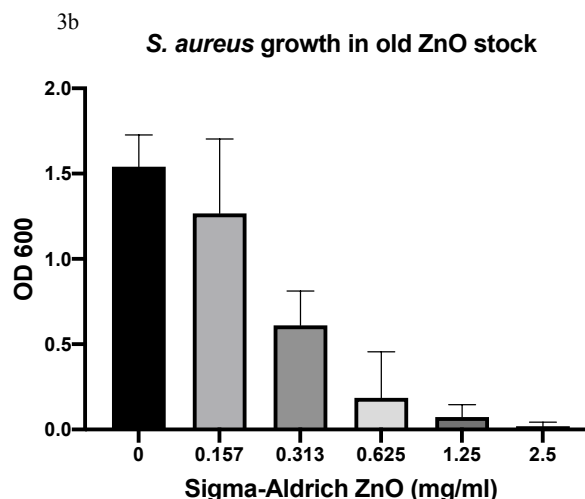
Figure 3b *S. aureus* **growth in old commercial-grade ZnO stock at the indicated concentrations.** Zinc oxide stock made at least 6 months prior to completing assay. Error bars represent mean +/- SD of three independent trials.

The next objective we wanted to test was how the size of the zinc oxide particles affects bacterial growth. Previous studies used particles smaller than the bacteria to inhibit growth. It is hypothesized that the bacteria ingest at least some of the particles, and internalization of the particles contributes to bacterial cell death. We wanted to know whether larger particles that cannot be ingested also have inhibitory effects. To answer this question, we ran assays with particles provided by John Reeks, a PhD student at TCU in Dr. Yuri Strzhemechny's lab from the Department of Physics and Astronomy. Fig 4a depicts the commercial-grade zinc oxide nanoparticles. As seen in the SEM image, these particles (blue arrow) are significantly smaller than the bacterial cell (yellow arrow) and they could be internalized and cause cell death. In the SEM images using the synthesized particles (blue arrows), it is clear they are significantly larger than the bacteria (yellow arrows) and therefore not possible for them to be ingested (Fig 4c, e, and g). Based on our results, we see very clear growth inhibition for all three of these synthesized samples. The image depicted in Fig 4d has an MIC around 0.625 mg/ml. This is a lower MIC than the one for the commercial-grade nanoparticles which is at 1.25 mg/ml (Fig 4b). Fig 4d shows that bacterial growth stabilizes around 0.313 mg/ml for the zinc oxide particle depicted in Fig 4e. For the zinc oxide particles in Fig 4g, the results indicate a more gradual decline than the other two synthesized particles as higher doses are required to see bacterial inhibition (Fig 4h). It is evident that these large particles are effective at inhibiting bacterial growth. This shows that the bacteria do not have to ingest zinc oxide particles for there to be inhibition as previous literature has suggested although physical contact between the particles and bacteria may still influence efficacy.
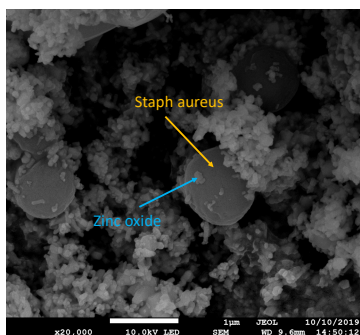
4a

Figure 4a Image of *S. aureus* bacteria with commerical-grade ZnO particles. Image taken with a scanning electron microscope. Scale bar is 9.6 mm.
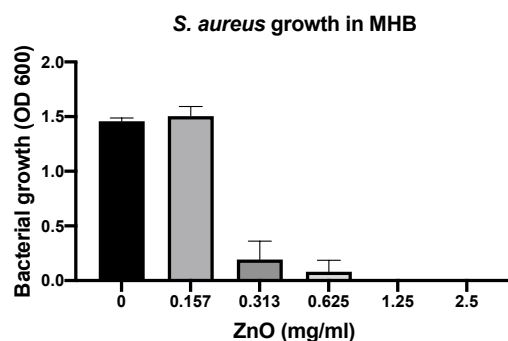
4b

