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# Antimicrobial Activity of Silver(I) Cyanoximates Against Streptococcus mutans UA159 Planktonic Growth and Biofilm Formation

Sarah Hameer
shameer@rollins.edu

# Antimicrobial Activity of Silver(I) Cyanoximates Against *Streptococcus mutans* UA159 Planktonic Growth and Biofilm Formation

A Senior Honors Project Submitted for Fulfillment of Requirements for Honors in the Major of Biology

Faculty Sponsor: Dr. Santiago-Narvaez

Sarah Hameer
Rollins College, Winter Park, FL

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**Abstract**

*Streptococcus mutans* is identified as the causative agent of human dental caries, with biofilm formation serving as its most important virulence factor. Biofilms are typically more challenging to treat than planktonic or free-floating bacteria, adding to the already challenging issue of antibiotic resistance. This has led to an increased need for the discovery of alternative antibacterial compounds other than antibiotics. Among these compounds, silver(I) cyanoximates have been demonstrated to have antibacterial activity against a variety of microorganisms, specifically those that colonize in-dwelling medical devices. To assess the antibacterial properties of two silver(I) cyanoximate compounds, silver(I) nitrosodicyanomethanide (Ag(ACO)) and silver(I)α-oximido-(2-benzoyl)acetonitrile (Ag(BCO)), growth curves, minimum inhibitory concentration (MIC) assays, minimum biofilm inhibitory concentration (MBIC) assays, and biofilm disruption assays were performed against *S. mutans* UA159. Both compounds inhibited planktonic growth and significantly decreased or disrupted biofilm formation at concentrations between 8 µg/mL and 128 µg/mL. The study of compounds that can target biofilm forming organisms contributes to the overall understanding of how to successfully target *S. mutans* and may lead to better treatment and prevention of dental caries as well as other biofilm related infections.

## Introduction

The oral cavity contains a plethora of surfaces that house numerous kinds of bacteria, including *Streptococcus mutans*, which has been identified as the causative agent of dental caries, or tooth decay. The majority of bacteria in the mouth preferentially colonize the surfaces of teeth, forming a biofilm known as dental plaque (Hamada and Slade, 1980). The formation of plaque plays a key role in the development of dental infections like tooth decay and periodontal disease (Loesche, 1986). There are a number of antimicrobial agents that have the ability to inhibit bacterial growth and biofilm formation, but the continued use of these antibiotics can result in drug resistance (Zhang et al., 2019). Biofilms like dental plaque are more challenging to treat than free-floating, planktonic bacteria. The tougher nature of biofilms in combination with the increasing prevalence of antibiotic resistant microorganisms, calls for the discovery and exploration of new antibiotic alternatives that could have applications in the clinical setting for preventative care of oral diseases.

### *S. mutans' Role in Tooth Decay*

*S. mutans* is a Gram-positive microorganism and a component of dental plaque, along with other microorganisms, saliva, and leftover food particles. Dental plaque is a multi-species biofilm, and the metabolic pathway of *S. mutans*, a component of this microbial community, is what makes this bacterium a major contributor to the formation of dental caries, or tooth decay. *S. mutans* is considered a major pathogen because of its acidogenic and aciduric properties (Zhang et al., 2019). This means that this bacterium has the ability to produce acid as well as survive in an acidic environment. *S. mutans* does not have a respiratory chain and uses glycolysis to generate ATP. Lactic acid is the major end product of glycolysis by *S. mutans* when excess sugar is present (Dashper and Reynolds, 1996).

Any carbohydrate found in plaque that oral bacteria can use as an energy source has cariogenic potential. *S. mutans* is able to utilize sucrose, fructose, glucose, and other sugars, but sucrose is the only dietary carbohydrate that can be used to form extracellular polysaccharides in the plaque, which is why it is considered the most cariogenic carbohydrate in the human diet (Forssten et al., 2010). Sucrose is one of the most preferred carbohydrate sources of *S. mutans,* and many oral bacteria have specific transport systems and enzymes involved in the metabolism of sucrose (Zeng and Burne, 2013).

Previous studies have found that *S. mutans* can implant more easily in the human oral cavity when there is frequent chewing of sucrose heavy foods, and that plaque formation is heavier with a high-sucrose diet than a high-glucose diet (Hamada and Slade, 1980). Thus, an increase in dietary sugar results in added acid production, acidifying the plaque (Forssten et al., 2010). This acidification initiated by *S. mutans* results in an overall decrease in pH of dental plaque found on the surfaces of teeth. The acid causes damage to tooth structure via the demineralization of tooth enamel, leading to the development of carious lesions. *S. mutans* is able to directly adhere to the hydroxyapatite matrix of the tooth. Localized release of acid through *S. mutans*' fermentative metabolism can decrease the pH of the tooth surface to below the critical pH of hydroxyapatite demineralization (Forssten et al., 2010). Diets that are high in sucrose result in increased acid that can exceed the capacity of the saliva to remove and neutralize excess acid, which results in more frequent acidification of the plaque (Forssten et al., 2010).

Modern diets that are high in sugar influence the process of biofilm or plaque formation and play a large role in the occurrence of dental caries. Both content of carbohydrates and frequency of consumption affect cariogenicity. Dental caries can be prevented by eliminating

cariogenic bacteria like *S. mutans* from the oral cavity, increasing the resistance of teeth, and modifying diet (Hamada and Slade, 1980).

