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University of Michigan Engineering Honors Program Capstone Final Report

Antimicrobial Resistance

Antimicrobial resistance is an emerging problem in medicine, giving rise to "superbugs," or bacteria that are resistant to the majority of common antibiotics. In a report by the CDC examining the threat of antibiotic resistance, it was estimated that in the United States alone more than 2.8 million people get sick from bacteria that are resistant to antibiotics every year, and of those 35,000 die [1]. Globally, more than 700,000 deaths are attributed to antimicrobial resistant infections each year (likely undercounted), and it is projected that by 2050 that number will rise to 10 million deaths per year, more than currently die from cancer [2]. The burden of these infections is staggering, comparable to that of tuberculosis, influenza, and HIV/AIDS *combined,* with 39% of the burden being caused by bacteria that is resistant to last-line antibiotics, such as carbapenems and colistin [3]. This evidence points to the fact that microbial resistance has already reached alarming levels, but combined with the fact that the identification of resistant bacteria is outpacing the introduction of new antibiotics, it would not be an exaggeration to say that worldwide antibiotic resistance could quickly lead to a near-apocalyptic scenario in terms of human health and medicine [4].

The root cause of antibiotic resistance is in the variation in the genetics of bacteria. When antibiotics are used to eliminate infectious bacteria, it kills all the bacteria except those bacteria that, by natural or acquired genetic variation, are resistant to the drug. Although the infection is effectively treated by killing the non-resistant bacteria comprising the majority of the infectious population, those with resistance survive and reproduce, leading to a new generation of resistant bacteria. Furthermore, resistant bacteria are able to confer resistance to bacteria that are non-resistant through horizontal gene transfer, meaning that populations of bacteria don't even need to be exposed to antibiotics and have their non-resistant subset eliminated in order for their populations to become uniformly resistant. This greatly increases the rate at which microbial resistance spreads, allowing uniform resistance to be created among many species of bacteria. New infections caused by these now superbugs are not able to be treated with the previously used antibiotics. Additionally, the problem gets accelerated when antibiotics are overprescribed and overused, as the more bacteria that are exposed to antibiotics, the more quickly resistance can be spread among the population of that species until it becomes a mostly uniform trait.

Concerningly hospitals, by the fact that antibiotics must be used regularly in the environment, have become hotbeds for microbial resistance. Patients hospitalized for unrelated conditions risk acquiring a healthcare associated infection, which often are life-threatening. What's more, bacterial biofilms form on the surfaces of prosthetics and implants, which once inside the host the infection is nearly untreatable, with strong conferred resistance to antibiotics. Despite infection control protocols, 75% of the burden of resistant infections are healthcare associated [3]. Even in places where patients are most vulnerable and care is most available, we cannot contain the spread of these dangerous bacteria. The prophesized "antibiotic apocalypse" is much closer than it has ever appeared. Without new approaches to treating these rapidly emerging superbugs, we soon may have no defenses left to protect against them.

Antimicrobial Nanoparticles

The main goal of the research surrounding biological applications of nanoparticles is to design a biomimetic[1] nanosized inorganic particle, imitating naturally occurring phenomenon such as self-assembly[2] found in organic compounds such as proteins that could potentially help reveal unique applications in the fields of medicine, materials, and more [5]. The main properties of nanoparticles being examined in research are summarized below.

Nano-sized

Numerous organic molecules are nanosized, being 1-100 nm in their principal dimension, with many of the essential macromolecular "building blocks" of life being nanoscale. Organic components that are within this size range undergo Brownian motion[3] and are able to self-assemble into more complex structures. Being at this very small size, they are able to take advantage of the ambient thermal energy in their environment, meaning the energy required from the cell for these particles to form higher order hierarchical materials is heavily reduced as compared to if they were not able to self-assemble, allowing the cellular mechanisms for the formation of these materials to reach upwards of 95% efficiency [6]. Brownian motion is intrinsic to all nanosized molecules and particles, meaning that it is possible to replicate the self-assembly of organic components with artificially created nanoparticles, making the creation of these structures a relatively easy procedure. Since our project does not focus on creating nanoparticle assemblies and instead on the synthesis of single nanoparticles, we will not be investigating the property of self-assembly. However, it is important to understand this characteristic in the case that future work on this project or similar projects involves particle assemblies in order to a range of more complex medicines.

Inorganic

As discussed in the above section, artificially created inorganic nanoparticles will be subject to Brownian motion and undergo self-assembly, mimicking a similar process seen in the creation of proteins in cells [5]. The creation of biomimetic nanostructures has already been proven, and the

applications of such materials is abundant [7-12]. As one example, the replication of the structure of seashells into an inorganic form has led to the creation of a range of biomimetic composites with similar if not improved toughness [12]. Our project aims to create inorganic metal oxide nanoparticles that are able to interact with biological organisms while being compatible in a natural environment, specifically infectious bacteria inside of the human body. One of the research areas of the Kotov Lab is the modification of inorganic nanomaterials to be more biomimetic by introducing the property of chirality[4]. Chirality is discussed in the next section.

Chiral

The basis for research into introducing chirality into inorganic systems is that molecular interactions can change when the stereochemical symmetry is modified, leading different structures to have different effects, sometimes drastically different. In the case of chiral structures, even though the formula is the exact same, the chiral orientation of the structure can lead to different biological interactions. Previous research has revealed that this pattern continues in inorganic nanoparticles with chiral particles having different characteristics from their achiral counterparts [13-19]. There are two common methods to produce chiral nanoparticles. One involves hitting the nanoparticles with circularly polarized light so that momentum is transferred from the photons to the particles to induce chirality. The other method involves attaching a chiral ligand to the surface of a nanoparticle so that the ligand lends the property of chirality to the nanoparticle.

