# *Part 2: Do Silver Nanoparticles Inhibit Bacterial Growth?*

**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.

**Purpose:** This lesson allows students to test the microbial growth inhibition ability of the silver nanoparticles which were synthesized in Part 1. The students will also learn and practice aseptic techniques to inoculate and grow bacteria. Students will graph and analyze results to describe the impact of silver nanoparticles on bacterial growth. Through this lesson they will understand that nano-sized particles in consumer products can impart new properties of practical value.

**Time Required:** Two 50 minute class periods plus 24 hours for growth of cultures.
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.

**Grade Level: high school biology**
The broth growth lab may be used with an advanced biology class.

**Teacher Background:** Nanoparticles are made of thousands of atoms of an element and are extremely small, ranging from 1 to 100 nanometers (nm) in size. A nanometer is 1 billionth of a meter or $1x10^{-9}$. Nanoparticles often have different properties than those associated with the element at the macroscale. For example, silver, gold, and copper interact with light differently at the nanoscale which in turn affects the color. Nanoscale silver has been used as an antibacterial agent, and can even kill harmful strains of bacteria which are resistant to antibiotics. Silver nanoparticles have been used in the dressing of wounds, surgical masks, food packaging, water treatment, and even socks which prevent the growth of bacteria that cause foul odor. Because of the widespread us of nanosilver, it is important for students to understand its applications as well as potential problems. See the resource section for general information on Ag nanoparticles as well as the environmental implications.

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.



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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.

**Equipment and Materials:**
This lab procedure assumes that the students are familiar with using aseptic technique.
It is recommended that the students work in lab groups of four students.
***Needed for all three procedures:***
Synthesized silver nanoparticles (teacher made or student synthesized in part 1)

- 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
- Incubating oven (optional)

***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*. Wear nitrile/latex gloves when working with *E. coli*

**Advanced 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.

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

**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.

# Student Guide
# (with answers)

## *Part 2: Do Silver Nanoparticles Inhibit Bacterial Growth*

**Introduction:** In this lab you will explore how effective silver nanoparticles are in inhibiting bacterial growth. You will use the silver nanoparticles you synthesized in Part 1 or nanoparticles provided by your teacher.

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.

***Materials:***

***Needed for all three procedures:***

Synthesized silver nanoparticles (teacher made or student synthesized in part 1)

- 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
- Incubating oven (optional)

***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.* Wear nitrile/latex gloves when working with *E. coli*



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**Procedure:** There are three options for this lab and your teacher wil direct you to the one you will do.

***1. 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. Using the same 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. Place lids on petri dishes and tape closed.
6. Wash hands with soap and water.
7. Incubate Petri dishes upside down at room temperature or place in an oven 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.

***2. 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(with aseptic pipette), 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. Cover Petri dishes with lids and tape closed. 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 in an oven 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.

***3. Growth in Nutrient Broth Procedure***

1. Disinfect table.
2. Zero the spectrophotometer at 600 nm using a sterile broth cuvette as the blank.



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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 labeled ape 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. (12 total)
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 in an oven 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.)

**Analysis:**

## 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 | |



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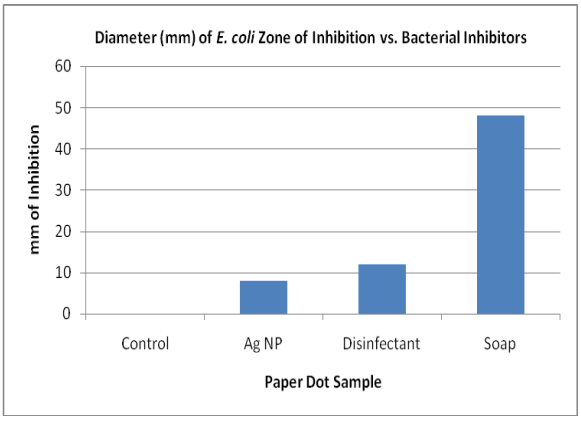
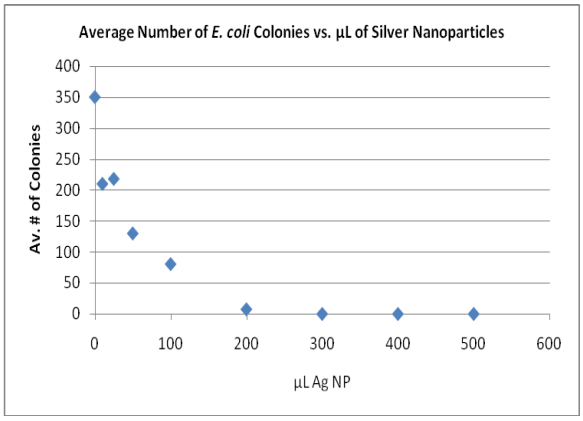
**Table 3. Growth in Nutrient Broth**

