Do Silver Nanoparticles Inhibit Bacterial Growth?

PA State Standards:

3.7.10, 12A	Identify and safely use a variety of tools, basic machines, materials, and techniques to solve problems and answer questions.
3.1.10, 12E	Describe how fundamental science and technology concepts are used to solve practical problems.
3.2.10, 12B	Evaluate experimental information for appropriateness and adherence to relevant science processes. Evaluate experimental data correctly within appropriate limits. Judge that conclusions are consistent and logical with experimental conditions.

Introduction:

Silver nanoparticles (AgNPs) are imbedded in clothing, band-aids, and food packaging to inhibit bacterial growth. In this activity, students test the antibacterial properties of their lab synthesized silver nanoparticles.

The antibacterial properties of the silver nanoparticles may be tested by measuring the zone of inhibition on an agar plate, by counting colonies on agar plates, or by measuring optical density in agar broth. The procedure for each of these activities is described. In the zone of inhibition activity, it is recommended that the students compare the antibacterial properties of the table disinfectant and the hand soap to the silver nanoparticles.

Guiding Question:

What is aseptic technique? Why must we use aseptic technique in this activity?

Equipment and Materials:

Needed for all three procedures:

- Synthesized silver nanoparticles
- 100 uL micropipetter with tips
- *E. coli* in nutrient broth, at an optical density of 0.03 at 600 nm
- Table disinfectant
- Marking pens
- Bunsen burner

Needed for zone of inhibition procedure:

- Sterilized filter paper dots
- Ethanol with bent glass elbow
- Forceps
- Dilute hand soap solution
- 8 sterile nutrient agar Petri dishes
- Sterile deionized water
- Metric ruler with mm calibration

Needed for colony counting procedure:

- 14 sterile nutrient agar Petri dishes
- Ethanol with bent glass elbow

Needed for growth in nutrient broth:

- Spectrophotometer
- 13 Sterile cuvettes containing 5 mL sterile nutrient broth – set aside one cuvette of sterile broth as a blank to zero the spectrophotomer
- Sterile synthesized AgNPs

Safety

Use aseptic technique when handling the *E. coli.*

Procedure:

Zone of Inhibition Procedure

1. Disinfect table.
2. Use marking pens to label the bottoms of Petri dishes – two control, two silver nanoparticle, two hand soap, and two disinfectant dishes.
3. Aseptically pipette 100 uL of the *E. coli* stock solution to a control Petri dish. Use a sterile bent glass elbow to aseptically spread the bacteria over the surface of the agar. Close the dish. Following aseptic techniques, pipette 100 uL of *E. coli* to each of the remaining seven dishes.
4. Dip tips of forceps in ethanol and flame to sterilize. Use sterile forceps to pick up a sterile paper dot. Soak the dot in sterile deionized water. Place the paper dot in the center of the control inoculated Petri dish. Repeat this procedure to place the appropriate paper dots in the Petri dishes.
5. Tape the lids on the Petri dishes.
6. Wash hands with soap and water.
7. Incubate Petri dishes upside down at room temperature or at 37° C for 24 hours.
8. Observe Petri dishes through the plastic. Do NOT open the Petri dishes. Using the ruler, measure the zone of inhibition around the paper dots and record in the data table.

