Teacher's Guide

Using Biosynthesized Silver Nanoparticles to kill Antibiotic Resistant *E. Coli*.

Grade Level: High school; undergraduate

Subject area(s): Biology; chemistry; biotechnology

Time required: 5-9 days (50 minute periods)

Learning Objectives: Through experimentation, students will understand applications of green chemistry and the antimicrobial aspects of silver. They will apply qualitative and quantitative analysis to results.

Summary: In this lab students will become familiar with biosynthesized silver nanoparticles, or bSNP's. As part of the lesson, students will learn the potential of using biosynthesized silver nanoparticles for use as antimicrobials against antibiotic-resistant bacteria (*Escherichia coli* in this case). Students will create biosynthesized silver nanoparticles in lab using plant extracts of peppermint, Aloe vera, and geranium to reduce $AgNO_3$ to metallic silver (Ag^0). Students will conduct tests to compare bSNP efficacy on streptomycin- and ampicillin-resistant *E. coli* by comparing the 'zone of inhibitions' to those of standard antibiotics.

This lab is designed to introduce students to biosynthesized silver nanoparticles and their medical applications. It is intended to show the antimicrobial properties of biosynthesized silver nanoparticles and their potential applications. Students will conduct both qualitative and quantitative analysis of bSNP efficacy as compared to the use of ampicillin and streptomycin. Students will conduct an activity directly related to leading edge nanotechnology research.

Pre-requisite Knowledge: Students should have experience with chemical synthesis, lab procedures, and bacterial growth.

Teacher background: Silver nanoparticles (SNPs) have long been recognized to have antimicrobial properties (see sources below for further information). These properties have been exploited to combat a multitude of organisms, particularly multi-drug resistant pathogens. Examples of such uses range from topical ointments for preventing infections in burn patients to coatings used in heart stents to ward off endocarditis.

Some concerns with this growing technology are the expense of the process and the SNP chemical synthesis methods. Up until recently, the multi-step synthesis methods used to manufacture SNPs have been extremely expensive and required highly toxic chemicals that pose both environmental and ecological risks.

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Scientists have recently discovered an exciting avenue of using biological methods as a means to synthesize SNPs. These methods provide several benefits not yet seen in commercial manufacturing of SNPs by providing large scale single step production at relatively low cost, and enabling silver production without the use of harsh chemicals.

Recent research has shown a variety of plants with medicinal properties can be used to create bSNP's. This activity will focus on three plants: Aloe vera, geranium, and peppermint. Scientists have shown that extracts from these three plants can be used to create bSNP's that exhibit antimicrobial properties. The teacher may want to read through the articles in the resource section to gain a better understanding of current methods and uses of bSNP's.

Students will also be introduced to methods in which bacteria can gain antibiotic resistances. This activity will focus on two methods: transformation in which *E. coli* will pick up 'naked' DNA or plasmids containing antibiotic resistance; and conjugation in which bacteria will "mate" and transfer plasmid DNA. New research is examining how bacteria develop resistance to SNPs (see Graves articles below). Further exploration of this topic can be completed at the discretion of the teacher.

Sources:

1. S. Ponarulselvam et. al., *"Synthesis of silver nanoparticles using leaves of Cathatanthus roseus Linn. G. Don and their antiplasmodial activities",* Asian Pacific Journal of Tropical Biomedicine 2(7): 574–580, July, 2012. http://wwwelsevier.com/locate/apjtb
2. Z. Sadowski, Wroclaw University of Technology *"Biosynthesis and application of silver and gold nanoparticles"* (July, 2014) (Couldn't find this) http://www.intechopen.com
3. M. Rashid et. al, Aligarh Muslim University *"Biosynthesis of Self-Dispersed Colloidal Particles Using the Aqueous Extract of P. peruviana for sensing d;-Alanine".* (July, 2014) (www.hindawi.com/journals/isrn/2014/670780/)
4. G. Sangeetha et. al. Karpagam University. "*Green synthesized ZnO nanoparticles against bacterial and fungal pathogens*" (December 2012) Progress in Natural Science: Materials International, Volume 22, Issue 6, Pages 693–700
5. U. Kumar Parida et. al. KIIT University. *"Preparation and characterization of green gold nanoparticles conjugate with OMP85 protein"* Advanced Materials Letters , in press. www.vbripress.com
6. Rapid evolution of silver nanoparticle resistance in *Escherichia coli*. Graves, J.L., Tajkarini, M., Cunnighman, Q., Campbell, A., Nonga, H., Harrison, S.H. and Barrick, J.E.. February 2015 6 (42): 1-13. Frontiers in Genetics Accessed at: http://www.ncbi.nlm.nih.gov/pubmed/25741363
7. A Grain of Salt: Metallic and Metallic Oxide Nanoparticles as the New Antimicrobials**.** https://www.jscimedcentral.com/Nanotechnology/nanotechnology-spid-joint-school-nanoscience-nanoengineering-1026.pdf