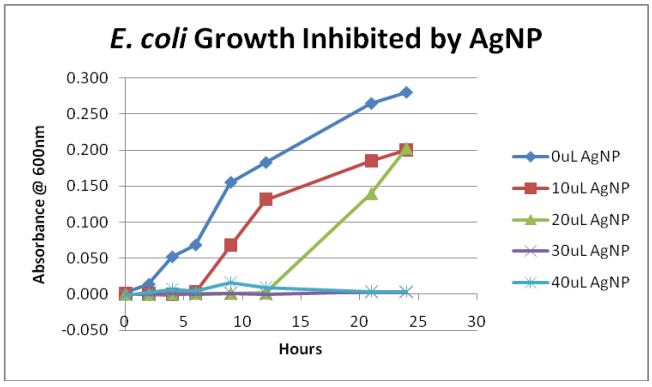
| Tube | Amount of AgNPs (µL) | Absorbance at 600 nm | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 hrs | Avg. 0 hrs | 2 hrs | Avg. 2 hrs | 12 hrs | Avg. 12 hrs | 24 hrs | Avg. 24 hrs |
| 1 | 0 | 0.000 | 0.002 | 0.014 | 0.014 | 0.180 | 0.183 | 0.303 | 0.281 |
| 2 | 0 | 0.004 | | 0.014 | | 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. Prepare a graph showing the relationship between the independent and dependent variables. Samples below:



2. Describe the relationship between independent and dependent variables.
*a. 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.

*b. 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.

*c. 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. What is the control in the experiment? What is the purpose of this control?
*a. 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.

*b. 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.

*c. 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. Why is the aseptic technique critical for accurate reults? Give there examples of aseptic practices used in this experiment.
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.

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

6. Why are silver nanoparticles more effective than bulk silver in inhibiting bacterial growth?
The high surface to volume ratio of the nanoparticles allows for increased contact with the bacteria.

**References:**


1. Pal, S., Tak, Y., & Song, J. (2007). Does Antibacterial Activity of Silver Nanoparticle Depend on Shape of Nanoparticle? A Study on Gram-negative *E. coli*. *Appl. Environ. Microbiol*., 1712-1720.
2. 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.
3. 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**.


**Resources:**

- Silver as an antimicrobial agent: http://microbewiki.kenyon.edu/index.php/Silver_as_an_Antimicrobial_Agent#Current_uses
- Silver nanoparticles and the environment: http://pubs.acs.org/doi/abs/10.1021/es2001248
- Properties and Applications: http://www.sigmaaldrich.com/materials-science/nanomaterials/silver-nanoparticles.html
- Nanoparticles information: http://www.nanoparticles.org/
- Synthesis and Applications: http://www.sciencedirect.com/science/article/pii/S1878535210000377
- Environmental Impacts: http://cohesion.rice.edu/centersandinst/icon/emplibrary/ICON-Backgrounder_NanoSilver-in-the-Environment-v4.pdf and http://www.empa.ch/plugin/template/empa/*/70057/---/l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National Nanotechnology Infrastructure Network
www.nnin.org
Penn State Center for Nanotechnology Education and Utilization
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
For more information contact Tara Fitzsimmons at fitzsit@juniata.edu


National Science Education Standards