Colony Counting Procedure

1. Disinfect table.
2. Use marking pens to label the bottoms of Petri dishes with the varying volumes of silver nanoparticles solutions to be used – 0 uL AgNPs, 10 uL AgNPs, 25 uL AgNPs, 50 uL AgNPs, 100 uL AgNPs, 200 uL AgNPs, and 300 uL AgNPs. Label two Petri dishes for each volume of silver nanoparticles, for a total of 14 Petri dishes.
3. Aseptically pipette 10 uL AgNPs to the appropriately labeled Petri dish. Use a sterile bent glass elbow to aseptically spread the nanoparticles over the surface of the agar. Be careful to spread the nanoparticles over the entire surface. Repeat this procedure, pipetting the appropriate volumes of nanoparticles to each of the Petri dishes.
4. Aseptically pipette 100 uL of *E. coli* stock solution to the first Petri dish, 0 uL AgNPs. Use the sterile bent glass elbow to spread the *E. coli* over the surface of the agar. Be careful to spread the bacteria over the entire surface. Repeat this procedure to aseptically inoculate and spread 100 uL *E. coli* onto each of the remaining Petri dishes.
5. Tape the lids on the Petri dishes. Allow Petri dishes to sit for 10 minutes undisturbed.
6. Wash hands with soap and water.
7. Incubate the Petri dishes upside down at room temperature or at 37° C for 24 hours.
8. Observe colonies through the plastic – do NOT open the Petri dishes. Count the colonies on each Petri dish and record in the data table.

Growth in Nutrient Broth Procedure

1. Disinfect table.
2. Zero the spectrophotometer at 600 nm using a sterile broth cuvette as the blank.
3. Use small pieces of marking tape to label the cuvettes of sterile nutrient broth with the varying volumes of silver nanoparticles to be used – 0 uL AgNPs, 10 uL AgNPs, 25 uL AgNps, 50 uL AgNPs, 75 uL AgNPs, and 100 uL AgNPs. Label two cuvettes for each volume of nanoparticles. Put each piece of tape near the top of the cuvette.
4. Aseptically pipette 10 uL of sterile AgNPs to the appropriate sterile cuvette of broth. Repeat this procedure for the varying volumes of the AgNPs solutions, aseptically pipetting the sterile AgNPs to the sterile broth cuvettes.
5. Aseptically pipette 100 uL *E.coli* to a cuvette with 0 uL AgNPs and gently finger vortex to mix. Record the absorbance of this solution at 600 nm in the data table at time 0 hours. Repeat this procedure, aseptically inoculating *E. coli* into each of the cuvettes and recording the time zero absorbances.
6. Wash your hands with soap and water.
7. Incubate at 37° C for 24 hrs. Gently finger vortex & take absorbance readings at 24 hrs. (If possible, take readings at recorded time intervals during the 24 hrs. Before each reading, gently finger vortex.)

Data

Table 1. Zone of Inhibition

Plate	Test Reagent	Diameter of Zone of Inhibition (mm)	Average Zone (mm)
1	Control		
2	Control		
3	AgNPs		
4	AgNPs		
5	Soap		
6	Soap		
7	Disinfectant		
8	Disinfectant		

Table 2. Colony Counting

Plate	Amount of AgNPs (uL)	Colony Count	Average Colony Count
1	0		
2	0		
3	10		
4	10		
5	25		
6	25		
7	50		
8	50		
9	100		
10	100		
11	200		
12	200		
13	300		
14	300		

Table 3. Growth in Nutrient Broth

Tube	Amount of AgNPs (uL)	Absorbance at 600 nm							
		0 hrs	Avg. 0 hrs	___ hrs	Avg. ___ hrs	___ hrs	Avg. ___ hrs	24 hrs	Avg. 24 hrs
1	0								
2	0								
3	10								
4	10								
5	25								
6	25								
7	50								
8	50								
9	75								
10	75								
11	100								
12	100								

Analysis

1. Prepare a graph showing the relationship between the independent and dependent variables.
    * Zone of inhibition – What type of graph should be used?
    * Colony counting – What type of graph should be used?
    * Growth in broth – This should include six lines on one set of axes.

2. Describe the relationship between the independent and dependent variables.
    * Zones of Inhibition – Compare the AgNPs results to the soap, disinfectant, and deionized water results.
    * Colony counting – Explain the shape of the graph.
    * Growth in broth – Include a second graph of average absorbance at 24 hours vs. uL AgNPs and explain noticeable trends.

3. What is the control in this experiment? What is the purpose of this control?

4. Why is aseptic technique critical for accurate results? Give three examples of aseptic practices used in this experiment.