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Vocabulary and Definitions:

1. **Nanotechnology** - Technology applied to the nanoscale, usually defined as 0- 100nm.At this scale, many materials exhibit unique properties that can be used to create new materials and devices.
2. **Silver nanoparticle (SNP)** - Particles of silver ranging in size from 10 to a few hundred nm in size. Current research suggests a multitude of uses for these particles including use as an antimicrobial. Silver is thought to disrupt cell membranes in bacteria.
3. **Biosynthesized nanoparticle** (Green Nanoparticles)- Employs either microorganism cells or plant extract for nanoparticle production.
4. **Antimicrobial**- an agent that kills microorganisms or inhibits their growth.
5. **Ampicillin**- An antibiotic belonging to the penicillin family of antibiotics. It destroys bacteria by inhibiting cell wall synthesis.
6. **Streptomycin**-An antibiotic belonging to the aminoglycosides family. Streptomycin destroys bacteria by inhibiting protein synthesis.
7. ***Escherichia coli* bacteria**-The bacterium commonly found in the lower intestines of animals. This bacteria is usually non pathogenic and is commonly used in lab.
8. **Bacterial conjugation**- A mechanism bacteria used to exchange DNA. During this process a bacterium will extend a pili to a neighboring bacterium and send plasmid DNA into that bacteria. This plasmid DNA can contain beneficial genes such as antibiotic resistances.

Materials:

- Introductory bacterial conjugation kit (Carolina Biological Science Supply); see Advance Preparation section below.
- Mortar and pestle (optional for grinding plants)
- Razorblades, sharp knives, or dissecting scalpel to finely cut leaves
- Whatman Paper #1
- 100-500ml Erlenmeyer flask
- 10 – 50 mL graduated cylinder
- 0.001 M $AgNO_3$ (20 mL per lab group)
- Peppermint plant, Aloe Vera plant, geranium plant (at least 20 grams of a plant per group)
- Hot plate
- Distilled water (100 mL per lab group)
- 3 hole paper punch
- Economy antibiotic disk, Streptomycin, 10 mcg (Carolina Scientific)
- Economy antibiotic disk, Ampicillin, 10 mcg (Carolina Scientific)
- Shaking water bath (optional)
- Autoclave or bleach solution
- Eye dropper (1 mL capacity)

- Scale to weigh plants
- Funnel
- Sterile agar plate – 1 per group
- Incubator
- Glass beads
- Permanent marker
- Parafilm
- Safety goggles, gloves, aprons or lab coats

Source/Website	Material
Sigma Aldrich (http://www.sigmaaldrich.com)	• $AgNO_3$
Carolina Biological (http:// www.carolina.com)	• Glass beads item #215821 • Ampicillin, streptomycin disks; item # 806493 and 806497 • Introductory bacterial conjugation kit (Used to obtain antibiotic resistant bacteria. Teacher may use other methods to obtain bacteria); Item # 211125 or 211125P
Garden Center	• Peppermint plant • Geranium • Aloe vera plant

Safety Information: Safety goggles, lab coats and gloves should be used throughout the lab. $AgNO_3$ is a hazardous material. Check with you school's chemical safety officer or regulations prior to use. The *E. coli* bacteria used in this activity is a nonpathogenic species. An autoclave or bleach solution should be used to kill off the specimens when the activity is complete. Proper aseptic technique should be used.

Advance Preparation:

1. The teacher should prepare 20ml of 0.001 M aqueous solution of $AgNO_3$ for each lab station prior to students beginning the lab.

2. The "Introductory Bacterial Conjugation Kit" requires significant time to set up. The teacher should read through the procedures to prepare the lab. It is up to the teacher to determine whether or not students want to complete the "Introductory bacterial conjugation kit" or just use the end result for use in this activity. If the teacher has other means of obtaining antibiotic resistant bacteria then the kit need not be used.

Suggested Teaching Strategies or Troubleshooting Tips: This lab can be completed in groups of 3-5. Circulate as students work to answer questions and monitor safety.

Part I of the lab deals with bacteria conjugation as a method used to create antibiotic resistant bacteria. The teacher may want to spend more time on this concept and have students

complete the entire "Introductory Bacterial Conjugation Kit" lab. A teacher may wish to save time and merely create the conjugated strain of *E.coli* for student use. Be sure to autoclave materials when completed. If an autoclave in unavailable use a bleach solution to kill off all samples at the completion of the lab.