There are many examples of chirality in biology, such as amino acids, lipids, sugars, and hormones all having certain chiral orientations. In fact, there exists homochirality among some naturally produced molecules, with nearly all natural amino acids in the L configuration, and most natural sugars occurring in the D configuration [20]. With nature seeming to favor the production of chiral molecules, it is possible that drugs produced with certain chiral orientations will interact with the biological components of cells and bacteria in different and possibly more effective ways than if the drug were achiral.

For example, ibuprofen is formed as a racemic[5] mixture, meaning that S-ibuprofen and R-ibuprofen are created in equal amounts when synthesized. However, only S-ibuprofen is active as an anti-inflammatory whereas R-ibuprofen is not. This means that if someone was to take only the active enantiomer for pain, they would need to take twice the amount of the racemic mixture in order to achieve similar levels of drug effect [21]. The *American Chemical Society* provides us with an even more extreme case of differences between enantiomers: thalidomide. Thalidomide is a drug that was first introduced as a sedative and hypnotic, but later was marketed as an over the counter drug to treat morning sickness in pregnant women. It is created as a racemic mixture, with equal parts R-thalidomide and S-thalidomide. Unfortunately, only R-thalidomide has the desired sedative effects. S-thalidomide, tragically, is teratogenic. In the few years after

thalidomide was sold to pregnant women as a racemic mixture about ten thousand babies worldwide were born with birth defects, and only half of those would survive, many with other nonlethal defects and limb abnormalities. Even if the drug was dispensed only with the R enantiomer, in biological environments the isomers interconvert, meaning separating them is pointless [22]. This "thalidomide disaster" shed light on how drastically different the drug effects of the enantiomers of a single drug can be. Even though molecules of different chiral orientations may share the same atomic composition, they may interact with their environment in profoundly different ways.

Differently Shaped

In addition to the above properties, nanoparticle research also aims to create particles of different sizes and morphologies. A nanoparticle that can be used to demonstrate this idea is zinc oxide. The precipitation of zinc oxide particles induced by the addition of the weak base ammonium hydroxide yields spherical particles whereas the addition of strong base sodium hydroxide yields nanorod shaped particles [23]. The purpose of creating particles with different three dimensional geometries would be so that each category of particles interacts with and inhibits different cellular mechanisms of bacteria. One of the main targets of antibiotics is bacterial enzymes involved in the production of the cell wall and in the production of proteins. If the antibiotics are able to inhibit these enzymes and their production, then the bacteria would inevitably die. Previous research involving zinc oxide nanoparticles showed that this type of particle was able to block β-galactosidase in a manner that mimics natural methods of enzymatic inhibition, and that differently shaped particles had differing levels of effectiveness (with nanopyridimal being the optimal shape, comparing "favorably" to the best natural β-galactosidase inhibitors) [24]. Due to the fact that differently shaped nanoparticles interact with bacteria in unique ways, it is possible to produce a wide variety of new antibiotics that are effective against many different species of resistant bacteria with only minor changes to the synthesis procedures. Antimicrobial nanoparticles possessing these varying physical characteristics to differentially interact with bacteria have the potential to dramatically expand the current library of antimicrobial compounds.

Hypothesis and Project Goals

It is our hypothesis that if particles are modified to be chiral they will be more effective at killing the bacteria due to their increased biomimicry. The overall goal of our project is to examine bactericidal metal oxide nanoparticles and determine if the addition of chiral ligands affect their killing activities. If we find that these particles prove to be effective at killing bacteria, these particles could find application as a new generation of antibiotics with no built up resistance among the current infectious microbe population. More importantly, if we find that adding chiral ligands enhances the killing activity of the nanoparticles then we may have discovered a

potential way to increase inorganic nanoparticle interaction with biological systems through the introduction of the property of chirality.

Other goals we hope to achieve are listed below:

- Develop a facile synthesis method to easily produce metal oxide particles that are nanosized. This method includes the addition of chiral ligands, specifically amino acids of both L and D orientation. We aim to explore the effects of varying reaction conditions on the properties of the nanoparticles, such as pH and temperature.

- Examine the cytotoxic effect of these nanoparticles on mammalian cells. If these nanoparticles are capable of killing bacteria at high levels without harming mammalian cells, this improves the capability of these nanoparticles to be applied to biological and health care systems.

Selected Particles

As previously mentioned nearly all L amino acids are produced in nature, meaning that D amino acids are more expensive to use. In addition, both versions of the amino acids produce similar levels of signal when analyzed; there is no added benefit here of using the more expensive ligand. Because of this, we mainly used L amino acids to reduce the cost of our experiments and only used D amino acids if we needed to compare the signals of our particles in order to see if they produce a mirror CD spectrum.

Initially, we synthesized chiral iron oxide nanoparticles with a variety of amino acid ligands[6]. An iron metal oxide nanoparticle made for a good candidate because iron is naturally occuring in the body, so we are not introducing a completely foreign metal to the body with no methods of either breaking down or secreting the particles. This is why we would not, for example, use a metal such as gold. The body has no mechanisms for breaking down larger amounts of nanoscale gold. Although high karat gold leaves are edible and commonly used as decorations in fancy restaurants and confectioneries, nanoscale gold has different properties and is unable to be incorporated into the body. Even though the gold nanoparticles would be able to reach their desired location in the body without being broken down, once there the particles would stay in the body unable to decompose the particles. So because iron oxide nanoparticles do not degrade before they have their desired effects and do not aggregate for long periods of time in the body, they make an excellent candidate for our project.