5. Suggest possible sources of error in your experiment. These sources of error may NOT be mistakes in reading the measurements or in calculations.

Acknowledgements

National Nanotechnology Infrastructure Network
www.nnin.org

Penn State Center for Nanotechnology Education and Utilization
For more information contact Amy Brunner at abrunner@engr.psu.edu or visit www.cneu.psu.edu

Penn State Center for Science and the Schools
For more information contact Bill Carlson at wsc10@psu.edu

Science in Motion at Juniata College
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References

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Sondi, I., & Salopek-Sondi, B. (2004). Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. *Journal of Colloid and Interface Science*, 177-182.

Vigneshwaran, N., Nachane, R., Balasubramanya, R., & Varadarajan, P. (2006) A novel one-pot 'green' synthesis of stable silver nanoparticles using soluble starch. *Carbohydrate Research*, **2012-2018**.

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Teacher Notes

Time for Completion:
This lab procedure assumes that the students are familiar with using aseptic technique. The student synthesis of silver nanoparticles takes one class period (45 minutes). The students may synthesize their own silver nanoparticles – this procedure is easy – or the teacher may synthesize the nanoparticles before class. Each of the three antibacterial procedures takes one period to get the inoculated agars incubating. The 24 hour bacterial growth observations and analysis take one period. It is recommended that the students work in lab groups of four students.

Target Grade Level:
The zone of inhibition lab may be used with a middle school through high school biology class. The colony counting lab may be used with a high school biology class. The broth growth lab may be used with an advanced biology class.

Objectives:
1. Students will practice aseptic techniques to inoculate and grow bacteria.
2. Students will graph and analyze results to describe the impact of silver nanoparticles on bacterial growth.

Major Concepts:
*Nano-sized particles in products can impart new properties of practical value.
*Aseptic technique
*Inhibiting bacterial growth

Preparation of Solutions and Tips:
Prepare nutrient agar (CAS 9002-18-0, Ward's Scientific, Cat. # 38-1002) using the instructions on the jar label. Sterilize in the autoclave and pour into sterile plastic Petri dishes. Cool to solidify, tape lids, and store in refrigerator until used.

Prepare nutrient broth (Carolina Biological Supply Co. Cat. # 78-5361) using the instructions on the jar label. Place 5 mL of nutrient broth in spectrophotometer cuvettes, cover with small pieces of aluminum foil, and autoclave. Store in refrigerator until used.

Broth tubes and agar zone of inhibition Petri dishes may be incubated at room temperature overnight or in a 37°C incubator. Broth tubes also may be incubated in a 37°C water bath.

For each of the Petri dish procedures, the 8.5 cm diameter Petri dishes may be used; each takes 20 mL of agar. To save money, the 5.5 cm diameter Petri dishes may be used; each takes 10 mL of agar. If the larger Petri dishes are used, inoculate 100 μL of *E. coli* stock solution. The student lab handout is written using these amounts. If the smaller Petri dishes are used, inoculate 25 μL of *E. coli* stock solution.

Broth tubes must be gently finger vortexed BEFORE taking a reading in the spectrophotometer. The bacteria will settle to the bottoms of the tubes and must be suspended to get an accurate optical density reading.

E. coli K12 stock (American Type Culture Collection # 788969) may be any nonpathogenic strain and grown in sterile nutrient broth to an optical density of 0.03 to 0.04 at $\lambda = 600$ nm. If the bacteria are too concentrated, dilute with sterile nutrient broth. Store the stock *E. coli* broth in the refrigerator until used.

Prepare paper dots by punching from filter paper and sterilizing in the autoclave.

Sterilize student synthesized silver nanoparticles by autoclaving at 121°C for 5 minutes for the broth growth activity.

If the 24 hours observations cannot be taken in 24 hours, put all of the broth tubes or agar plates into a refrigerator to slow the bacterial growth.

All contaminated agar samples should be autoclaved before placing in biological waste.