*Virulence Factors*

Virulence is defined as the ability of an organism to infect a host and cause a disease. Virulence factors are molecules that assist bacteria in colonizing a host and ultimately causing an infection. *S. mutans* has various virulence factors that contribute to its pathogenicity and enable it to induce the development of carious lesions. The main virulence factors of *S. mutans* that are associated with cariogenicity are adhesion, acidogenicity, acid tolerance, and biofilm formation (Banas, 2004).

Adhesion of oral microorganisms is facilitated by the acquired enamel pellicle, which provides a site for the bacteria to attach. The acquired enamel pellicle covers the enamel of the teeth and has different functions such as serving as a protective barrier from acid substances, promotion of the enamel remineralization process, reduction of tooth friction, and prevention of the drying of tooth surfaces, but it also allows for the formation of a dental biofilm (Alejandra and Daniel, 2019). The ability for *S. mutans* to form biofilms along with its acidogenic and aciduric properties heavily contribute to its virulence and cariogenicity. Biofilm formation is considered an important virulence factor in certain microorganisms because it contributes to microbial resistance to conventional antibiotics, host immune responses, environmental stressors, predation, and other forces (Santos et al., 2018).

*Biofilm Formation*

Biofilms are aggregates of microorganisms that grow on living or nonliving surfaces. These surfaces can include teeth, gums, mucosa, rocks, and implanted medical devices. Biofilm formation is the preferred method of growth for many microorganisms because of the many

advantages it provides: resistance to drugs, nutritional sources, adhesion/cohesion capabilities, protection from environmental stress, immune avoidance, etc. (Santos et al., 2018). Biofilm formation occurs in various stages (Figure 1). The first stage of biofilm formation involves the reversible attachment of free-floating, planktonic bacteria to a surface. As more bacteria attach to the initial colonizers on a surface, a monolayer and a matrix begin to form, resulting in irreversible attachment (Vasudevan, 2014). The matrix holds all of the bacterial cells together in a mass and attaches this mass onto the surface that is being colonized (Kaplan, 2010). The extracellular matrix often contains components from the host; for example, in the context of an *S. mutans* biofilm, the extracellular matrix contains saliva glycoproteins. The components of the extracellular matrix help to maintain biofilm architecture and stabilize it through intermolecular interactions, providing a protected, nutritionally rich ecological niche that enhances microbial survival (Santos et al., 2018).

Bacteria continue to join the biofilm over time, leading to biofilm maturation. At this point, the biofilm is a heterogeneous community in terms of bacterial metabolism; certain cells within the microbial community will be metabolically active, while others are metabolically inactive, or dormant. Metabolic heterogeneity, specifically the presence of dormant or persister cells, is a key characteristic of biofilms that allows for resistance to antimicrobials (Lewis, 2010). Mature biofilms form pillar and mushroom shaped structures that contain channels, which allow for the exchange of nutrients and oxygen as well as the expulsion of waste (Kaplan, 2010). Once attachment, microcolony formation or proliferation, and maturation has happened, dispersion or detachment of the biofilm can occur (Santos et al., 2018). Biofilm dispersal happens when portions of the established biofilm detach and colonize a new surface. This can occur due to stressors such as limitation of nutrients, and also allows for the transmission of pathogenic

bacteria from environmental reservoirs to human hosts (Kaplan, 2010). Dispersion is a key stage of the biofilm lifestyle because it allows for and increases bacterial survival and transmission of disease.

Biofilms can be single- or multi-species. Multi-species or polymicrobial biofilms are more complex than single-species biofilms because of the cooperation that occurs between microorganisms (Santos et al., 2018). All biofilms produce exopolysaccharides, which are the main component of the extracellular matrix. The extracellular matrix plays a large role in microbial adhesion to surfaces, as well as in cohesion, while also hindering the ability of compounds like antimicrobials to diffuse through a biofilm (Koo et al., 2013). Different microorganisms produce different kinds of exopolysaccharides; for example, *Streptococcus mutans*' exopolysaccharides consist of mainly glucans, polysaccharides derived from D-Glucose and linked by glycosidic bonds (Koo et al., 2010).

The biofilm nature of *S. mutans* aids in the process of tooth decay because of its ability to adhere to the enamel salivary pellicle, which is a glycoprotein layer that covers a tooth's surface. As mentioned earlier, *S. mutans* produces extracellular glucans, a virulence factor that serves as a key component of the biofilm's extracellular matrix. Glucans act as "glue" by encouraging adherence and accumulation of cariogenic bacteria (Chen et al., 2016). Glucans play an essential role in cell-cell and cell-surface adhesive interactions in plaque, with water-soluble glucan being the main glucan responsible for plaque progression (Forssten et al., 2010). Since *S. mutans* forms a multi-species biofilm, it is more resistant to environmental stressors, predators, detergents, and antibiotic treatment than single-species biofilms (Townsley and Shank, 2017).