However, even though in speculation iron oxide nanoparticles should be safe to use in the body and some studies show that they are biocompatibile with certain cells [25], there are some studies that show that they could be lethal [26]. However, we hope that with our synthesis method and

use of an amino acid ligand our particles will be more compatible than the ones in the previous studies. Because many organic compounds are chiral, it is possible that modifying these particles to give them a chiral orientation may be the key to their overall compatibility by mimicking those compounds that are naturally occurring in the cells. But even if we were to be able to easily synthesize a completely safe iron oxide nanoparticle, from what we found they were unable to produce consistent results when analyzed with instruments that could measure their optical activity, size, etc. This is most likely due to the fact that working with iron in a laboratory setting can be very difficult. Iron oxidizes rapidly due to oxygen in the atmosphere, so any samples of iron we have could have different amounts of oxidation giving us different results when analyzed. Even if we follow the exact same procedure and control the external conditions, at least to the best of our abilities, we could produce two samples that vary in their characteristics. It is for this reason that we had to halt the development of these particles and instead direct our focus to metal oxides that would be easier to work with and had similar levels of biocompatibility.

This led us to choose cerium oxide nanoparticles as a new candidate for our project. Besides use as an antibiotic to kill infectious bacteria, cerium oxide eliminates reactive oxygen species (ROS). These are unstable molecules that contain oxygen, like peroxides and superoxides, which react easily with other cellular molecules and can damage DNA, RNA or proteins. Some nanoparticles exhibit antibacterial activity through ROS generation, however this nonspecific killing activity will harm mammalian cells in addition to killing bacteria. Using cerium would circumvent this issue. One remaining issue is that, similar to gold, the body does not have cerium naturally occurring with itself which could present problems when applying it inside of mammalian biological systems. The research surrounding the biocompatibility of cerium oxide nanoparticles is limited, with some studies showing that tissue damage by the particles is limited with almost no systemic toxicity [27] whereas others showed an increased toxic behavior in nanoscale cerium oxide as opposed to microscale versions of the particles [28]. With conflicting studies showing very different amounts of biocompatibility, our choice to proceed with cerium was based on the fact that when analyzed, cerium showed much greater consistency in particle characterization. With greater optical activity and more uniform physical characteristics, we will continue to work with cerium oxide nanoparticles and perform our own tests on the biocompatibility of cerium oxide against mammalian cells. If cerium is able to kill bacteria but not mammalian cells, then it will make a good candidate for our project as a new antibiotic to combat antibiotic resistance.

Nanoparticle Synthesis Methods

What follows is a brief description of the synthesis process used to create both iron and cerium oxide nanoparticles.The first nanoparticles synthesized for this project in the lab were iron oxide and were produced using the wet precipitation method. The wet precipitation method involves

the addition of aqueous reagent solutions. The reaction occurs in the aqueous phase to produce nanoparticle precipitates that can then be removed from the liquid and dried to obtain nanoparticle powders.

Iron Oxide

Precipitation of iron oxide nanoparticles involves the dissolution of an iron source such as ferrous or ferric chloride in water, where the ions will dissociate and the iron will form a metal hydroxide with the water via deprotonation. This iron hydroxide then undergoes dehydration (water elimination) producing iron oxide, the desired product. For iron, the dehydration reaction occurs optimally under basic conditions (pH ≈ 9) so we also add sodium hydroxide into the solution to encourage the production of iron oxide. Before the addition of our base, however, we must dissolve our chosen amino acid into the solution with the iron. This serves two purposes. One, to bond with the iron and act as a ligand, modifying the particles to be chiral once precipitated. And two, to ensure the formation of a monodispersed solution by preventing the aggregation of the particles. Since the goal of the project is to create nanoscale particles, we must add some kind of ligand to prevent the creation of particle clusters, hence why amino acids are crucial to this synthesis. Overall, precipitation is a relatively simple synthesis method that allows us to quickly produce many different types of particles in order to best discover which particles are the best candidates.

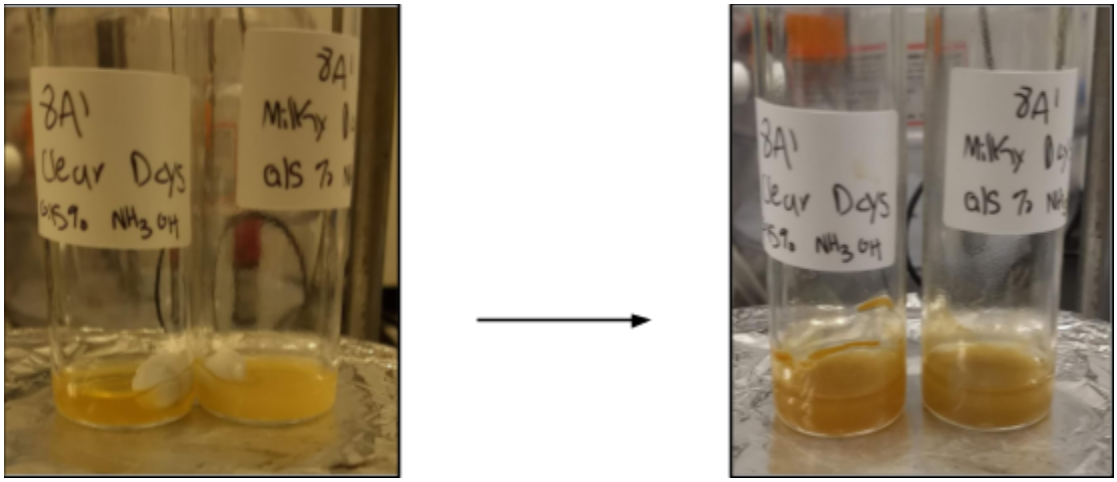

Figure 1. Iron oxide precipitation synthesis reaction at start of reaction (left) versus at end of reaction after 2 hours (right).