Sample Data:

Table 1. Zone of Inhibition

Plate	Test Reagent	Diameter of Zone of Inhibition (mm)	Average Zone (mm)
1	Control	0	0
2	Control	0	
3	AgNPs	8	8
4	AgNPs	7	
5	Soap	46	48
6	Soap	50	
7	Disinfectant	11	12
8	Disinfectant	13	

Table 2. Colony Counting

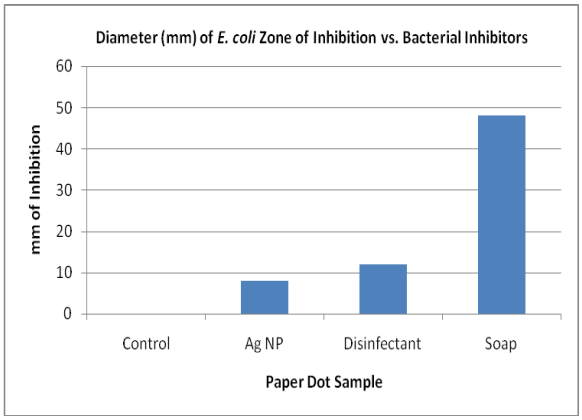
Plate	Amount of AgNPs (μL)	Colony Count	Average Colony Count
1	0	324	350
2	0	376	
3	10	192	210
4	10	228	
5	25	188	218
6	25	248	
7	50	92	130
8	50	168	
9	100	78	81
10	100	83	
11	200	5	8
12	200	10	
13	300	0	0
14	300	0	

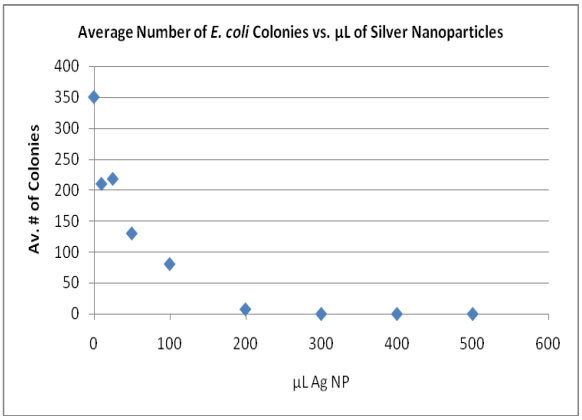
Table 3. Growth in Nutrient Broth

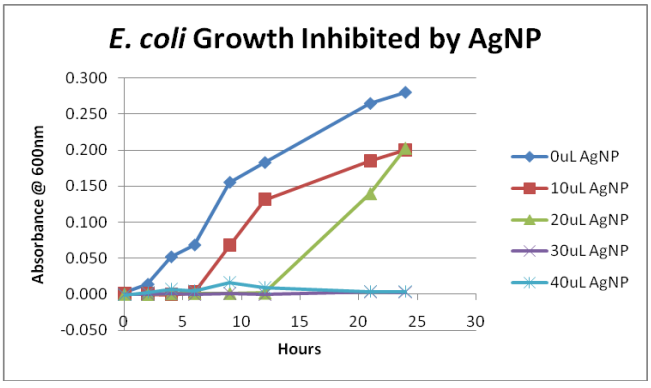
Tube	Amount of AgNPs (µL)	Absorbance at 600 nm							
		0 hrs	Avg. 0 hrs	2 hrs	Avg. 2 hrs	12 hrs	Avg. 12 hrs	24 hrs	Avg. 24 hrs
1	0	0.000	0.002	0.014	0.014	0.180	0.183	0.303	0.281
2	0	0.004		0.014		0.186		0.258	
3	10	0.001	0.001	0.001	0.001	0.142	0.132	0.191	0.201
4	10	0.000		0.000		0.121		0.210	
5	25	0.001	0.001	0.000	0.001	0.003	0.002	0.241	0.203
6	25	0.000		0.001		0.001		0.164	
7	50	0.009	0.006	0.005	0.006	0.003	0.004	0.004	0.004
8	50	0.002		0.007		0.005		0.003	
9	75	-0.001	-0.001	0.004	0.004	0.008	0.005	0.008	0.005
10	75	0.000		0.003		0.002		0.002	
11	100	0.001	0.000	0.004	0.003	0.007	0.007	0.007	0.008
12	100	-0.001		0.002		0.007		0.008	

Answers to Questions:

1.