*Antibiotic Resistance*

Antibiotic resistance is a major challenge being faced worldwide. Antibiotics work by disrupting essential bacterial processes and targeting structures that are unique to bacterial cells. The main structures and processes targeted by antibiotics include the synthesis of peptidoglycan, which makes up the bacterial cell wall, DNA replication, and protein synthesis. All of these processes are essential to bacterial survival, and have unique components that are not found in human cells, which allow antibiotics to successfully target only the bacteria. Multidrug resistant organisms have arisen, causing infections that are extremely difficult to treat or even untreatable. In recent years, there has been a dramatic increase in infections caused by resistant microorganisms that do not respond to conventional treatment, and even last-resort antibiotics have proven unsuccessful (Frieri et al., 2017). The number of resistant pathogens is continuing to increase due to the unnecessary and improper use of antibiotics, and the search for alternative methods of treatment has become crucial.

Biofilms in particular are tougher to treat with antibiotics than free floating bacteria because of the presence of persister cells. Bacteria found in biofilm communities tend to have higher rates of horizontal gene transfer and more frequent rates of mutation than those of planktonic cells (Gebreyohannes et al., 2019). Bacteria within a biofilm can easily transfer antibiotic resistance genes to other microbes within the community, adding another layer of difficulty when it comes to treatment. It is challenging for antibiotics to penetrate through biofilms due to their density, and once they do, they are only able to target metabolically active cells. After treating a biofilm with antibiotics, persister cells are the only living cells that remain, and once antibiotic levels decrease, these cells become metabolically active and repopulate the biofilm (Lewis, 2010). Thus, higher doses of these drugs over a longer period of time are needed

to treat biofilms. This method of treatment can still be ineffective, leading to infection persistence (Frieri et al., 2017).

Antibiotic agents have been proven to be useful in decreasing the development of dental caries. Although *S. mutans* is highly susceptible to many antibiotics like penicillin, ampicillin, and erythromycin, it is not practical for them to be used for caries control in the long run (Hamada and Slade, 1980). The discovery of alternative antiplaque or antimicrobial agents that can reduce the rate of accumulation of plaque, reduce or remove existing plaque, or inhibit the growth of bacterial species implicated in disease would have more practical long-term applications (Forssten et al., 2010).

*Silver Compounds as Antibiotic Alternatives*

The search for alternative antimicrobial agents is becoming increasingly important due to the growing prevalence of antibiotic resistant microorganisms. Natural alternatives to antibiotics already exist, but the challenge lies in figuring out how to implement them in a clinical setting (Ghosh et al., 2019). Advances in fields like synthetic chemistry and biotechnology have made the search for antibiotic alternatives possible. Broad-spectrum antibiotics kill bacteria indiscriminately, removing both pathogenic and nonpathogenic microbes, and can lead to the evolution of antibiotic resistant strains. This phenomenon has increased the necessity for more selective antibiotics or antibiotic alternatives.

Silver, a transition metal, has long been used for a wide range of medical applications, such as topical creams, dental amalgams, and medical devices like catheters and heart valves because of its antibacterial effects (Mijnendonckx et al., 2013). Different forms of silver are utilized in the medical setting such as silver nitrate, silver sulfadiazine, and colloidal silver (Sim et al., 2018). There are various ways that silver can be incorporated into products and used in the

clinical setting. A common application of silver is in the surface coating of medical apparatuses, where implanted devices can be coated with silver nanoparticles to benefit from its antimicrobial effects (Sim et al., 2018). Applications of silver aside from its use for indwelling medical devices include their incorporation into topical treatments and wound dressings.

Silver is biologically active in its monoatomic ionic state, which is when it is soluble in aqueous environments (Sim et al., 2018). Various mechanisms have been discovered that describe how different forms of silver can target microbes. Silver ions can be used to create holes in the bacterial cell wall, silver ions can enter the cell and inhibit cellular respiration while disrupting metabolic pathways and generating reactive oxygen species, and silver can disrupt the process of DNA replication (Sim et al., 2018).

Silver ions and silver-based compounds in general are highly toxic to microorganisms, but present low toxicity for human cells, which is one of the essential characteristics of an antibiotic agent. Due to size, shape, surface charge, concentration, and colloidal state, silver nanoparticles are able to adhere to and penetrate microbial cells, while modifying microbial signal transduction pathways (Dakal et al., 2016). The bacteriostatic and bactericidal effects of silver in general are due to interactions of the compound with bacterial DNA, inactivation of bacterial respiration enzymes, generation of reactive oxygen species, and disruption of membrane permeability, transcription, and translation (Lotlikar et al., 2019). Research involving nanoparticles, which are nano-sized molecules that are usually less than 100 nanometers in diameter, has gained significant interest in the search for new antimicrobial agents (Kandi and Kandi, 2015). The use of silver nanoparticles in particular has recently been developed as a way to combat multidrug resistant microorganisms as an alternative to conventional antibiotics.

Silver based compounds have been found to be effective against both planktonic and biofilm bacterial growth, increasing their attractiveness as an antibiotic alternative. Attachment is a very important aspect of biofilm formation, and silver has been found to prevent bacterial adhesion without affecting host cell integration in the case of indwelling medical implants such as catheters and dental devices, making it an ideal antimicrobial agent (Eckhardt et al., 2013).

