**Antibacterial Reactivity of Ag(I) Cyanoximate Complexes**

Adapted from: “Light Insensitive Silver(I) Cyanoximates As Antimicrobial Agents for Indwelling Medical Devices.”*Inorg. Chem.* **2010**, *49*, 9863-9874 (DOI: 10.1021/ic100830x)

**Learning Goals:**

After this laboratory experiment, students will be able to:

1. Prepare one of a series of Ag(I) cyanoximate complexes and perform appropriate characterization of identity and purity
2. Measure antimicrobial activity in a semi-quantitative way using the Kirby-Bauer assay, including design and implementation of appropriate control experiments.
3. Evaluate a series of complexes as potential antimicrobials for dental applications based on the criteria of heat stability, water insolubility, and antibacterial activity.
4. Identify most cost-effective complex.

**Introduction:**

Many issues are associated with the rapidly expanding group of U.S. citizens known as the “Baby Boomers.” Health issues are one of the primary concerns of an aging population and associated with that is the increasing practice of joint replacement especially of the knee and hip. The use of dental implants is increasing as those procedures have become less expensive and more practiced resulting in more implants and fewer uses of removable dental appliances.

Infections may arise from the introduction of implants into the human body due to the presence of bacteria such as *Streptococcus mutans* which is a primary cause of dental caries (cavities). Other bacterial sources may be introduced during hip and knee replacements. In this experiment, we will use *e. coli*. and possibly other species.Bacteria introduced into the body in these procedures are very difficult to treat with standard systemic administration of antibiotics so it would be best to introduce some type of antibacterial agent along with the implant or incorporated into the glues used to secure the implant. You may be familiar with the addition of silver nanocrystals in athletic clothing to reduce bacterial growth and odor in those applications. Implants, however, require the antimicrobial agent to be stable at temperatures of sterilization around 121℃ or higher and also be water-insoluble and light stable, but still impart antimicrobial activity. Cyanoximes with the general formula, HO-N=C(CN)-R bonded to various metals, specifically Ag(I) represent a class of compounds with the desired properties. The R-group may be a wide variety of substituent groups, such as heterocycles, alkanes and aromatic moieties.

Your opportunity in this lab is to select one of three possible R groups, synthesize the cyanoxime and then react the cyanoxime with silver to form a new complex. The three R groups available to you are nitrile, carboxamide, and ethyl ester.

Upon successful synthesis, you will then characterize your compound utilizing standard spectroscopic and melting point techniques. Furthermore, you will need to test your compound for heat and light stability (what do you know about silver compounds?) and consider the solubility in water. Finally, you will need to check your compound for antibacterial activity. After you have conducted the synthesis, characterization and antibacterial testing, you will present your findings to your instructor in the method your instructor requires. Make sure your report contains sufficient detail such that your work could be reproduced by a technically trained person. And, congratulations! You may have made the next wonder drug!

**Timeline:**

**Week 1** ‒ **Set up ligand synthesis**

**Week 2** – **Isolate and characterize ligand. Prepare Ag(I) complex.**

**Week 3** ‒ **Characterize metal complex**

-Melting point test for heat resistance

-Qualitative test for solubility

-Set up antibacterial test: make disc (and control experiments). Note that antibacterial assays will need to be checked 24-48 hours after incubation.

**Week 4** ‒ **Collect antibacterial test data.**

-Gather results from classmates.

 -Calculate most effective complex per dollar

**Procedure:**

**Part I: Synthesis of Ag(I) cyanoximate complexes (Periods 1 and 2)**



**Step A: Cyanoxime ligand synthesis H(1-3) using the Meyer Reaction under acidic conditions**

1. Add 4 mmol of your nitrile precursor (**1-3**)and 4.4 mmol solid NaNO2 to a 25 mL round bottom flask.
2. Add 5 mL of distilled water. Seal your flask with rubber septa and sparge the solution with nitrogen for 5-10 minutes to remove air.
3. While sparging, cool the solution to 0°C by stirring in an ice-water bath.
4. Add 6 mmol of glacial acetic acid dropwise using a syringe.
5. After acetic acid has been added, allow the sealed reaction mixture to sit at room temperature overnight or up to one week.
6. To work up, acidify with 3 M HCl to pH ~3 and saturate the solution with ~ 1 g solid NaCl. Extract the aqueous layer with 3 x 5 mL of diethyl ether. Rotovap combined ether layers to dryness. Remove trace amounts of solvent and acetic acid with a vacuum pump, if available.