Figure 4b *S. aureus* growth with commerical-grade ZnO particles. Error bars represent mean +/- SD of three individual trials. Results refer to Figure 4a.
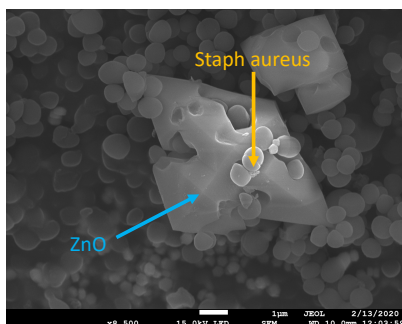
4c

Figure 4c Image of *S. aureus* bacteria with synthesized ZnO particles. Image taken with a scanning electron microscope. Scale bar is 1 mm.
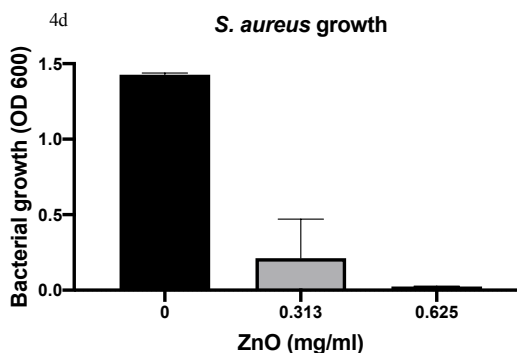
4d

Figure 4d *S. aureus* growth with synthesized ZnO particles. Error bars represent mean +/- SD of three individual trials. Results refer to Figure 4c.
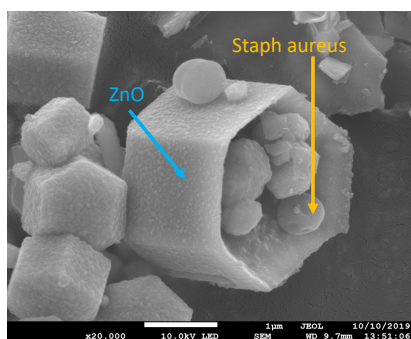
4e

Figure 4e Image of *S. aureus* bacteria with synthesized ZnO particles. Image taken with a scanning electron microscope. Scale bar is 9.7 mm.

4f

Figure 4f *S. aureus* growth with synthesized ZnO particles. Error bars represent mean +/- SD of three individual trials. Results refer to Figure 4e.

4g

Figure 4g Image of *S. aureus* bacteria with synthesized ZnO particles. Image taken with a scanning electron microscope. Scale bar is 10 mm.
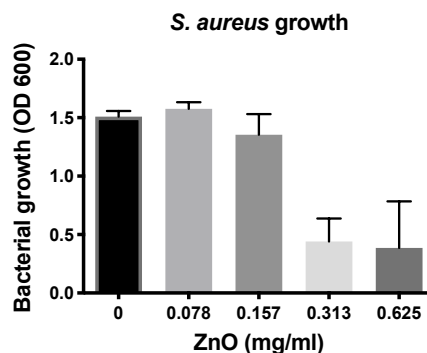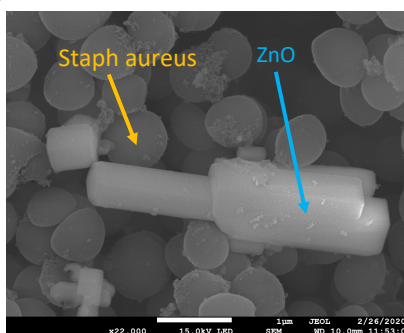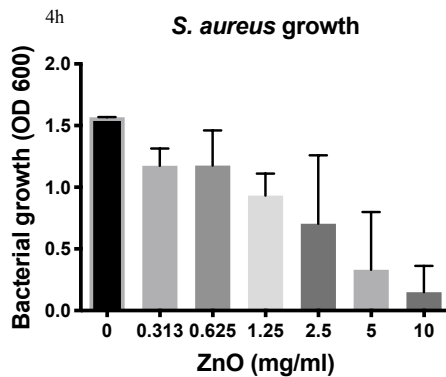
4h

Figure 4h *S. aureus* growth with synthesized ZnO particles. Error bars represent mean +/- SD of three individual trials. Results refer to Figure 4g.