*Use of Silver in Clinical Dentistry*

Silver has known anticaries properties and has been used in the dental setting for centuries. Silver nitrate was one of the first silver compounds used to arrest cavities. It was claimed to form a barrier, preventing decay in the dentine, the second layer of tooth found below the enamel (Peng et al., 2012). Since then, silver has been incorporated into various dental materials in the form of silver fluoride and silver diamine fluoride and is also a main component of dental amalgam, which used to be the most common material used to fill cavities. Use of silver compounds in the clinical dental setting has been a slight cause for concern when it comes to tissue ulceration or white changes in the mucosa upon its use, however, these effects are very mild and short-lived (Peng et al., 2012). Given the transient nature of these effects, the anticaries benefits of silver may outweigh them.

The use of silver nanoparticles in clinical dentistry is gaining popularity because of their established effectiveness as antimicrobial components of various medical devices. Specific to the dental field, silver nanoparticles have been incorporated into various prosthetic materials, adhesives, and implants to prevent biofilm formation and limit tooth decay (Noronha et al., 2017). Silver nanoparticles and silver compounds in general also have the capability to produce a synergistic effect with other antimicrobial agents, creating a world of possibilities when it comes to the application of silver in the future of oral healthcare.

*Silver(I) Cyanoximates*

Silver(I) cyanoximates have been established as antimicrobial agents for indwelling medical devices due to their properties of light insensitivity, poor water solubility, high thermal stability, lack of toxicity to organic ligands, and *in vitro* antimicrobial activity (Gerasimchuk et al., 2010). Both silver and cyanoximes have a range of biological activities on their own, and produce a heightened antimicrobial effect when combined. These silver compounds have been found to inhibit biofilm growth of Gram-positive and Gram-negative bacteria, and some are equally as effective against planktonic and biofilm growth, despite the more resistant nature of biofilms (Lotlikar et al., 2019). The specific mode of action of silver(I) cyanoximates is not well known, but studies have been performed comparing silver nanoparticles and silver(I) cyanoximates that have suggested potential differences in the modes of action of these two antimicrobial agents (Lotlikar et al., 2019).

Silver(I) cyanoximate compounds such as silver(I) nitrosodicyanomethanide (Ag(ACO)) and silver(I)α-oximido-(2-benzoyl)acetonitrile (Ag(BCO)) can have practical applications in the clinical dental setting for the prevention of dental caries. These compounds can be incorporated into dental materials like light-curable acrylate polymeric composites that are used as fillers and adhesives (Lotlikar et al., 2019). There is also potential for silver(I) cyanoximates to be used for the prevention of infection after dental procedures or surgeries. A synergistic effect has been observed when certain silver(I) cyanoximate compounds are added together with conventional antibiotics, which indicates a high potential of these compounds to be used as antimicrobials (Lotlikar et al., 2019).

*Future Implications of this Research*

This research is important in the field of oral health and could have significant implications on dentistry in the clinical setting. The overall goal of our study is to provide an increased understanding of how to target this microbe in an effort to prevent oral diseases such as dental caries and periodontal disease. In recent years there has been a shift from treatment to prevention when it comes to oral care. There are certain mechanical oral hygiene procedures that help to control plaque, but they are not sufficient, which is why the supplementation of antimicrobial agents to dental healthcare products has been very valuable (Forssten et al., 2010).

Expanding on current knowledge and gaining a more in-depth understanding of silver(I) cyanoximate compounds and their effects on different types of *S. mutans* growth—particularly biofilm growth—may ultimately lead to their application in preventative dentistry. Various studies have been conducted in an effort to discover antibiotic alternatives that are effective against *S. mutans*. However, little is known about the specific effects and mechanism of action of silver(I) cyanoximate compounds on planktonic and biofilm growth of *S. mutans*. The objective of this project is to determine the effects of certain concentrations of two silver(I) cyanoximate compounds, Ag(ACO) and Ag(BCO), on *S. mutans* growth via bacterial growth curves, minimum inhibitory concentration (MIC) assays, minimum biofilm inhibitory (MBIC) assays, and biofilm disruption assays.

**Materials and Methods**

*Growth Conditions*

*S. mutans* UA159 was struck out onto Brain Heart Infusion (BHI) (Sigma-Aldrich, St. Louis, MO) agar to obtain single, isolated colonies. Plates were incubated for 24-48 hours at 37°C

in a 5% (v/v) $CO_2$/ 95% air atmosphere. An isolated colony was then suspended into BHI broth and incubated for 24 hours to create an overnight culture.

*Preparation of Compounds*

Ag(ACO) and Ag(BCO) compounds were resuspended in dimethyl sulfoxide (DMSO) to create two 1000 µg/mL stock solutions, one for each compound. Two-fold serial dilutions were performed from 128 µg/mL to 0.125 µg/mL for each compound to be used for growth curves, MICs, MBICs, and biofilm disruption assays. The Ag(BCO) ligand, the Ag(ACO) ligand, and silver nitrate were also resuspended in DMSO to make 1000 µg/mL stock solutions, which were serially diluted at the same concentrations as Ag(ACO) and Ag(BCO) to be used for growth curve controls. The same was done with ciprofloxacin and ampicillin to create 100 µg/mL stock solutions, which were then serially diluted to be used as controls.