Once you have purified and isolated your cyanoxime ligand, determine the yield and percent yield of this step. Characterize your ligand using FT-IR spectroscopy and melting point. Collect characterization data before carrying out the metalation with Ag(I).

**Step B: Cyanoximate synthesis of Ag(1-4) (Periods 2 and 3)**

Metalation of each corresponding cyanoxime ligand will be carried out using the following procedure:

1. In a 25 mL round bottom flask or other small flask, dissolve about 0.1 grams of your cyanoxime ligand in a mixture of 2 mL ethanol (EtOH) and 2 mL of distilled water.
2. Heat the cyanoxime ligand solution to 50 °C with stirring.
3. In a separate 25 mL round bottom flask, dissolve 0.5 equivalents (with respect to your cyanoxime ligand) of potassium carbonate, K2CO3 in 2 mL of distilled water (make sure your stoichiometric ratios of ligand to K2CO3 are as close to 1 to 0.5, respectively, as possible).
4. Add the hot cyanoxime ligand solution dropwise to flask containing the aqueous solution of K2CO3. The reaction mixture will change colors immediately.
5. Place your reaction mixture for about 2 minutes into an ultrasound bath to accelerate the evolution of CO2. If an ultrasound bath is not available, allow the reaction to run for longer time (about 20-30 minutes).
6. In a separate flask or beaker, dissolve 1.0 equivalents (with respect to your cyanoximate ligand) of silver nitrate, AgNO3, in about 2 mL of distilled water.
7. Add the aqueous AgNO3 solution to the flask containing your cyanoximate ligand.
8. After 20-30 minutes of stirring, collect the colored precipitate by vacuum filtration, wash with 3 x 2 mL of distilled water, and allow to dry.

Obtain the yield and percent yield of your silver complex. Characterize your complex by FT-IR spectroscopy.

**Part II. Applications (Periods 3 and 4)**

For your compound to perform in its application it must be heat and light stable and be relatively water insoluble. It must also be able to inhibit bacterial growth.

**Heat Stability Studies**

You should consider what defines heat stability in your application and how you might test for heat stability. Think about the physiological environment in which your compound will exist and what might be the minimum and maximum limits of heat stability. Measure the melting point of your compound and use that information to help you determine the heat stability of the complex.

**Aqueous Solubility Studies**

All compounds are soluble to some extent in water. Chemists typically use Ksp values to provide some sense of solubility of a compound. You may want to consider if there are ranges of water solubility that could be tolerated or what might happen if your compound eventually completely dissolves? What if it permanently remains at the site, might that cause problems? Propose an experiment for how you would determine Ksp and explain your proposal in your lab report.

**Antibacterial properties**

The antibacterial properties of your silver compounds will be tested using the Kirby-Bauer technique. You will infuse a piece of filter paper with your compound, and place that piece of paper on a petri dish growing *e. coli* bacteria.

*Assay for antimicrobial activity*

1. Prepare 5-10 mL of a ~10 mg/mL solution of your complex in dimethylformamide (DMF). Weigh your complex using the analytical balance and prepare your solution in a volumetric flask. Calculate the actual concentration based on the actual mass you used.

2. Pipette 20 uL (Or add one drop) of your Ag(I) solution onto a 6 mm paper disk and allow to dry. Prepare two additional disks for control experiments. (See instructions below.)

3. Your instructor will provide a petri dish containing agar growth medium and a culture tube of *e.* *coli.* and/or another bacterial strain in broth. As shown in the figure below, use a cotton swab to plate the bacteria from the test tube onto the growth medium surface. Ensure that the surface is completely coated by swabbing back and forth across the entire plate, then turning the plate 120° and swabbing again. Turn another 120° and complete a third swab. Be sure to dip your cotton swab in the broth frequently in order to ensure a thorough coating.



4. Use tweezers to place your paper disk(s) on the agar surface. Be careful that the disk only touches the plate in the place it is to remain. Heat your tweezers over a flame or in a bacticinerator after you are finished.