Next, we wanted to determine whether media affected zinc oxide efficacy. The first media type that we tested was RPMI + 5% LB, which is composed of elements closely related to what is found in mammalian cells. We conducted side-by-side MIC assays, one with MHB, a more traditional bacterial culture media, and one with RPMI + 5% LB. In MHB, we see an MIC around 1.25 mg/ml which is what we expected to see with the commercial-grade particles (Fig 5a). In RPMI + 5% LB, however, we see a more gradual decline, and bacterial growth stabilizes around 1.25 mg/ml, but *S. aureus* growth is never completely inhibited in RPMI + 5% LB even at 2.5 mg/ml. One thing to note is that *S. aureus* does not grow as well in RPMI + 5% LB (compare the OD for both conditions at 0 mg/ml of zinc oxide). To normalize for differences in growth in the two conditions and make it easier to compare them, percentage growth, which sets the amount of growth in 0 mg/ml of zinc oxide to 100%, is indicated above the particular zinc oxide concentrations. This makes it easier to assess the results as we see about the same percent in growth around 18% at 0.313 mg/ml in MHB and 1.25 mg/ml in RPMI + 5% LB (Fig 5a and 5b).
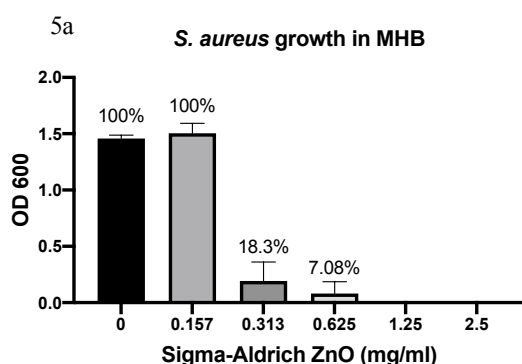

Figure 5a *S. aureus* growth in MHB at indicated concentrations. Zinc oxide stock made from commercial-grade Sigma-Aldrich particles. Error bars represent mean +/- SD of three independent trials. Percentages represent percent growth of *S. aureus* for indicated zinc oxide concentration.
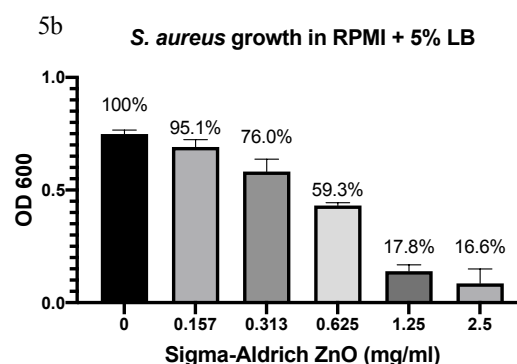
Figure 5b *S. aureus* growth in RPMI + 5% LB at indicated concentrations. Zinc oxide stock made from commercial-grade Sigma-Aldrich particles. Error bars represent mean +/- SD of three independent trials. Percentages represent percent growth of *S. aureus* for indicated zinc oxide concentration.