*Growth Curves*

The optical density (OD600) of the overnight culture was measured with a DeNovix spectrophotometer (DS-11+ (M/C)) and diluted to an optical density between 0.05 and 0.1. Three 96-well plates were inoculated: one containing the positive and negative controls and the remaining two containing the Ag(ACO) and Ag(BCO) compounds, respectively. Half of the control plate was inoculated with UA159 alone and the other half with UA159 plus 20µg/mL ciprofloxacin. Plates containing Ag(ACO) and Ag(BCO) were inoculated with UA159 plus increasing concentrations of each compound from 0.125 µg/mL to 128 µg/mL. OD600 of all plates was read and recorded after inoculation, serving as the 0-hour time point. Plates were incubated for 2 hours at 37°C in a 5% (v/v) $CO_2$/ 95% air atmosphere after which OD600 was read and recorded again. From this point on, OD600 was recorded every 1.5 hours. Plates were left in the incubator overnight and the final OD600 values were recorded the following morning

at the 25-hour time point. OD600 was plotted against time for all treatment conditions to generate growth curves. The same protocol was followed to generate control growth curves of UA159 in the presence of the Ag(BCO) ligand, Ag(ACO) ligand, and silver nitrate.

*Minimum Inhibitory Concentration (MIC) Assay*

UA159 overnight cultures were normalized to 0.3 in BHI broth. The normalized culture was diluted 1:100 and this diluted culture was used to inoculate 96-well plates. Plates containing Ag(ACO) and Ag(BCO) were inoculated with increasing concentration of the compounds from 0.125µg/mL to 128µg/mL in each column. A control plate was inoculated with increasing concentration of ciprofloxacin from 0.625 µg/mL to 40 µg/mL. A sample from the 1:100 diluted culture was serially diluted from $10^{-2}$ to $10^{-5}$ and plated in duplicate to determine bacterial load. After inoculation, all plates were incubated overnight at 37°C in a 5% (v/v) $CO_2$/ 95% air atmosphere. The following morning, turbidity of the wells in the 96-well plates was observed and recorded to determine the MICs. Dilution plates were left in the incubator for 48 hours and then colonies were counted in order to calculate CFU/mL.

American Type Culture Collection (ATCC) strains of *Staphylococcus aureus* 29213 and *Escherichia coli* 25922 were used to perform control MIC assays with ampicillin. Overnight cultures of *S. aureus* and *E. coli* were normalized to 0.3 in Luria-Bertani (LB) broth (Sigma-Aldrich, St. Louis, MO). The normalized culture was diluted 1:100 in LB and the diluted culture was used to inoculate 96-well plates. Two columns served as media and growth controls, containing LB only and LB plus the diluted cultures, respectively. The remaining wells were inoculated with increasing concentrations of ampicillin from 0.048 µg/mL to 25 µg/mL. A sample from the 1:100 diluted culture was serially diluted from $10^{-2}$ to $10^{-5}$ and plated in duplicate to determine bacterial load. After inoculation, plates were incubated overnight at 37ºC.

Turbidity of the wells was observed and recorded after incubation to determine MIC values. Dilution plates were left in the incubator for 24 hours and colonies were counted to calculate CFU/mL for both *S. aureus* and *E. coli*.

*Minimum Biofilm Inhibitory Concentration (MBIC) Assay*

MBIC assays were performed similarly to MIC assays in 96-well plates. Overnight cultures were diluted in the same way, however, for the MBIC assays, tryptone yeast plus 1% (w/v) glucose (TYG) broth and TY plus 1% sucrose (TYS) broth were used instead of BHI. After inoculation, the 96-well plates were incubated at 37ºC overnight. The next day, the supernatant was removed and wells were washed with distilled water and allowed to dry overnight. Biofilms were then stained with 0.1% crystal violet, washed with distilled water, and left to dry for a few hours. To solubilize the crystal violet, 30% acetic acid was added to all wells. Solubilized crystal violet was transferred to new microtiter plates and absorbance was measured with a microplate reader at 575 nm (BioTek Synergy HTX Multi-Mode Reader), using acetic acid as the blank. Graphs displaying average absorbance values for all samples were generated to determine the MBIC of each compound.

*Biofilm Disruption Assay*

UA159 overnight cultures were normalized to 0.3 in BHI. Microplates were inoculated with TYS broth and the normalized culture, with one column serving as a media only control. Plates were incubated at 37ºC overnight to allow for biofilm growth. The next day, the supernatant was removed and plates were inoculated with fresh media containing increasing concentrations of Ag(ACO) and Ag(BCO) from 0.25 µg/mL to 128 µg/mL. Plates were incubated for another 24 hours at 37ºC. After incubation, the supernatant was removed and wells were washed with distilled water and allowed to dry for a few hours. Remaining biofilms were

stained with 0.1% crystal violet, washed with distilled water, and left to dry for another few hours. Crystal violet was solubilized with 30% acetic acid. The solubilized crystal violet was transferred to new microtiter plates and absorbance was measured with a microplate reader at 575 nm (BioTek Synergy HTX Multi-Mode Reader), using acetic acid as the blank. Graphs displaying average absorbance values for all samples were generated to assess which concentrations of each compound resulted in the most significant biofilm reduction.