5. Place the lid on the petri dish. Label the lid with your name and the date. If you used multiple disks, make notes on the lid of the dish about each disk. Because the lid and dish move independently, make marks on the edge of the lid and dish so that you know how they should line up. Seal the edge of the dish and lid with parafilm.

1. Incubate the plate at 37°C for 24-48 hours with the lid side up. After 24 hours, check your petri dish and measure the diameter (including the paper disk) of the *growth inhibition zone* to the nearest millimeter where the opaque bacteria were unable to grow.

*E. coli*

Growth inhibition diameter

7. Cotton swabs and used petri dishes should be autoclaved before disposal. Ask your instructor about how best to collect the waste. Wash the bench area where you were working with 70% isopropanol. Be sure to wash your hands after working with the bacteria.

*Control experiments*

One important issue to keep in mind is to have control experiments so that you can determine only the effects of the complex. You need to consider what other factors may be interfering with your determination of antibacterial activity. How do we know that your complex and not some other aspect of your procedure is responsible for the antibacterial activity?

Design at least two control experiments to be approved by your instructor. Be sure to label above the dish an identifier that lets you know which piece of filter paper is which. Measure the growth inhibition zone after the same incubation time as your complex.

*Data organization*

1. Collect the growth inhibition data for each of the complexes and control experiments that were completed for all students in your laboratory.
2. Prepare a table showing the average and standard deviation for experiments that were replicates.
3. Determine which complex is the most effective for bacterial growth inhibition.

**Cost Analysis**

The dollar costs of the reagents used in this procedure are listed below (as of August 2015). Calculate the approximate cost of the different complexes, including observed yield. Use this information to determine which complex is the most effective *per dollar*.

|  |  |  |  |
| --- | --- | --- | --- |
| **Product** | **CAS** | **Amount** | **Price** |
| [malononitrile](http://www.sigmaaldrich.com/catalog/product/aldrich/m1407?lang=en&region=US) | 109-77-3 | 100 g | 23.30 |
| [2-cyanoacetamide](http://www.sigmaaldrich.com/catalog/product/aldrich/108448?lang=en&region=US) | 107-91-5 | 100 g | 24.00 |
| [ethyl cyanoacetate](http://www.sigmaaldrich.com/catalog/product/aldrich/e18425?lang=en&region=US) | 105-56-6 | 250 g | 41.60 |
| [sodium nitrite](http://www.sigmaaldrich.com/catalog/product/sial/237213?lang=en&region=US) | 7632-00-0 | 500 g | 65.60 |
| [acetic acid (glacial)](http://www.sigmaaldrich.com/catalog/product/sial/320099?lang=en&region=US) | 64-19-7 | 2.5 L | 99.70 |
| [hydrochloric acid](http://www.sigmaaldrich.com/catalog/product/sial/320331?lang=en&region=US) | 7647-01-0 | 2.5 L | 92.00 |
| [sodium chloride](http://www.sigmaaldrich.com/catalog/product/sial/793566?lang=en&region=US) | 7647-14-5 | 500 g | 37.40 |
| [potassium carbonate](http://www.sigmaaldrich.com/catalog/product/sial/791776?lang=en&region=US) | 584-08-7 | 500 g | 73.70 |
| [silver nitrate](http://www.sigmaaldrich.com/catalog/product/sial/209139?lang=en&region=US) | 7761-88-8 | 25 g | 116.00 |
| [ethanol](http://www.sigmaaldrich.com/catalog/product/sial/459844?lang=en&region=US) | 64-17-5 | 1 L | 112.00 |
| [acetonitrile](http://www.sigmaaldrich.com/catalog/product/sial/271004?lang=en&region=US) | 75-05-8 | 1 L | 108.50 |
| [pyridine](http://www.sigmaaldrich.com/catalog/product/sial/270970?lang=en&region=US) | 110-86-1 | 1 L | 164.00 |
| [2-picoline](http://www.sigmaaldrich.com/catalog/product/aldrich/109835?lang=en&region=US) | 109-06-8 | 1 L | 57.50 |
| [dimethyl sulfoxide](http://www.sigmaaldrich.com/catalog/product/sigma/d4540?lang=en&region=US) | 67-68-5 | 1 L | 91.50 |
| [diethyl ether](http://www.sigmaaldrich.com/catalog/product/sial/673811?lang=en&region=US) | 60-29-7 | 1 L | 137.00 |