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Analysis of the Fungicidal Nature of ZnS on *Sordaria fimicola*

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ABSTRACT

Nanoparticles are distinctly different from their micro-sized colleagues. ZnS nanoparticles were synthesized by exposure to ultrasonic radiation while mixing $ZnCl_2$ and Na_2S. The particles were then placed onto one side of a nutrient agar at varying concentration. Because the fungicidal nature varies with particle size, a comparison between the micro ZnS particles and the synthesized ZnS nanoparticles. One side of the nutrient agar was loaded with the ZnS particles. Pregrown *Sordaria fimicola* will be centrally positioned onto this plate. The growth of these blocks was measured and provided a quantitive measurement of the particles ability to retard grown. It was found that the two nanosized ZnS particles had a significantly higher ability to retard growth than ZnS micro particles. There was a marked difference between the growth control side of the agar and the treatment side of the agar. The synthesized ZnS nanoparticles in fact show a higher efficacy to retard fungal growth than the purchased ZnS microparticles.

Keywords: *fungicide, nanoparticles, Sordaria fimicola, sonochemical, ultrasonic precipitation, ZnS.*

INTRODUCTION

Nanoparticles are extremely small particles ranging from 1 nanometer to 100 nanometers. There are many different reasons that nanoparticles are being studied so extensively. The main reason is due to the small particle size and the effect this has in given reactions. By lowering the size of particles in a sample the area exposed vastly increases. By increasing the exposed area the kinetics of a reaction can be changed as well. It's not just the kinetics of a reaction that is changed, the structural and electrical properties of the materials also change. Nanoparticles have a wide range of uses including anti-microbial treatment as well as degrading Methylene Blue (Khaled, Nasr, Rola, 2013). This exhibits its remarkable ability to act on all things from methylene blue to microbes.

There has been considerable research growth recently in trying to create new nanomaterials (Mondel, Bhattacharya, 2013). Nanoparticles use has increased and research into nanoparticles should follow. With more advanced technology available to study and produce nanoparticles, it's one of the most rapidly advancing fields of the sciences (Tadaaki, 2015).

ZnO nanoparticles tend to show antimicrobial properties. ZnO nanoparticles was loaded onto cotton and the antibacterial property was shown (Qun, Shui-Lin, Wan-Chao, 2007). The purpose of this experiment, is to test the antimicrobial properties of ZnS nanoparticles.

Bacteria evolves at a rapid rate making the search for antibacterial an endless race. Bacteria have an exponential reproduction rate causing them to grow from one to 1028 in only ten generations. If only one bacterium in a colony is resistant to antibiotics this bacteria would regenerate its population in a short time period. Since the generation time varies with different types of bacteria it could take anywhere from an hour and forty minutes to six days 16 hours for one of the fastest and one of the slowest bacteria's respectively to go through 10 generation cycles. Since bacteria and most fungi reproduce asexually they can pass on this trait and soon entire colonies are antibiotic resistant (Todar 2012). The prevalent use of antibiotics is causing bacteria and fungi to become resistant to antibiotics. With the World Health Organization stating that "this serious threat is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country. Antibiotic resistance–when bacteria change so antibiotics no longer work in people who need them to treat infections–is now a major threat to public health" (WHO 2014).

Microbes are being constantly exposed to antibiotics, and soon the microbes become antibiotic resistant. Due to the feed industry which in "2011 U.S. livestock producers purchased 29.9 million pounds of antimicrobials" (Levy 2014). This 29.9 million pounds of antimicrobials is being used on a large scale causing a multitude of microbes to become antibiotic resistant. Antibiotic use and resistance go hand in hand, "we found that antibiotic consumption is associated with the development [of] antibiotic resistance" (Bell 2014).

Antibiotic resistance has dire consequences with a "minimum 25,000 patients in Europe and 23,000 in the USA die each year from infections caused by resistant bacteria" (Carlet 2014). If this study was successful the key would be to use ZnS as a curative treatment. This will in turn lower the deaths contributed to antibiotic resistant microbes. This study is vital in furthering both the medical field, and the antibiotic industry.

There are many reasons to use ZnS nanoparticles for its antimicrobial properties. ZnS nanoparticles exhibit a zone of inhibition, approximately 29.8mm in diameter. Whereas, there was no zone of inhibition on zinc sulfide micro-particles. This zone of inhibition shows that there is some sort of retarding nature on bacterial growth due to particle size, which demonstrated the anti-microbial property of nano zinc sulfide particles (Suyana, et al. 2014). Previous research suggests that zinc sulfide micro-particles had no effect when zinc sulfide showed a zone of inhibition demonstrates that these nonreactive bulk particles when in nano-size are effective antibacterial agents (Suyana, et al. 2014). Since the experiment showing the antifungal activity of ZnS nanoparticles was published in 2014 it is still considered a recent study, a study confirming these researchers' results would be pertinent to the scientific community.