Even though the lighting between both pictures is slightly different, it is still possible to see that the color of the solution had changed as the reaction progressed, especially in regards to the solution on the right. Color change is an important indicator that metal oxide is precipitating during the reaction. However, if the precipitation does not dissolve back into the solution this signals the formation of aggregates, which is not desired.

Cerium Oxide

After our attempts at iron oxide, we then moved on to cerium oxide as a new candidate. The synthesis of cerium oxide nanoparticles followed the wet precipitation method as well, with changes being made to the procedure to reflect the different chosen metal. For this synthesis we included the use of hexamethylenetetramine (HMT) which works to stabilize the production of cerium hydroxide. The way HMT does is through strong chelating forces between itself and the cerium ions formed from dissociation, causing coordinate bonds to form between the cerium metal and HMT. This spaces out the bonds in the cerium hydroxide lattice, ensuring that the molecules are in a favorable configuration to be dehydrated [29]. In later syntheses, we modified the procedure to use sodium citrate as a chelating agent in place of HMT. To dehydrate the metal hydroxide complex formed during metal salt dissolution, we used sodium borohydride. This chemical is a reducing agent that reduces the metal ions to develop metal oxides.

Furthermore, our project explored the concept of nanoparticle doping with these particles. Doping involves the introduction of other elements into the particle structure in order to increase the stability by preventing aggregation and precipitation, but also can assist in controlling the functionality of the particles as well (something that we struggled to do with iron oxide). In our experiments on doping, we created numerous solutions in which we dissolved differing amounts of cerium nitrate and cobalt chloride. In those solutions that contain more cobalt, we assume that the particles we are synthesizing is cerium doped cobalt oxide and in those solutions that contain more cerium, we assume the particles are cobalt doped cerium oxide. With this synthesis method however, we have no way of ensuring that the only particles we produce involve the doping of the intended element (for example, there will be some cerium doped cobalt forming in solutions that contain more cerium). Future exploration with this synthesis will have to involve investigating ways to separate the products of the reaction.

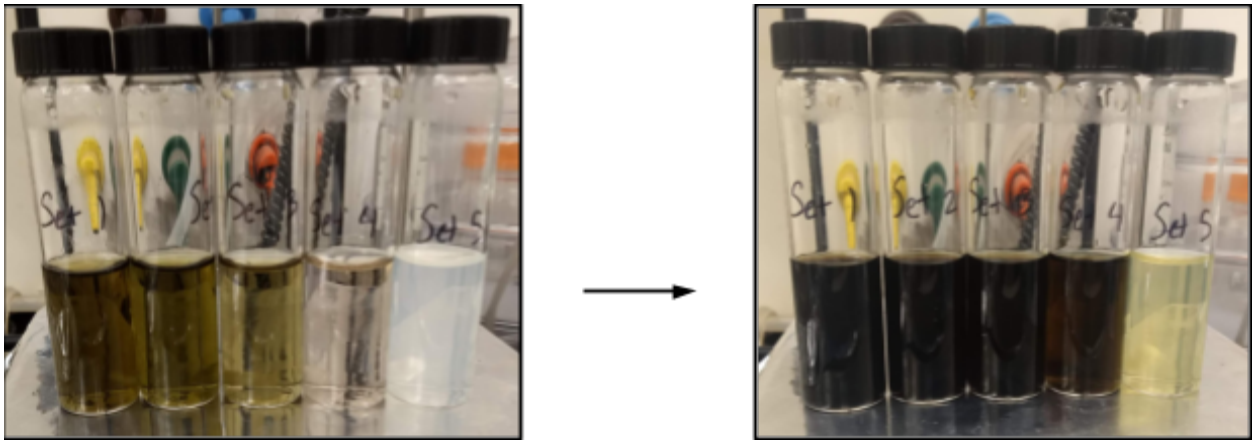

Figure 2. Cobalt doped cerium oxide/cerium doped cobalt oxide precipitation synthesis reaction at start of reaction (left) versus at end of reaction after 2 hours (right). From left to right in each picture the amount of cobalt chloride added to solution decreases, with the left and right vials containing only cobalt and cerium, respectively, with no doping.

As noted before with iron oxide, there is a noticeable color change taking place over the course of this reaction. This indicates that nanoparticle formation is taking place in the vial, but could also mean that particle aggregates are forming. Even if we add ligands like amino acids who help prevent aggregation of particles, it is likely that not all metal oxides are bonded with the ligand. Those that don't will still be susceptible to aggregation, meaning that we can only reduce this unwanted behavior—not completely prevent it. After the reaction has ended, the samples are then washed. When the particles are redispersed during this process in water, it is possible that aggregates can be broken down via sonication or similar methods into individual nanoparticles again.