Note: If students fail to create viable bacteria or bSNP's during parts I and II, you may want to have pre-made samples ready to be used.

Part II-VI of the lab will have students researching and creating biosynthesized silver nanoparticles from plants to be used as an antimicrobial. The teacher may wish to spend more time on this topic and delve deeper into the biochemistry of silver nanoparticles. This activity is an introduction to these concepts.

Suggested Instructional Procedure:

Time	Activity	Goal
Day 1	**The day before the lab**	
45 min	Introduce students to the topic of silver nanoparticles. Assign students in groups of 3-4. Have students read selected journals, research and share with class the uses and methods used to create silver nanoparticles in the medical field. Have students focus on both commercially created and biosynthesized. *This lab is intended to show the antimicrobial properties of biosynthesized silver nanoparticles. If students have not been exposed to methods of antibiotic defenses of bacteria, extra time should be taken to discuss the topic.* *This lab is intended to connect Biosynthesized Silver Nanoparticles to the medical field. The lab compares efficacy of different antibiotics as compared to biosynthesized silver nanoparticles to kill antibiotic resistant E. coli.*	Review methods that bacteria exhibit to become antibiotic resistant. Introduce how silver nanoparticles are used in the medical field. Introduce the concept of biosynthesized nanoparticles (sometimes referred as green nanoparticles) as compared to commercially manufactured silver nanoparticles
Day 2	**The day of the student lab part I**	
5 min	Students discuss how bacteria become antibiotic resistant. Lead class discussion about the significance of antibiotic resistances.	To ensure students understand how bacteria become antibiotic resistant.

	(see resources for links)	
35-40 min	Using "Introductory bacterial conjugation kit", students will grow ampicillin and streptomycin resistant *E. coli*. Follow kit procedures. Keep a sample of final *E. coli* sample (resistant to ampicillin and streptomycin) for use in later lab.	Students will complete this portion of the lab to create antibiotic resistant bacteria to treat with silver nanoparticles.
5min	Clean up.	
Day 3	**The day of the student lab part II**	
5 min	Students answer warm-up questions. "What are biosynthesized silver nanoparticles and how are they different from commercially produced silver nanoparticles?" "Discuss three uses for silver nanoparticles with your partners." Distribute *Student Worksheets* to students.	To ensure students understand what silver nanoparticles are, how they can be created and their uses specifically as antimicrobial treatment.
35-40min	Students follow procedures and complete Part II of the lab.	To allow students to create bSNP's for use as an antimicrobial.
5min	Clean up.	
Day 4	**The day of the student lab part III**	
5 min	Students answer warm-up questions. "What are silver nanoparticles?" "How can bSNP's be used as antimicrobials?" "What can we expect today as we inoculate petri dishes with our antibiotic resistant *E. coli* and test ampicillin, streptomycin, and bSNPS'? "How can we determine results?"	To ensure students understand what bSNP's are and how they can be used…specifically as antimicrobials.
35-40min	Instruct students to take out Student Worksheets. Students follow procedures and complete Part III of the lab	To allow students to apply their bSNP's to treat antibiotic resistant *E. coli*.
5min	Clean up.	
Day 5	**The day of the student lab part VI**	
45 min	Students observe results and draw conclusions from previous day's experiment.	

Directions for the Activity:
Ask students questions to provoke thought and review what they already know. Possible answers area in red.

1. What are silver nanoparticles? How big are they*? SNP's are particles of silver that range in size between 10 and a few hundred nm and can be used as antimicrobials. An example of SNPs use is for burn victims. Doctors apply a salve containing silver to fight against any potential infections.*
2. What is bacterial conjugation and how can it be used as a method to transfer antibiotic resistances? *Bacterial conjugation occurs when a host bacterium extends a structure known as a pili into a neighboring bacterium. After this pili penetrates the neighboring bacterium plasmid DNA can be transferred. Any genes located on the new plasmid will be used by the bacterium, i.e. antibiotic resistances.*
3. What are potential problems with commercial silver nanoparticle manufacturing ? *The process of creating silver nanoparticles uses toxic chemicals that can end up in the environment. The process is expensive and time consuming.*
4. What does it mean for nanoparticles to be biosynthesized? What are some benefits to this method? *This process employs either microorganism cells or plant extract for nanoparticle production. It is comparatively inexpensive as compared to current commercial methods, doesn't contain the toxic chemicals, and is a one step process.*

Procedure:

Part I

1. Obtain the "Introductory Bacterial Conjugation Kit" and follow kit procedures. The teacher may wish to expedite this portion of the activity and cut out some procedures. Completion of this activity will create viable antibiotic resistant *E. coli*. These bacteria will be resistant to ampicillin and streptomycin and will be used in part III-VI of the lab.