These results indicated to us that zinc oxide does in fact have different activity in different media. We then decided to test zinc oxide's activity in phosphate-buffered saline (PBS), a media type that is essentially a water-based salt solution. MIC assays cannot be done in PBS because there are no nutrients to support *S. aureus* growth. Therefore, to assess zinc oxide activity in PBS, a survival assay is conducted where *S. aureus* survival under the different conditions is determined by enumerating numbers of surviving bacteria over time. This assay was done side-by-side with MHB to compare bacterial survival between MHB and PBS. In MHB alone, bacterial growth steadily increases over time (Fig 6a). However, in the presence of zinc oxide in MHB, bacterial numbers remain stable over time (Fig 6a) indicating that zinc oxide inhibits growth but does not impact overall survival. This differs from PBS as *S. aureus* survival decreases in both conditions, in PBS alone and when zinc oxide is present; however, in the presence of zinc oxide, bacterial survival declines faster and nearly all the bacteria are dead by 4 hours in comparison to 8 hours without zinc oxide (Fig 6b).

6a

Figure 6a *S. aureus* survival over time in MHB. Bacterial colonies were counted from the indicated time points. Error bars represent mean +/- SD of three individual trials.
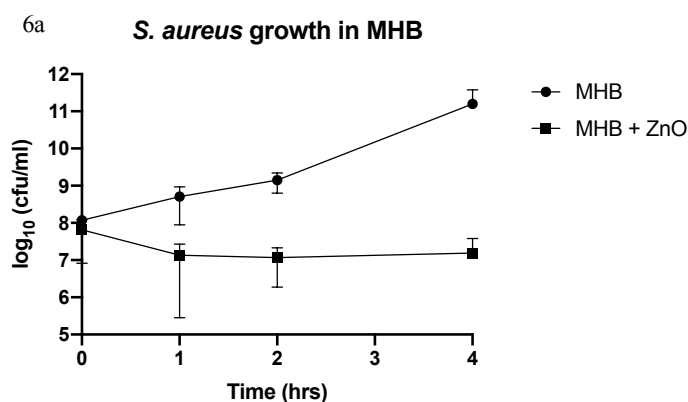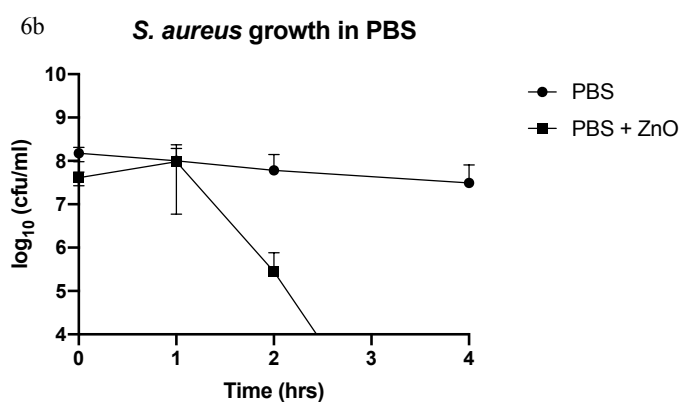
6b

Figure 6b *S. aureus* survival over time in PBS. Bacterial colonies were counted from the indicated time points. Error bars represent mean +/- SD of three individual trials.

The last objective was to determine whether the zinc oxide particles were releasing something soluble into the media that could account for its antimicrobial activity. Because we did not know how long it would take for whatever is being released into the media to accumulate, we tested the conditioned media after one hour, 24 hours (1 day), and two weeks of the zinc oxide particles sitting in MHB. As seen in Fig 7a, after just one hour of zinc oxide particles sitting in MHB, there is clear bacterial inhibition. Similar amounts of inhibition are also seen for 24 hours (Fig 7b). However, when zinc oxide particles are sitting in MHB for two weeks, there are higher levels of bacterial growth inhibition (Fig 7c). Our results suggest that there is something soluble being released in the media that is contributing to bacterial inhibition and physical contact between the bacterial cells and zinc oxide particles is not necessary to see some inhibition. These results also suggest that the amount of time the particles sit in the media may contribute to the level of bacterial growth inhibition.
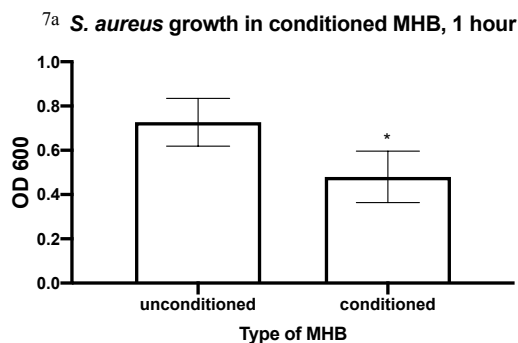