Washing

After synthesis of metal oxide nanoparticles is completed, there will be left over unreacted molecules as waste. If we were to analyze the samples as is, we would be unable to guarantee that we would be able to collect accurate data as the unreacted molecules would be present in our analysis as noise. For example, unreacted amino acids would superficially increase the perceived signal coming from our particles when in reality their signal might not be as high as measured. Also, the reaction will continue to progress in the container beyond the set time limit unless the reacted compounds are separated and the unreacted molecules are disposed of. In order to remove these molecules, we must "wash" the solutions. This involves centrifuging so that the particles are sedimented out and removing the supernatant containing the waste. This can be repeated as many times as needed, and instead of using water to redisperse the particles our project often used alcohols such as ethanol, methanol, and isopropanol. If there are any unreacted

molecules that are not soluble in water, they may be soluble in alcohol and be removed when the supernatant is removed. For instance, amino acids are soluble in water so they get removed during the first wash, but if there are other unreacted molecules that won't dissolve in water they might dissolve in alcohol, so doing an additional wash with alcohol can remove these from the sample. When the samples are done being washed, they may be redispersed in water using a sonicator and either put aside to be analyzed or sent to the lyophilizer to be dried.

Instrumental Analysis Methods

To characterize the properties of our nanoparticles, a variety of instruments were used. To measure the chirality of our nanoparticles, circular dichroism spectrophotometers (Jasco J-1700 and J-815) were used. A quartz cuvette filled with diluted nanoparticle solution was loaded into the spectrophotometers and absorbance was measured from 190 nm to 900 nm where chiroptical activity of our nanoparticles is expected. To determine the surface charge and dynamic light scattering size distribution profile, we used a ZetaSizer (Malvern Panalytical). To determine the exact size and morphological characteristics of our nanoparticles, we used transmission and scanning electron microscopes. Microscopy samples were loaded onto electron microscopy grids and fully dried before being visualized.

Antimicrobial Activity Materials and Methods

Experiments examining antimicrobial activity in planktonic bacteria cultures were conducted in 96 well plates filled with dilute (optical density = 0.01) bacteria and varying concentrations of nanoparticles that were allowed to grow for 18 hours. Growth of bacteria was observed using plate readers. Bacteria grown in bleach were used as negative controls and bacteria grown without bleach or nanoparticles were used as positive controls. The bacteria we have examined (so far) are *E. coli* (UTI89) and *S. aureus* (USA300). USA300 is a strain of MRSA, which is antibiotic resistant. Graphs of optical density measurements over time were created to determine the relative levels of growth inhibition exhibited by the various nanoparticles synthesized in this project.

Results and Discussion

Nanoparticle Characterization

Results of a typical circular dichroism (CD) measurement are shown in Figure 3. The top spectra is for L/D cysteine in isolation. From the spectra, we see one distinct peak for both L and D cysteine at around 200 nm. When the L/D cysteine is ligated to the surface of the nanoparticles, we see new peaks appearing in the spectra readouts. For the non-doped cerium oxide nanoparticles, new peaks appear at around 260 nm. These peaks represent absorption beyond just the chiral ligand, instead indicating that the chiral ligands have bonded to the surface of the

nanoparticle. The presence of these peaks can be used to confirm the creation of chiral particles. To ensure the validity of the data obtained from the spectrophotometers, samples were diluted until the high tension voltage for all samples was below 600 V in the region of interest (> 200 nm).

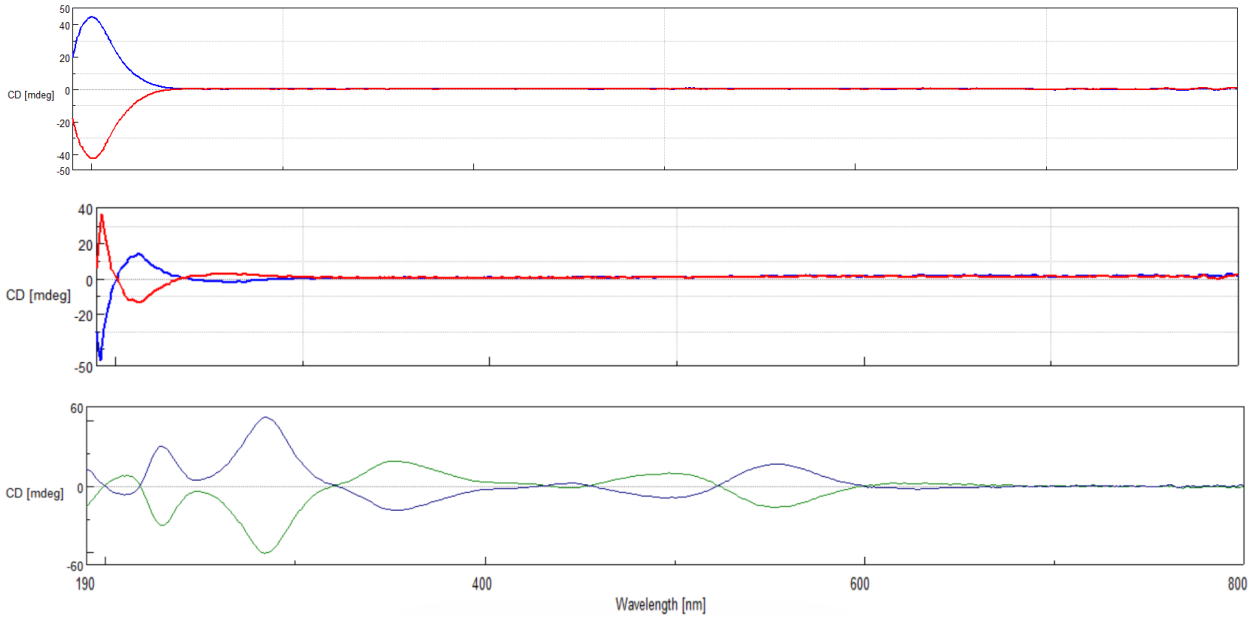

Figure 3. CD spectra of L/D cysteine (top), cerium oxide nanoparticles with L/D cysteine (middle), and cerium oxide nanoparticles doped with cobalt with L/D cysteine (bottom).