2. *Zone of Inhibition:* The diameters of the zones of inhibition indicate how well the test reagents inhibit bacterial growth. The test reagents from most effective to least effective are soap, disinfectant, AgNPs, and deionized water. AgNPs do inhibit bacterial growth. It is hypothesized that the zone of inhibition by the AgNPs is small because the heavy masses limit diffusion through the agar.

Colony Counting: With no AgNPs present, the greatest number of bacterial colonies grow. As the [AgNPs] increases, the number of surviving colonies decreases exponentially. At 300 μL AgNPs, all of the bacteria are killed and there are no surviving colonies.

Growth in Broth: The bacteria grow the quickest with no AgNPs present. With the addition of 10 μL AgNPs, growth is inhibited for six hours. With the addition of 20 μL AgNPs, growth is inhibited for twelve hours. With the addition of 30 μL of AgNPs, there is no bacterial growth in 26 hours.

3. *Zone of Inhibition:* The deionized water on the paper dot is the control. The control shows how the bacteria grow around the paper dot with no inhibitor present when using the same lab procedures for all test Petri dishes.

Colony Counting: The agar dish with no added AgNPs is the control. The control shows how many bacterial colonies grow with no AgNPs present when using the same lab procedures for all test Petri dishes.

Growth in Broth: The broth with no added AgNPs is the control. The control shows how much bacterial growth will occur with no AgNPs present when using the same lab procedures for all test cuvettes.

4. Bacterial contamination would probably result in too much bacterial growth. Incorrectly using the pipettes could result in too many or too few bacteria inoculated. If the spread silver nanoparticles do not completely cover the surface of the agar, the bacterial colonies will grow uninhibited in the areas not covered by nanoparticles. If the broth cuvettes are not vortexed before taking an absorbance reading, the growth values will be low. The paper dots could fall off the agar when the Petri dishes are inverted for incubation, giving inconclusive results.

Additional Information

Silver nanoparticles are used to control spoilage of foods, to fight bacterial infections, and to limit bacterial growth in clothing. In minute concentrations, silver is nontoxic to human cells. The high surface area to volume ratio of the nanoparticles allows for increased contact with the bacteria with minimum exposure to silver.

The lethal effects of silver nanoparticles on bacterial cells are suspected to be due to a combination of four processes. Silver nanoparticles bind to the cell walls and cause pits. Silver may bind to respiratory proteins and inhibit respiration. Silver may bind to

sulphur-containing proteins in the cell membrane, interfering with the protein structures, and changing the membrane permeability. Silver may bind to phosphate groups in the DNA, interfering with transcription and replication.

Silver nanoparticles have been used in the food packaging industry to protect foods from external degradation sources, increasing food shelf life. AgNPs are also being used in coatings of food handling machinery to decrease contamination, thus decreasing the amount of down time necessary for maintaining poultry and beef handling equipment. NASA and the military are using clothing infused with silver nanoparticles because the nanoparticles minimize bacterial skin irritations and associated odors. This clothing is highly practical for use in an environment where washing clothing on a regular basis is not possible. Silver nanoparticles are being used in pillows, mattresses, paper used in contaminated areas, biomedical applications linked to HIV detection, refrigerators, freezers, dishwashers, soaps, vacuum cleaners, adhesive strips, and hospital scrubs.

Extension

Students could research products already on the market that contain silver nanoparticles. The research could include opportunities to bring examples of these products (i.e. band-aids, food storage containers, socks) into the classroom. Students could discuss the claims and costs of the products.