Figure 7a *S. aureus* **growth in MHB conditioned media only (without ZnO particles) from 1 hour.** Freshly made 20 mg/ml zinc oxide was left in MHB for one hour before removing particles and looking at *S. aureus* growth in conditioned media. Error bars represent mean +/- SD of six individual trials.
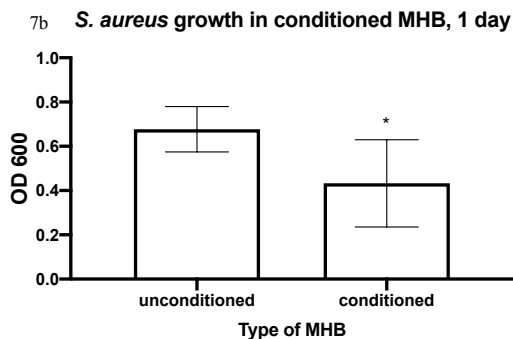
Figure 7b *S. aureus* **growth in MHB conditioned media only (without ZnO particles) from 1 day.** Freshly made 20 mg/ml zinc oxide was left in MHB for one day before removing particles and looking at *S. aureus* growth in conditioned media. Error bars represent mean +/- SD of five individual trials.

Figure 7c *S. aureus* **growth in MHB conditioned media only (without ZnO particles) from 2 weeks.** Freshly made 20 mg/ml zinc oxide was left in MHB for two weeks before removing particles and looking at *S. aureus* growth in conditioned media. Error bars represent mean +/- SD of five individual trials.
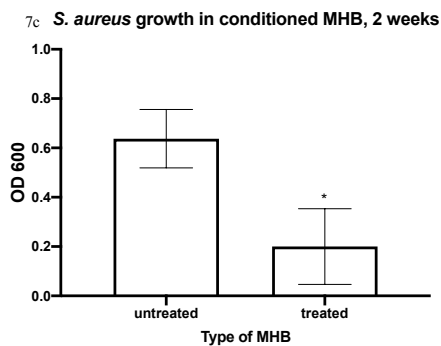
Lastly, we tested the conditioned media of different zinc oxide concentrations to see if that also made a difference in zinc oxide's antimicrobial activity. At every concentration, the bacterial growth is at an OD of around 0.4 (Fig 8a-e) in comparison of an OD of around 0.6 for *S. aureus* grown with unconditioned media. These results suggest while there is something soluble being released from the zinc oxide particles that is inhibiting bacterial growth, this inhibition is not as great as what is seen when zinc oxide particles are present.
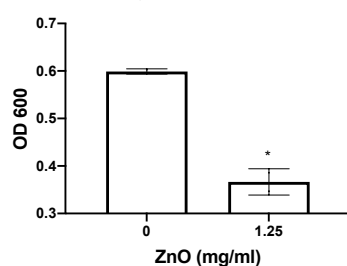
8a **S. aureus growth in conditioned MHB**

Figure 8a *S. aureus* **growth in MHB conditioned media only (without ZnO particles) at the 1.25 mg/ml of zinc oxide.** Media was conditioned with commercial zinc oxide particles for at least one hour before added to *S. aureus* to assess growth. Error bars represent mean +/- SD of two independent trials.