For the spectra of the doped cerium oxide nanoparticles, we see the appearance of numerous other peaks beyond what is seen for non-doped cerium oxide. This indicates that doping cobalt into the structure of the nanoparticle has important consequences for the properties of the nanoparticles. We aim to explore exactly how the properties of the nanoparticles are altered by the presence of doped cobalt more in the future.

Figure 4 demonstrates results typically seen from measuring the surface charge and dynamic light scattering size of our nanoparticles. As seen in this graph, the zeta potential peaks around +28mV and follows a binomial distribution of charge. What we hope to see when we collect zeta potential data is a single large peak and charge (+ or −) that suits the intended purpose of the substance. For example, if we wanted a substance that would interact with DNA, which is negatively charged, we would hope to see a single *positive* zeta potential peak. In our case, we do not know whether it is better to have positively or negatively charged particles, but considering the cell membrane potential of bacteria with a build up of negative charge on the inside of the cell, it is likely that a slight positive charge for our particles would be ideal.

The dynamic light scattering size distribution profile of our nanoparticles shows our particles are generally 100-1000 nm. Based on this profile, our particles would not be classified as nanosized. However, based on electron microscopy data, we know this to be untrue. One reason for this could be the formation of aggregates before reading the sample. One way to counteract this would be by sonicating the sample thoroughly before inserting it into the machine. Another reason this could be occurring with our samples is nanoparticle sintering. This property will be explained in more detail in a later section. Either way, for more exact size information electron microscopy should be used for these nanoparticles.

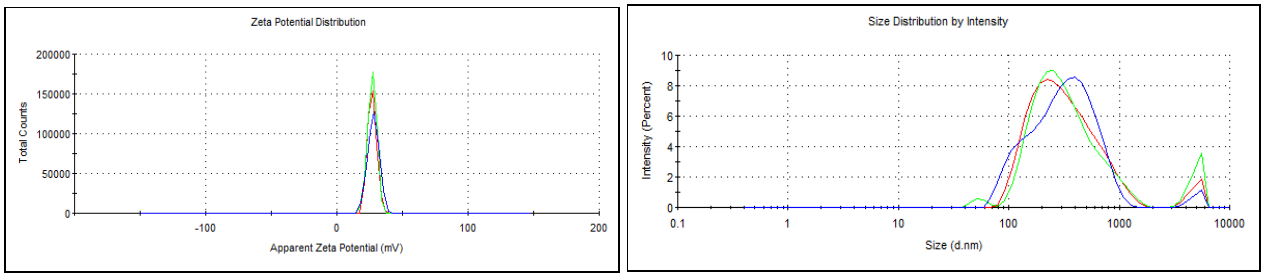

Figure 4. Zeta potential (left) and size distribution (right) data for non-doped cerium oxide nanoparticles synthesized with L-glutamic acid as the amino acid ligand.

A transmission electron microscopy (TEM) image for L-glutamic acid non-doped cerium oxide nanoparticles is presented in Figure 5. Here we get a clearer idea of how large our particles actually are. It is much easier to pick out individual particles in this image, with one circled that is attached to a larger cluster. The diameter of this particle is about 5 nm, which confirms that our synthesis method is capable of producing nanosized particles.

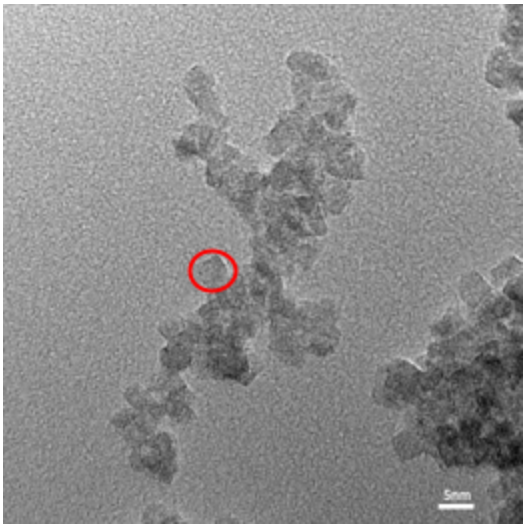

Figure 5. TEM image of non-doped cerium oxide nanoparticles with L-glutamic acid as the amino acid ligand. Scale bar is 5 nm. (Credit: Sumeyra)

One important thing to notice about the image in Figure 5 is that the nanoparticles are not monodisperse in solution. Instead, the nanoparticles exist as clusters of distinct nanoparticles. This is likely the reason that the dynamic light scattering size distribution profile shows the nanoparticle size as larger than it actually is.