**Results**

*Growth Curves*

To demonstrate the general effects of the Ag(BCO) and Ag(ACO) compounds on *S. mutans* growth, growth curves were generated by tracking *S. mutans* growth over a 25-hour period in the presence of an increasing concentration of each compound. Higher concentrations of both compounds demonstrated a negative effect on growth. For Ag(BCO), a significant decrease in growth compared to the UA159 control was seen at 128 µg/mL (Figure 2A). A significant decrease in growth compared to the UA159 control in the presence of Ag(ACO) was observed at concentrations of 64 µg/mL and 128 µg/mL (Figure 2B).

Growth curves were also performed in the presence of the Ag(BCO) ligand, Ag(ACO) ligand, and silver nitrate to serve as controls. All compounds demonstrated a negative effect on growth at concentrations of 64 µg/mL and 128 µg/mL (Figure 3). Silver nitrate also demonstrated a slight reduction in growth at 32 µg/mL compared to the UA159 control (Figure 3C).

*Minimum Inhibitory Concentration (MIC) Assay*

MIC assays were performed to gather qualitative data suggesting whether or not Ag(BCO) and Ag(ACO) prevent planktonic growth of *S. mutans*. A 96-well microtiter plate was

inoculated with bacteria and increasing concentrations of each compound. After 24 hours of growth, turbidity of the wells was observed and recorded. The MICs or lowest concentrations of Ag(BCO) and Ag(ACO) that produced clear wells, indicating no bacterial growth, were 8 µg/mL and 16 µg/mL, respectively (Figure 4). Control MIC assays were performed using ampicillin and ATCC strains of *S. aureus* 29213 and *E. coli* 25922. The lowest concentrations of ampicillin that inhibited *S. aureus* and *E. coli* planktonic growth were 0.195 µg/mL and 1.5625 µg/mL, respectively (Figure 5).

*Minimum Biofilm Inhibitory Concentration (MBIC) Assay*

In order to determine the concentrations of Ag(BCO) and Ag(ACO) needed to inhibit *S. mutans* biofilm growth, MBIC assays were performed. Biofilms were cultivated in the presence of the compounds and then stained with crystal violet. Absorbance was measured, which was then used to calculate percent increase or decrease in biofilm growth and determine the MBIC values for each compound. Ag(BCO) and Ag(ACO) caused a significant decrease in *S. mutans* biofilm growth at 16 µg/mL and 32 µg/mL, respectively (Figure 6). At 16 µg/mL, Ag(BCO) caused a 27.6% decrease in *S. mutans* biofilm growth (Table 1). At 32 µg/mL, Ag(ACO) caused an 84.31% decrease in *S. mutans* biofilm growth (Table 1). Following these significant decreases in biofilm growth, percent decrease values continued to grow larger with increasing concentrations of both Ag(BCO) and Ag(ACO). The highest reduction in biofilm growth in the presence of each compound was determined to be a 69.69% reduction at 64 µg/mL for Ag(BCO), and an 86.7% reduction at 128 µg/mL for Ag(ACO) (Table 1).

*Biofilm Disruption Assay*

Biofilm disruption assays were performed to determine if the compounds, Ag(BCO) and Ag(ACO), are capable of disrupting already established *S. mutans* biofilms. Biofilms were

cultivated in the absence of the compounds, and then the compounds were added at increasing concentrations to the established biofilms. After treatment with the compounds, the remaining biofilms were stained with crystal violet and absorbance was measured to calculate percent increase of decrease in biofilm robustness. Significant decreases in biofilm thickness were observed starting at 16 µg/mL for both Ag(BCO) and Ag(ACO) compounds (Figure 7). At this concentration, Ag(BCO) caused a 31.86% decrease in biofilm thickness and Ag(ACO) caused a 28.99% decrease in biofilm thickness (Table 2). The overall trend of biofilm disruption for both Ag(BCO) and Ag(ACO) was an increase in disruption at higher concentrations of each compound. The highest level of disruption for Ag(BCO) was 43.72% at 128 µg/mL, and that of Ag(ACO) was 50.18% at 128 µg/mL (Table 2).

**Discussion**

The effects of two silver(I) cyanoximate compounds, Ag(BCO) and Ag(ACO), on *S. mutans* growth were assessed via growth curves, MIC assays, MBIC assays, and biofilm disruption assays. Overall, these experiments provided information about the antimicrobial activity of these compounds against both free-floating, or planktonic, and biofilm growth of *S. mutans*, the causative agent of dental caries. We expected that both Ag(BCO) and Ag(ACO) would decrease or have negative effects on both planktonic and biofilm growth of *S. mutans*, given the established antimicrobial properties of silver (Ag) and cyanoximes. Our results support this hypothesis, demonstrating a negative trend in *S. mutans* growth with increasing concentration of the respective compound for each type of experiment we performed.