8b **S. aureus growth in conditioned MHB**

Figure 8a *S. aureus* **growth in MHB conditioned media only (without ZnO particles) at the 2.5 mg/ml of zinc oxide.** Media was conditioned with commercial zinc oxide particles for at least one hour before added to *S. aureus* to assess growth. Error bars represent mean +/- SD of two independent trials.

8c **S. aureus growth in conditioned MHB**
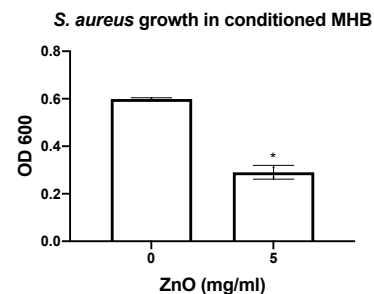
Figure 8a *S. aureus* **growth in MHB conditioned media only (without ZnO particles) at the 5 mg/ml of zinc oxide.** Media was conditioned with commercial zinc oxide particles for at least one hour before added to *S. aureus* to assess growth. Error bars represent mean +/- SD of two independent trials.

8d **S. aureus growth in conditioned MHB**
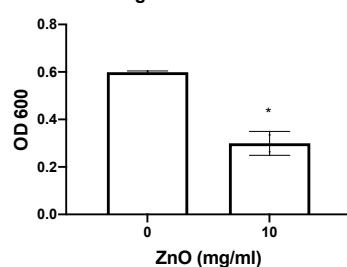
Figure 8a *S. aureus* **growth in MHB conditioned media only (without ZnO particles) at the 10 mg/ml of zinc oxide.** Media was conditioned with commercial zinc oxide particles for at least one hour before added to *S. aureus* to assess growth. Error bars represent mean +/- SD of two independent trials.
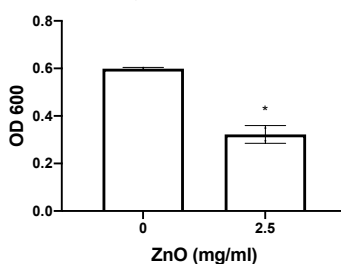
8e **S. aureus growth in conditioned MHB**

Figure 8a *S. aureus* **growth in MHB conditioned media only (without ZnO particles) at the 20 mg/ml of zinc oxide.** Media was conditioned with commercial zinc oxide particles for at least one hour before added to *S. aureus* to assess growth. Error bars represent mean +/- SD of two independent trials.

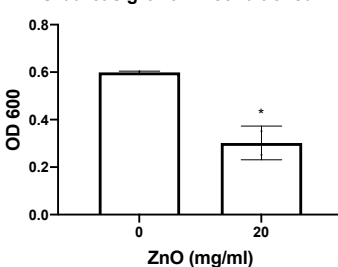DISCUSSION

Our goal was to better understand the mechanisms behind zinc oxide's antimicrobial activity by testing the importance of particle size, the influence of different media types, and the necessity of physical contact. One theory of zinc oxide's antibacterial action is that the small zinc oxide particles are internalized by the bacteria, interfere with the bacterial processes, and thus cause cell death[1]. Since we saw bacterial inhibition with the large particles, we concluded that internalization of the particles by the bacteria is not necessary for there to be inhibition as this current theory suggests. This indicates that there could be something else that is contributing to cell death. However, there were some caveats with the synthesized large particles. While running assays with the large particles, we found that over time, some of the samples would lose some of their efficacy therefore producing varied results. This problem made it difficult to accurately deduce an MIC. This issue could be due to some impurities that remained during the process of synthesizing the particles. It could also be due to the fact that the particles start to degrade when left out in light as it could interfere with the surface chemistry of the particles. Since it is theorized that changing physical properties of the zinc oxide could change the efficacy of the particles, it is imperative to have a homogenous sample[6,7]. A better understanding of the zinc oxide particles and how surface chemistry changes with different factors is necessary in order to synthesize consistently efficient particles. Additionally, there is a new hypothesis that the zinc ions (Zn^{2+}) released from the zinc oxide particles are interfering with the phosphate ions in the media thus forming a new particle type[10]. An example of this is seen in Fig 4c. It is unclear whether the particle in the center is zinc oxide or zinc phosphate. Further research is necessary on this newfound theory in order to determine the effects of phosphate on zinc oxide's activity. Perhaps using different media types with and without phosphate could better enhance our

understanding. A planned future experiment in our lab is to test this by monitoring *S. aureus* growth with the zinc oxide particles in saline solutions with and without phosphate.