ZnO nanoparticles exhibit a negative correlation between size of particles and antibacterial properties, meaning the smaller the size of ZnO nanoparticles the better antimicrobial properties as well as thermal properties (Azizi et al. 2013). ZnS nanoparticles need to be as small as possible to maximize it's antimicrobial property. This study will focus on the preparation of ZnS nanoparticles, quantifying its antibacterial properties, and characterizing these particles

MATERIALS AND METHODS

Preparation of ZnS Nanoparticles (Suyana et al. 2014)

A 500 mL beaker was placed in an ultrasonic bath with 125 mL of 0.5 M aqueous solution of $ZnCl_2$. A solution of 125mL of 0.5 M Na_2S was added dropwise to the beaker of $ZnCl_2$ for an hour at room temperature while sonicating. An off white precipitate of ZnS formed during this process. The precipitate was washed with excess amounts of water to remove any unreacted species. The collected particles were dried in an oven at 70^0C for 24 hours.

Characterization of ZnS Nanoparticles

The produced ZnS nanoparticles were characterized using a Dynamic light scattering instrument, DLS. A solution of ZnS nanoparticles was placed into the detector and the DLS was ran determining the size of the particles. The samples used for the DLS were pulled from the top of the solution, as to avoid precipitate. Thus the values obtained refer to the average size of the particles suspended in solution rather than the average size of all the particles in solution.

Microbial Study:

A loaded nutrient agar was prepared to test the efficacy of the particles. 23 grams of nutrient agar was suspended in one L of water of near boiling water. The solution was sterilized by autoclaving it at 121^0C at a pressure of 15 pounds per square inch (100kPa) for 15 minutes. The sterile solution was placed into an oven at 45^0C to allow the solution to cool to a point just above the point it solidifies at. 25 mL of the agar was poured into 100 mm petri dishes to create a standardized growing condition for *Sordaria fimicola* wild type.

Agar plates with varying concentrations were prepared, to determine the particle concentrations that exhibited an inhibitory effect. A set of 5 agar plates loaded with 0.25 mL aliquots ranging from 10 millimolar up to 50 millimolar synthesized ZnS nanoparticles. The particles were only treated on half of the agar plate. *Sordaria fimicola* was implanted onto the plate using pregrown block of agar. After the plate was inoculated, it was incubated for 48 hours at 27^0C. The growth of the fungus was measured, by quantifying the distance from the implanted agar to the perimeter of growth on both the treatment side and control side. The difference between the treatment side and the control side was computed. Resulting in a negative value if the treatment had an inhibitory effect on the fungal growth.

This set concentration range was employed for both ZnS nanoparticle and micro-particle laden agars. These prepared agars at the given concentration was used to determine if these ZnS nanoparticles have an antibiotic property that is greater than ZnS microparticles. A set of three agar plates was prepared for each of these varying concentrations.

A two tailed analysis of variance, Anova, was used to compare the means. The data was was placed into a three column table. The first column being the concentration variable. The second column being the treatment variable (nano ZnS one, nano ZnS two, and micro ZnS). And the third column being growth difference between the treatment and control. Anova assumes that the data was obtained in a random unbiased manner, that the sample means correspond to the population mean, that the individual exposed to different treatment levels and different treatments have equal variance and are normal (Leblanc 2016). The data was obtained randomly and since Anova is relatively robust to violations of the normality and equal variance assumption as long as the sample size remains equal between groups (Leblanc 2016). Since the sample size are equal in all the sets indicates that slight deviations from those assumptions will not greatly affect the p-value obtained. Anova tests for equality between all sets of data, if the p-value is less than 0.05 than the alternative hypothesis is true, that the means of the different sample sets are significantly different. Significantly different means obtained required a post hoc test to identify different groups. Tukey HSD was chosen, as the sample size for all groups were equal.

RESULTS

Two synthesis trials were ran. This is due to the low yield obtained in the first run. Run #1 only produced a 20.92% yield. This lead to Run #2 to see if a better yield could be obtained, an increase in 11% yield occurred. This can be attributed to the washing process. Run #2 allowed for a more settled solution, resulting in less product expelled during the washing process.

Table 1. Data obtained during the preparation of ZnS nanoparticles.