Generation of growth curves allowed us to assess the general effects of Ag(BCO) and Ag(ACO) compounds on *S. mutans* growth before moving on to other assays. The growth curves we generated demonstrate the characteristic lag, log or exponential, and stationary phases of

bacterial growth. The lag phase is found at the beginning of the curve and is representative of the phase where bacteria are growing and dividing. This is followed by the log phase, which is a phase of exponential bacterial growth where more cells are dividing than dying. The stationary phase comes next, reaching a plateau in bacterial growth because the number of cells dividing roughly equals the number of cells that are dying. By tracking *S. mutans* growth over an extended period of time in the presence of an increasing concentration of each compound, we found that higher concentrations of both Ag(BCO) and Ag(ACO) demonstrated a negative effect on growth (Figure 2). A delay in the log or stationary phase of the growth curve would imply that bacterial growth was inhibited (Wang et al., 2016). Our data demonstrates delays in the log and stationary phases when the bacteria are in the presence of the compounds at high concentrations. The Ag(BCO) and Ag(ACO) data collected followed the expected trend of a decrease in growth in the presence of a higher concentration of compound. Both Ag(BCO) and Ag(ACO) demonstrated delays in these phases of bacterial growth at a concentration of 128 µg/mL, and Ag(ACO) also showed a delay at a concentration of 64 µg/mL (Figure 2).

As a control, growth curves were generated using the Ag(BCO) ligand, Ag(ACO) ligand, and silver nitrate, which serve as the synthetic precursors of the silver(I) cyanoximate compounds we are testing. We found that both ligands and silver nitrate demonstrated a delay in the log and stationary phases at 64 µg/mL and 128 µg/mL, with silver nitrate showing a slight delay at 32 µg/mL as well (Figure 3). Ag(BCO) and Ag(ACO) are silver(I) cyanoximate compounds, which are complexes of silver and cyanoximes, or a class of oxime-based versatile ligands (Lotlikar et al., 2019). Despite demonstrating inhibition of growth at the same concentrations as the ligands and silver nitrate controls, the Ag(BCO) and Ag(ACO) compounds are more practical antimicrobial agents than the control compounds alone. Silver nitrate on its own is an effective

antimicrobial, as indicated in our growth curves; however, leeching of silver ions is a problem when silver nitrate is used in the clinical setting because a large amount can be toxic, and if used in a polymer, leeching of metal ions will weaken the overall polymeric structure (Lotlikar et al., 2019). This is especially important in the dental setting, where these antimicrobial compounds can be incorporated into materials such as composite used for fillings, which needs to be strong in order to successfully execute a procedure. Cyanoximes, like the Ag(ACO) and Ag(BCO) ligands, have antimicrobial properties, growth-regulating properties, and are barely soluble at high temperatures (Lotlikar et al., 2019). This means that when a silver complex is formed using these ligands, as in the case of Ag(ACO) and Ag(BCO), there is a synergistic effect with minimal leeching of silver ions, making them more practical for use in the clinical setting.

Minimum inhibitory concentration (MIC) assays provide better insight and data regarding the effects of antimicrobial compounds on growth than growth curves. This assay served as our next step in determining the antimicrobial activity of these silver(I) cyanoximate compounds against planktonic *S. mutans* growth. The overall goal of MIC assays is to determine the lowest concentration of the tested antimicrobial agent that inhibits visible growth of the bacteria (Wiegand et al., 2008). Prior to running MIC assays with the Ag(BCO) and Ag(ACO) compounds, an ATCC control experiment was performed. Given that MIC assays are standardized to validate our experimental design, it was important to first conduct MIC assays with ATCC control strains of both Gram-positive *S. aureus* and Gram-negative *E. coli*. This experiment served as quality control, where we used ampicillin, an antibiotic known to inhibit growth of both *S. aureus* and *E. coli*. Known MIC values for commonly used antibiotics for ATCC or quality control microorganisms are published, and experimentally obtained MIC values for control strains should fall between the published range to be acceptable (Weigand et al., 2008). The MIC values we

obtained from our ATCC control experiment fell within the range of literature MIC values for both bacterial strains tested. These results validated our experimental design, allowing us to move forward with MIC assays using the Ag(BCO) and Ag(ACO) compounds.

MIC assays were performed to gather qualitative data suggesting whether or not Ag(BCO) and Ag(ACO) prevent planktonic growth of *S. mutans*. Microtiter plates were inoculated with bacteria and increasing concentration of compound. After 24 hours of growth, turbidity of the wells was observed and recorded. The lowest concentrations of Ag(BCO) and Ag(ACO) that produced clear wells, indicating no bacterial growth, were 8 µg/mL and 16 µg/mL, respectively (Figure 2). The MIC assays provide a more accurate depiction of the concentrations of each compound that inhibit planktonic growth than growth curve data, and all replicates we performed produced similar results. However, the more common mode of bacterial growth *in vivo* is in the form of biofilms, such as plaque in the oral cavity. Thus, we performed minimum biofilm inhibitory concentration (MBIC) assays to gain a better understanding of the silver(I) cyanoximates' effects on biofilm growth, which behave differently from free-floating cells and has been established as the natural mode of bacterial life (Sandberg et al., 2008).