The next objective we tested was how media type influences zinc oxide's antimicrobial action. Based on our results with MHB, RPMI + 5% LB, and PBS, it is clear that changing the media type impacts the efficacy of zinc oxide. Since MHB is a traditional bacterial culture media and the standard media used for antibiotic testing, it was used as the baseline with which to compare the other media types to. RPMI + 5% LB is a media type that is most similar to the environment of mammalian cells. Higher doses of zinc oxide are required to see significant bacterial inhibition than when the particles are immersed in MHB. Based on these results, we concluded that zinc oxide might be best used as a topical agent. However, more research is needed before we can make a definitive statement on how zinc oxide can be used as an antibiotic. Additionally, once we better understand the mechanisms behind zinc oxide's antimicrobial activity, we could possibly engineer the particles in a way where it could have a lower MIC in RPMI + 5% LB thus suppressing bacterial growth at much lower concentrations than what is seen in Fig 5b. When monitoring *S. aureus* growth over time in MHB and PBS, we also see how the change in media affects zinc oxide activity. In MHB, zinc oxide would be considered a bacteriostatic antibiotic as it stops bacterial growth but does not kill the cells. As can be seen in Figure 6a, the bacterial numbers hold steady in the presence of zinc oxide in MHB. In contrast, in PBS, bacterial numbers decline over time and this significantly increased in the presence of zinc oxide (Fig 6b). In these conditions, zinc oxide would be classified as bactericidal as *S. aureus* cells are dying. Further studies will be needed to determine exactly how media composition is playing a role in zinc oxide's effects.

The last objective we tested was whether the zinc oxide particles released something into the media that contributed to its antimicrobial activity. In order to test this, we centrifuged stock concentrations of zinc oxide so that the particles would be pelleted to the bottom. We then removed the supernatant and placed this with *S. aureus* cells. Based on our results, there is clear inhibition of S. aureus growth with conditioned media alone and we concluded that there is likely something in the media that is contributing to inhibition of bacterial growth. This indicates that physical contact is not necessary to see bacterial inhibition, although having the particles may increase the amount of inhibition. As seen in Fig 4b, when zinc oxide particles were present, there is absolutely no bacterial growth whereas there is still some growth with just the conditioned media (Fig 7,8). Whether this is due to physical contact with the particles or due to the fact that the particles are still present in the media continually producing a soluble antimicrobial product is not clear. One additional thing to note is that there is a greater amount of bacterial inhibition at 2 weeks conditioned media (Fig 7c) than compared to one hour and one day conditioned medias (Fig 7a and 7b). This suggest that the zinc oxide may be continuously releasing something soluble into the media over time. Further testing will need to be done at longer time points than 2 weeks to determine if even higher levels of inhibition are seen.

We then tested the conditioned media at different time points. The amount of inhibition remains relatively the same so release of the soluble inhibitory molecule occurs quickly. We then used different stock concentrations of zinc oxide to condition the media and the results showed that there was also very little difference in the amount of bacterial inhibition. This suggests that increasing zinc oxide concentration does not increase inhibitory activity, at least at the doses we used. One caveat to our approach is that when testing conditioned media, we just centrifuged the tubes to pellet the zinc oxide. We had no way to filter out all zinc oxide particles. Therefore,

there is no way to know for sure if any residual zinc oxide particles remained in the supernatant that we used to treat the *S. aureus* cells with. In order to circumvent this problem, we want to redesign this assay in such a way where we have a barrier that is permeable to the soluble substances but not permeable to the zinc oxide particles. On one side of this barrier there would be *S. aureus* cells so that whatever is being released from the zinc oxide particles can permeate the barrier and these soluble substances would be continuously released as the zinc oxide particle would remain on the other side of the barrier.