One reason that we propose that our nanoparticles do not appear monodisperse in solution is likely due to their ability to sinter at room temperature. Sintering is a process in which solid material is compacted to form a solid mass of material usually by applying heat or pressure without melting it to the point of liquefaction. Sintering of nanoparticles has been observed before and a few mechanisms have been proposed for how the process occurs in nanoparticles. One explanation is that due to their extremely small size, nanoparticles are capable of absorbing high amounts of ambient thermal energy where bulk materials do not possess this characteristic. This could allow for nanoparticle sintering to occur at room temperature whereas for bulk materials this usually occurs at elevated temperatures and pressures. It is possible that this is what is happening to our nanoparticles, and we plan to explore this in more detail in the future.

Antimicrobial Potential

So far, we have only examined the killing activity of non-doped cerium oxide nanoparticles. The results of a typical growth curve experiment for both *S. aureus* and *E. coli* are shown in Figures 6 and 7. From the graphs, it is clear that the nanoparticles exhibit different killing activity for the two bacteria. All nanoparticles exhibit little to no killing activity with *E. coli*. Against S. *aureus*, varying levels of killing activity are observed.

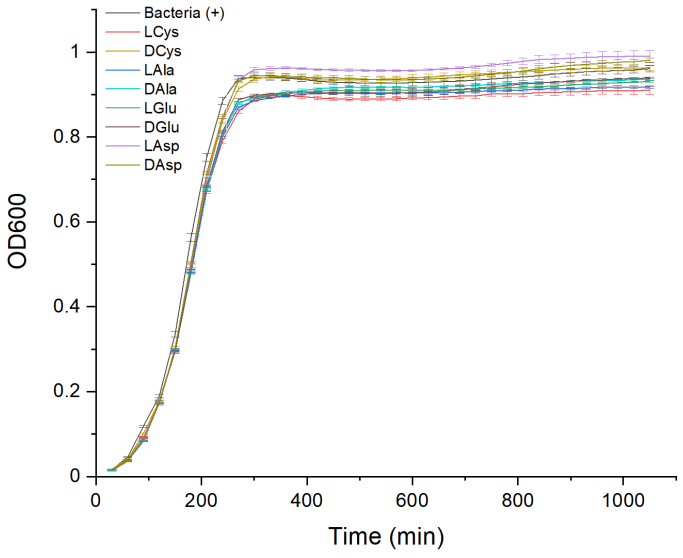

Figure 6. Results of a typical growth curve experiment using *E. coli* UTI89. Error bars represent standard error (n = 3). Optical density measurements were taken at 600 nm.

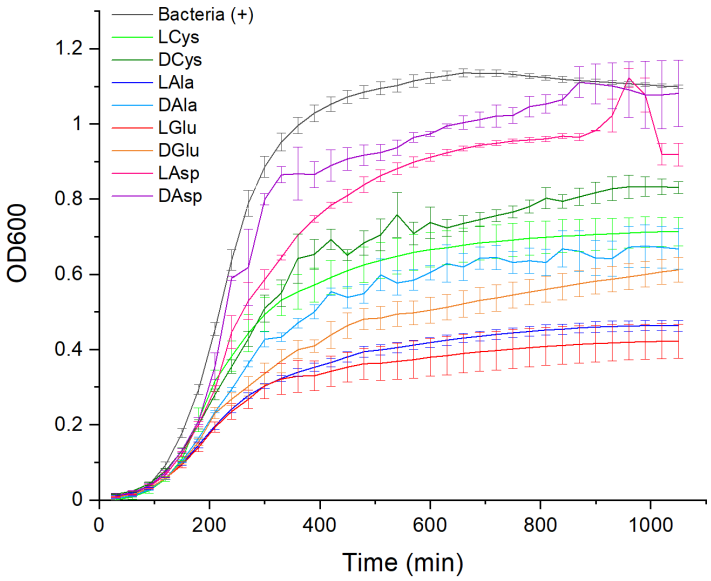

Figure 7. Results of a typical growth curve experiment using *S. aureus* USA300. Error bars represent standard error (n = 3). Optical density measurements were taken at 600 nm.

One potential explanation for the difference in killing activity between *E. coli* and *S. aureus* could be because *E. coli* is a gram-negative bacteria whereas *S. aureus* is a gram-positive bacteria. This difference can potentially elucidate the killing mechanism of our cerium oxide nanoparticles, where it is possible that the cerium oxide nanoparticles exhibit some kind of activity disrupting the cell wall of the bacteria. Or it is possible that the structure of the gram-negative cell wall prevents the nanoparticles from reaching the inside of the cell where the mechanism takes place. Either way, this finding needs to be confirmed by testing these nanoparticles against more gram-negative and positive bacteria to see if the pattern continues.

Beyond this observation, we also notice that the nanoparticles exhibit differential killing activity based on the type of amino acid ligand used. Figure 7 represents a typical growth curve experiment for *S. aureus*, where across multiple growth curve experiments we have observed the following general pattern of killing activity from highest activity to lowest: glutamate > alanine > cysteine > aspartate. We also observed that L amino acid ligated nanoparticles exhibit greater killing activity than their D amino acid counterparts. Interestingly, despite belonging to the same category of amino acid, glutamate and aspartate exhibit greatly different levels of killing activity. The structure of these amino acids are very similar and theoretically should demonstrate similar chemistry. We expect that they would demonstrate similar levels of killing activity. Since this is not the case, it is possible that another factor is contributing to the difference seen in the killing activities of the nanoparticles beside the chemistry of the chiral ligand. Further experiments are required to see if this pattern holds up across more experiments. Incorporating more amino acid

ligands into these experiments might also help elucidate a more exact pattern and reasoning behind the differential levels of killing activities observed for these nanoparticles.