Run # 1	
Used (g)	8.5219 ZnCl2
	4.8709 Na2S
Produced (g)	1.2726 ZnS
Theoritical (g)	6.0835 ZnS
% Yield	20.92%
Average Dispense Rate (mL/min)	2.19
Run #2	
Used (g)	8.5124 ZnCl2
	4.8709 Na2S
Produced (g)	1.9632 ZnS
Theoretical (g)	6.0869 ZnS
% Yield	32.25%
Average Dispense Rate (mL/min)	2.35

This study used a dynamic light scattering instrument to perpetuate the size of the particles. Table 2 gives values for the various particles. There were no suspended particles bigger than 40 nm or smaller than 10nm.

Table 2. Results from the DLS. Shows the average size of the particles. (Dil.) refers to diluted samples at a 1 to 1 ratio. Characterization of ZnS Particles

Micro ZnS	Volume-Wt	Number-Wt		
Mean	38.0 nm	35.9 nm		
Stdev	4.1 nm	4.8 nm		
%	100%	100%		
Fit Error	10.23			
Micro ZnS Dil.	Volume-Wt		Number-Wt	
Mean	11.0 nm	487.3 nm	10.8 nm	
Stdev	0.7 nm	48.1 nm	0.8 nm	
%	98.7%	1.3%	100%	
Fit Error	16.12			
ZnS Nano One	Volume-Wt			Number-Wt
Mean	11.0 nm	252.1 nm	414.5 nm	10.8 nm
Stdev	0.7 nm	8.2 nm	20.2 nm	0.8 nm
%	97.7%	0.5%	1.8%	100%
Fit Error	11.78			
ZnS Nano One Dil.	Volume-Wt		Number-Wt	
Mean	11.0 nm	434.9 nm	10.8 nm	
Stdev	0.7 nm	41.3 nm	0.8 nm	
%	99.3 %	0.7 %	100%	
Fit Error	14.51			
ZnS Nano Two	Volume-Wt		Number-Wt	
Mean	11.0 nm	539.2 nm	10.8 nm	
Stdev	0.7 nm	50.3 nm	0.8 nm	
%	99.0%	1.0%	100%	
Fit Error	27.18			
ZnS Nano Two Dil.	Volume-Wt	Number-Wt		
Mean	20.8 nm	19.7 nm		
Stdev	2.2 nm	2.4 nm		
%	100%	100%		
Fit Error	29.35			

During the tests there were some key indications that the fungal growth was inhibited by the ZnS nanoparticles. However the concentrations chosen seem to be too concentrated for this study. See Table 3, with a p-value of 0.648 shows the concentration is very unlikely to be a factor.

The following Anova, Table 3, indicates that the means across groups were not equal to each other. With a P-value of 0.003 for the different particle sizes it shows that the probability that, if the study was implemented correctly, the samples have only a 0.3% likely hood the values obtained were by chance alone. Which is sufficiently low that we could reject that the samples are the same. Then a Tukey HSD test showed the similar groups as viewed by Table 4. This post hoc test differentiated between the ZnS microparticles and the ZnS nanoparticles. There was a significant difference between the ZnS microparticles and both the ZnS nanoparticles. While there was not a significant difference between the varying ZnS nanoparticles.

Table 3. Anova for the ZnS. Underneath cases, Part. refers to the different particle size. Conc. refers to the varying concentration. The different particle size differed significantly (P = 0.003). While there was no significant difference between concentration.

Anova					
Cases	Sum of Squares	df	Mean Square	F	P
Part.	659.5	2	329.7	6.92	0.003

Conc.	119.3	4	29.84	0.06	0.648
Resid.	1429.9	30	47.66		

Table 4. Post Hoc Test on ZnS particle Size. This table shows the average growth of fungus with the varying particle size. Micro ZnS falls into its own group according to the post hoc Tukey test (group A). While both the nano ZnS particles into a separate group from the ZnS microparticles (group B). If one views mean growth micro ZnS on average inhibit fungal growth by 1.76 mm. While nano ZnS one and nano ZnS two inhibited growth by 9.33 and 10.34 mm respectively on average. This shows that ZnS nanoparticles are different in their antibiotic properties from the micro particle counterparts

	Micro ZnS	Nano ZnS One	Nano ZnS Two
Means	-1.76	-9.32667	-10.34
	-----A------	-------------------B---------------	

DISCUSSION

There was be a significant change in bacterial growth depending on the size of ZnS particles. The Anova had a p-value of < 0.05 I could make the statement that nanoparticles significantly inhibit growth as compared to the bulk material. This agreed with the given scientific literature. The results gathered help to perpetuate the ability of nanoparticles to retard the growth of micro bacteria. This study helped to support the findings that Zn nanoparticles create a zone of inhibition (Suyana et. Al. 2014). Since this study looked at loading a substrate with nanoparticles it would also help and verify the findings that Zn nanoparticles are effective when loaded on a substrate (Qun, Shui-Lin, Wan-Chao, 2007).