Part II

1. Separate students into groups of 3-5. Each group should be assigned to work with one of three plants; aloe vera, geranium, peppermint. The teacher will want to make sure to have at least two groups working on the same plant to compare data.
2. The students will then thoroughly wash ~20g of leaves from their assigned plant.
3. The students will finely cut 20g of leaves from their assigned plant and put it into an Erlenmeyer flask containing 100ml of distilled water.
4. Students will boil the mixture for 1.5 minutes and let it cool down to a temperature that they can safely handle.
5. Students will pour supernatant liquid (without disturbing sediment) through a Whatman #1 filter paper into a beaker or flask. Note: If students run out of time this solution may be frozen for up to one week or be stored until the next lab period.
6. Students will add 1ml of pure plant broth to 20ml of 0.001M $AgNO_3$.
7. Let mixture sit for 24hrs or until next lab period. Soak 2 circular pieces of paper from a three hole punch in mixture and let dry to create a bSNP disk.

Part III

1. Students will use a sterile agar plate to investigate and compare bSNP's to ampicillin and streptomycin as an antimicrobial.
2. Students will dry the circular pieces of paper containing bSNP's.
3. Students will use glass beads to spread 1ml of *E. coli* evenly onto a petri dish containing agar.
4. Students will wait until liquid has absorbed into the agar and apply antibiotic disks and bSNP disk evenly throughout the petri dish. Be sure to push disks into agar so that they don't move. Wrap petri dishes in parafilm and incubate at 37°c for 24 hrs.

Part IV

1. Students will analyze bacterial growth around all three disks by measuring 'zones of inhibition'. Students may wish to continue growth for an additional 24 hrs.

Cleanup

Instruct students to return all chemicals to the teacher for proper disposal.

Assessment: Assessing this lab can be done in a variety of ways. Partial credit may be given for part I, part II, part III, part IV, conclusion questions, and presentations.

Have students present a review of their findings with the class via a short presentation. Students should be able to answer the questions below. Possible answers in red.

What was the purpose of Part I of the lab?

Part I's purpose was to grow E. coli that was resistant to ampicillin and streptomycin.These bacteria were used to test the efficacy of bSNP's.

Part II of this lab created biosynthesized silver nanoparticles. What was the purpose of adding plant broth to $AgNO_3$? How did your plant assist in creating antimicrobial nanoparticles? Support your answer with information from a review of published research.

Plant broth was used as a reducing agent to create Ag^{+0}. The Ag was coated with plant molecules which allowed for small bundles of silver to exist without binding to other Ag. Answers may vary as to how the plant assists in killing bacteria. Scientists differ on the matter currently. Students should back their ideas with research.

How did bSNP affect bacterial growth as compared to antibiotics?

Student data should show that bSNP's inhibited growth significantly. Students

Additional Resources: You may wish to use these resources either as background or as a resource for students to use in their inquiry-based design.

- Antibiotic/Antimicrobial Resistance: https://www.cdc.gov/drugresistance
- Antibiotic Resistance Fact Sheet: http://www.who.int/mediacentre/factsheets/antibiotic-resistance/en/.

- Prokaryote Conjugation: http://www.nature.com/scitable/definition/conjugation-prokaryotes-290
- Bacterial Conjugation (Wikipedia): https://en.wikipedia.org/wiki/Bacterial_conjugation
- Bacterial Conjugation: http://www.ncbi.nlm.nih.gov/books/NBK21942/
- Application of Nanoparticles to Biology and Medicine: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC419715/
- Biological Synthesis of Nanoparticles from Plants and Microorganisms: http://www.cell.com/trends/biotechnology/fulltext/S0167-7799(16)0

Optional activity extension: Students who have a good grasp of the content of the lab can be further challenged with this assignment

- In your group, research other organisms that are currently being used to create nanoparticles. (What type of nanoparticles? What are they being used for? etc) Present your findings to the class with a slide presentation (using Power Point or equivalent). *Answers will vary*

Next Generation Science Standards
HS-PS1.A Structure and Properties of Matter
HS-PS1-2 Constructing Explanations and Designing Solutions
HS-PS1-8 Developing and Using Models
HS- PS2.B Types of Interactions
HS-PS2-6 Obtaining, Evaluating, and Communicating Information
HS-LS1-A Structure and Function
HS-LS1-3 Feedback mechanisms

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