Other Observations

The reaction is sensitive to changes in pH. This was discovered by accident when too much sodium hydroxide was added to one of the amino acid solutions and the reaction color was different than what was expected. We ran an experiment running multiple reactions simultaneously with varying pH from 0.5 to 14 and observed the color change in the reaction. Small amounts of highly concentrated hydrochloric acid or sodium hydroxide were added to achieve the desired pH. To measure the pH of the reaction mixtures, pH papers were used. The results of this experiment are shown in Figure 8.

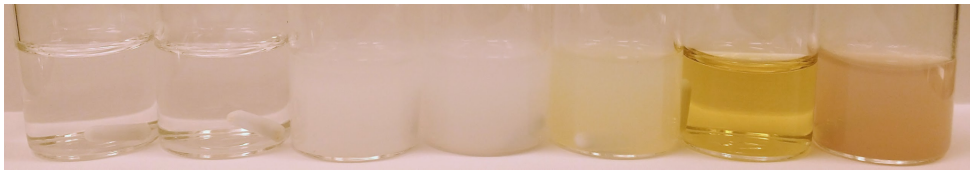

Figure 8. Demonstration of reaction sensitivity to pH indicated by color change. From left to right: pH = 0.5, 2, 5, 7, 9, 12, 14.

As can be seen in Figure 8, the color of the reaction is dependent on the pH of the reaction mixture, indicating that this reaction is sensitive to changes in pH. At low pH levels, no nanoparticle forms. At neutral pH, a white precipitate is formed. At a pH of around 9, a yellow-ish precipitate is formed. At a pH of 12, no precipitate is formed but the reaction color changes from clear to yellow. At very high pH, an orange precipitate is formed. It is possible that the differences observed in reaction color indicate a difference in the type of nanoparticle formed. As mentioned previously, zinc oxide nanoparticles form different shapes under different reaction pHs. It is possible that a similar mechanism is taking place here as well. Most surprisingly is the fact that the reaction solution at pH = 12 remains clear whereas the reaction below and above that pH shows precipitate formation. This phenomenon requires further experimentation to determine if nanoparticles are formed at that pH and if differing the reaction pH synthesizes different types of nanoparticles.

Conclusions

We have successfully developed a facile synthesis method to produce nanosized cerium oxide particles that exhibit some level of killing activity against bacteria. In particular, these nanoparticles seem to exhibit killing activity against *S. aureus* but not *E. coli*. It is possible this is due to the difference in the cell walls of the bacteria. The killing activity of the nanoparticles is also dependent on the amino acid ligand used in their synthesis. Further experiments are required to determine a reason for the observed differences in killing activity of these nanoparticles.

Future Work

There are many different directions this project can take depending on the results obtained in future experiments. A list of future experiments and areas to explore are listed below:

- Investigate the effects of reaction temperature and pH on the characteristics of the nanoparticle formed. Additionally, explore the effect of removing the chelating agent from the reaction mixture. From preliminary results, we have found that removing citrate has had negligible effect on the type of nanoparticle synthesized, however we wish to explore this in more detail.

- Investigate in more detail the effects of doping the nanoparticle structure with cobalt. Preliminary results indicate that these doped nanoparticles exhibit enhanced chiroptical activity, but we wish to explore if they would demonstrate enhanced killing activity as well. Exploration of doping with other metals would also be a possible future direction.

- Synthesize nanoparticles using a wider range of amino acid ligands to determine if it is possible to establish a pattern of killing activity based on the structure and chemistry of the ligand and if this information can help to guide future experiments involving chiral nanoparticles.

- Investigate the effect these nanoparticles have on preventing or treating bacterial biofilms. Biofilms are particularly difficult to treat due to the protective extracellular matrix that is formed that prevents antibiotics from reaching the bacteria. Nanoparticles have been proven in previous research to be particularly effective at treating biofilms. If our nanoparticles demonstrate the ability to treat biofilms, this would expand their usefulness in practical applications.

- Investigate the cytotoxic effect of these nanoparticles on mammalian cells. As mentioned previously, if these nanoparticles are capable of killing bacteria without being toxic to mammalian cells it improves the applicability of these nanoparticles to real-world biological and health care systems and scenarios.

- Explore alternative quantifications of nanoparticle bactericidicity. While growth curves offer crucial information such as the kinetics of the killing activity, we have found our results to be somewhat inconsistent using this method. We hope to further substantiate our results and data by providing other methods of quantifying the levels of killing activity of the nanoparticles, such as zone of inhibition tests. These experiments are often simple and can serve to strengthen the results and conclusions of this project.

Key Terms, Defined

All definitions obtained from Merriam-Webster online dictionary.

1. *Biomimicry*: the imitation of natural biological designs or processes in engineering or invention [30].

2. *Self-Assembly*: the process by which a complex macromolecule or a supramolecular system spontaneously assembles itself from its components [31].

3. *Brownian motion*: a random movement of microscopic particles suspended in liquids or gases resulting from the impact of molecules of the surrounding medium [32].

4. *Chiral*: of or relating to a molecule that is not superimposable on its mirror image. An example of this would be human hands: looking at our hands side-by-side reveals that they are mirror images with the mirror plane being vertical separating the hands, yet when our hands overlap they are not superimposable [33].

5. *Racemic*: of, relating to, or constituting a compound or mixture that is composed of equal amounts of dextrorotatory (D-) and levorotatory (L-) forms of the same compound and is not optically active [34].

6. *Ligand:* a group, ion, or molecule coordinated to a central atom or molecule in a complex [35].

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