Our results show that some of the mechanisms ascribed to zinc oxide action, internalization of the particles and necessity of physical contact, may not be strictly required although both of these may contribute to overall efficacy. In all likelihood there are multiple mechanisms utilized by the zinc oxide in order to elicit its antimicrobial effects. The future directions for this research will continue to focus on trying to better understand how zinc oxide inhibits bacterial in order to design these particles to make it more effective. For example, it is hypothesized that reactive oxygen species (ROS) are being released from the zinc oxide. In the future, we would like to test this theory with ROS susceptible bacterial mutants to determine if they are affected by possible ROS released by the zinc oxide. Another hypothesis suggests that Zn^{2+} is released into the media thus contributing to bacterial inhibition. This can be tested by designing a media that has high amount of Zn^{2+} in order to compare how bacterial growth changes in media with these ions. Another factor to consider is how zinc oxide activity changes in the presence of different bacterial types. *S. aureus* is a gram-positive bacterium; however, in the future, we would like to test zinc oxide in the presence of gram-negative bacteria, such as *E. coli*, to see if this influences zinc oxide's activity. We also hope to continue to examine how changing physical properties of zinc oxide may influence its activity in the hope we can

maximize its potential as an antimicrobial agent. It is imperative to find new ways to inhibit bacterial growth as we are reaching a point where we are running out of antibiotics. Examining non-traditional antibiotics such as zinc oxide could open up new antibacterial agents for antibiotic applications.

<div align="center">REFERENCES</div>

1. Kohanski, Michael A et al. "How antibiotics kill bacteria: from targets to networks." *Nature reviews. Microbiology* vol. 8,6 (2010): 423-35. doi:10.1038/nrmicro2333

2. Tagliabue, Aldo, and Rino Rappuoli. "Changing Priorities in Vaccinology: Antibiotic Resistance Moving to the Top." *Frontiers in immunology* vol. 9 1068. 30 May. 2018, doi:10.3389/fimmu.2018.01068

3. Ventola, C Lee. "The antibiotic resistance crisis: part 1: causes and threats." *P & T : a peer-reviewed journal for formulary management* vol. 40,4 (2015): 277-83.

4. CDC. Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2019.

5. Aminov, Rustam I. "A brief history of the antibiotic era: lessons learned and challenges for the future." *Frontiers in microbiology* vol. 1 134. 8 Dec. 2010, doi:10.3389/fmicb.2010.00134

6. Siddiqi, K. S., Ur Rahman, A., Tajuddin, & Husen, A. (2018). Properties of Zinc Oxide Nanoparticles and Their Activity Against Microbes. *Nanoscale research letters*, *13*(1), 141. https://doi.org/10.1186/s11671-018-2532-3

7. Sirelkhatim, A. et al. Review on Zinc Oxide Nanoparticles: Antibacterial Activity and Toxicity Mechanism. *Nanomicro Lett* **7**, 219-242 (2015).

8. "Staph Infections - Symptoms and Causes." Mayo Clinic, 6 May 2020, www.mayoclinic.org/diseases-conditions/staph-infections/symptoms-causes/syc-20356221.

9. Chambers, Henry F, and Frank R Deleo. "Waves of resistance: Staphylococcus aureus in the antibiotic era." *Nature reviews. Microbiology* vol. 7,9 (2009): 629-41. doi:10.1038/nrmicro2200

10. Herrmann, Rudolf et al. "Rapid degradation of zinc oxide nanoparticles by phosphate ions." *Beilstein journal of nanotechnology* vol. 5 2007-15. 5 Nov. 2014, doi:10.3762/bjnano.5.209