The particles were characterized by grabbing an aliquot of the solution used during the fungal growth portion of the lab. This sample was then ran through the dynamic light scattering instrument which characterized the size. The aliquot was grabbed from the solution. However since ZnS is highly insoluble in water there was white precipitate on the bottom of the solution. So a sample was grabbed from the clear liquid above the precipitate and leads to the corresponding values.

In the undiluted samples based off the number-weight measurement which is slightly more accurate than the volume-weight. Number-weight is more accurate because in volume-weight the bigger samples are more heavily weighted. ZnS microparticles obtained an average of 35.9 nm while ZnS nanoparticle #1 and ZnS nanoparticles #2 both obtained 10.8, this corresponds to the lower limit of sensitivity for this given instrument. The data gathered from the undiluted samples helps to correspond with the given scientific literature that the smaller the particle the more inhibiting they are on growth. The microparticle solution contained nanoparticles around 35.9 nm in diameter but due to the small inhibition on growth there was a lot less nanoparticles or the difference in size greatly influenced the fungicidal nature of these particles. This trend was discussed prior that the smaller the particles the bigger inhibition on growth.

However when these solutions were diluted by a factor of one to one, an interesting phenomenon occured. The ZnS microparticles dropped to an average of 10.8nm, ZnS nanoparticles #1 remained at 10.8 nm and ZnS nanoparticles #2 increased to 19.7 nm. A reasonable explanation as to why this happened cannot be deduced. However for all the samples, when diluted the fit error was higher in these than in the undiluted samples. Fit error refers to the likely hood that the data gathered falls into the given model. Since there is a higher Fit Error than the diluted, the sample readings are more likely to be flawed. However in over concentrated solution a particle will scatter light but this given light beam could be scattered multiple times and this is detrimental in the readings. So the characterization of these particles should be taken prudently as the undiluted sample may be over concentrated, and the diluted sample doesn't fit the model as well as the undiluted sample.

ZnS nanoparticles may prove to not be the most effective antibiotic, but due to a number of advantages such as being relatively cheap to produce and being generally safe to humans it could be potentially be substituted for traditional sources of antimicrobials. There would have to be a head to head test between the commercial products and the ZnS nanoparticles. But based off my results ZnS nanoparticles don't completely inhibit growth, while a chemical specifically aimed at killing off micro bacteria would do a better job.

On average the ZnS nanoparticles that were synthesized initially retarded the growth of fungus by 9.33 mm. This is a noticeable change that showed the inhibitory effect of ZnS on *Sordaria fimicola.* However ZnS nanoparticles synthesized second actually inhibited the growth of this fungus even more at 10.34 mm of inhibition. That compared to 1.76 mm of inhibition for the ZnS microparticles produced a p value of 0.003. This means that the average growth for the three sets of data had an extremely small value that it happened by chance. Which supports the statement that ZnS nanoparticles inhibit the growth of *Sordaria fimicola*.

It was expected that the higher the concentration of ZnS nanoparticles the more inhibitory of a response it produces. The expectation was that the most concentrated substrate at 50mM will retard fungal growth the most. The range selected appears to be maximally inhibitory to the fungus. The lowest concentration was already inhibiting the fungal

growth at a maximum rate. So ultimately the concentration should have been lowered and then maybe the concentration would have had an effect on the growth of the fungus.

Some of my sources of error range from commercial grade reagents to novice experience in microbiology. For the reagents if research or laboratory grade reagents were used this could have been a source of error avoided. Changing the grade of reagent would affect the percent yield but shouldn't affect the size of particles. Commercial grade reagents were used to simulate if these ZnS nanoparticles were commercially produced. Similarly if a procedure was tweaked it may not have resulted in the same conclusion.

I would implore future scientists to further this study. There could be alteration in the experimental design such as doping the nanoparticles with different dopants as this would increase the nanoparticles ability to react with light (Sreedhar D et. Al 2014). This would be interesting to see if it's interaction with light plays apart in the retarding nature of the particles. When exposed to a dopant the mixture has a higher band gap than undoped, meaning their optical properties are increased. And certain studies have hypothesized that it is the photocatalytic activity on nano Zn particles that make them effective as an antimicrobial agent. Therefore increasing their optical activity should increase the antimicrobial properties of these nanoparticles. Also another route one could take is to test Zn nanoparticles on different types of microorganisms. This test is performed on *Sordaria fimicola* is it effective on other eukaryotes.

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