In order to determine the lowest concentration of compound needed to inhibit *S. mutans* biofilm growth, MBIC assays were performed. This assay was performed in a similar manner to the MICs using a 96-well microtiter plate, however the results are quantitative instead of qualitative. Crystal violet staining of biofilms allowed us to measure absorbance, which was then used to calculate percent increase or decrease in biofilm growth. Crystal violet measures the amount of biofilm present and is useful to assess prevention and destruction of biofilms (Sandberg et al., 2008). We found that Ag(BCO) and Ag(ACO) caused a significant decrease in *S. mutans* biofilm growth at 16 µg/mL and 32 µg/mL, respectively (Figure 3). The MBIC values of both

Ag(BCO) and Ag(ACO) are higher than the MIC values. Given the tougher nature of biofilms and the limited ability of antimicrobials to diffuse through them, we expected to see these differences in MIC and MBIC values, demonstrating that biofilms have a higher resistance to the compounds we tested than planktonic cultures (Koo et al., 2013). A large problem when it comes to infections caused by biofilms is the increased resistance compared with free-floating bacteria, and often antimicrobial agents that are effective against planktonic bacteria are completely or significantly less effective against biofilms (Sandberg et al., 2008). Our results indicate that Ag(ACO) and Ag(BCO) compounds are nearly as effective against preventing *S. mutans* biofilm growth as they are against *S. mutans* planktonic growth.

While prevention of biofilm formation is an important factor to gauge when testing antimicrobial agents, it is also key to examine the effects of the compounds against already established biofilms. A good antimicrobial agent should not only be able to inhibit biofilm formation, but also destroy an already formed biofilm. This is highly relevant when it comes to dental treatments, given that biofilms are easily able to form on tooth surfaces and cleaning is not always sufficient to eliminate plaque one hundred percent of the time. Mechanical cleaning through the use of toothbrushes and floss are the most common methods for dental biofilm control, but these are only effective in areas where plaque is accessible (Martínez-Hernández et al., 2020). Given this information, anti-biofilm agents can be used and are recommended as a way to improve and build upon preventative mechanical measures being taken (Martínez-Hernández et al., 2020).

We performed biofilm disruption assays in order to assess the abilities of Ag(ACO) and Ag(BCO) to disrupt established *S. mutans* biofilms. This protocol is nearly the same as that of the MBIC assays, the only difference being that the addition of the compounds comes after 24 hours of biofilm growth rather than at the time of inoculation. Our results demonstrated significant

disruption of *S. mutans* biofilms starting at a concentration of 16 µg/mL for both compounds (Figure 7). Percent decrease was calculated as done previously for MBIC determination, revealing the highest percent decrease in biofilm thickness for Ag(BCO) and Ag(ACO) to be 43.72% at 128 µg/mL and 50.18% at 128 µg/mL, respectively (Table 2). Given the robust nature of biofilms, it can be difficult for antimicrobial agents to penetrate through and target these structures. As expected, the compounds were less effective against already established *S. mutans* biofilms and showed a larger decrease when used for the prevention of biofilms. However, a significant decrease in biofilm thickness was observed at higher concentrations of each compound, indicating the potential of these compounds as antimicrobial agents in the dental setting for preventative care and treatment.

Overall, Ag(BCO) was observed to be slightly more effective than Ag(ACO) when it comes to inhibition of planktonic and biofilm growth as well as biofilm disruption of *S. mutans*. This inconsistency in effectiveness between the two compounds can be explained by the differences in their chemical structures. Ag(BCO) contains an aromatic ring, which is very commonly used in drug design because of its well understood synthetic and modification paths (Polêto et al., 2018). This aromatic substituent found on Ag(BCO) is replaced with an amine in the same location on Ag(ACO), which we can infer makes the Ag(ACO) less potent or effective against *S. mutans* than Ag(BCO).

The next step to gain a better understanding of the antimicrobial activity of Ag(BCO) and Ag(ACO) is to determine the mode of action of these compounds. MIC, MBIC, and biofilm disruption assays do not indicate the mode of action of the compounds. With MIC assays for example, if the compound tested was bacteriostatic and not bactericidal, viable cells may be present after treatment even if the wells appear to have no visible growth (Weigand et al., 2008). In this

case, once the compound or drug is removed, bacterial growth is free to resume. These are important factors to consider when assessing the potential of a compound to serve as an antimicrobial agent in the clinical setting. Antimicrobial compounds, like Ag(BCO) and Ag(ACO), that appear effective based off of the results of MIC and MBIC assays can be further tested using an adenylate kinase assay to determine mode of action. Bacteriostatic compounds only limit growth, whereas bactericidal compounds kill bacteria. Adenylate kinase assays detect cell lysis, indicating that the cell is dead (Saputo et al., 2018). Performing adenylate kinase assays would determine whether the compounds tested are bacteriostatic or bactericidal.

Even with all of the data gathered from MIC, MBIC, and biofilm disruption assays, as well as once mode of action is determined, it is key to gain an understanding of the efficacy of the drugs *in vivo*. The studies we have performed can be taken further by testing Ag(BCO) and Ag(ACO) against *S. mutans* biofilms grown on hydroxyapatite to mimic the conditions found in the mouth and in an *in vivo* setting (Forssten et al., 2010). Based off of the growth curve, MIC, MBIC, and biofilm disruption data, we observed that both Ag(BCO) and Ag(ACO) have a negative effect on *S. mutans* planktonic and biofilm growth. Future directions including the determination of mode of action of the compounds, *in vivo* studies, and perhaps testing the compounds against various biofilm ages rather than just 24-hour biofilms will build upon data we have collected. This data will ultimately contribute to the overall understanding of how to successfully target *S. mutans*, with the hope of improving dental treatments and preventative dental care.

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**Figures**

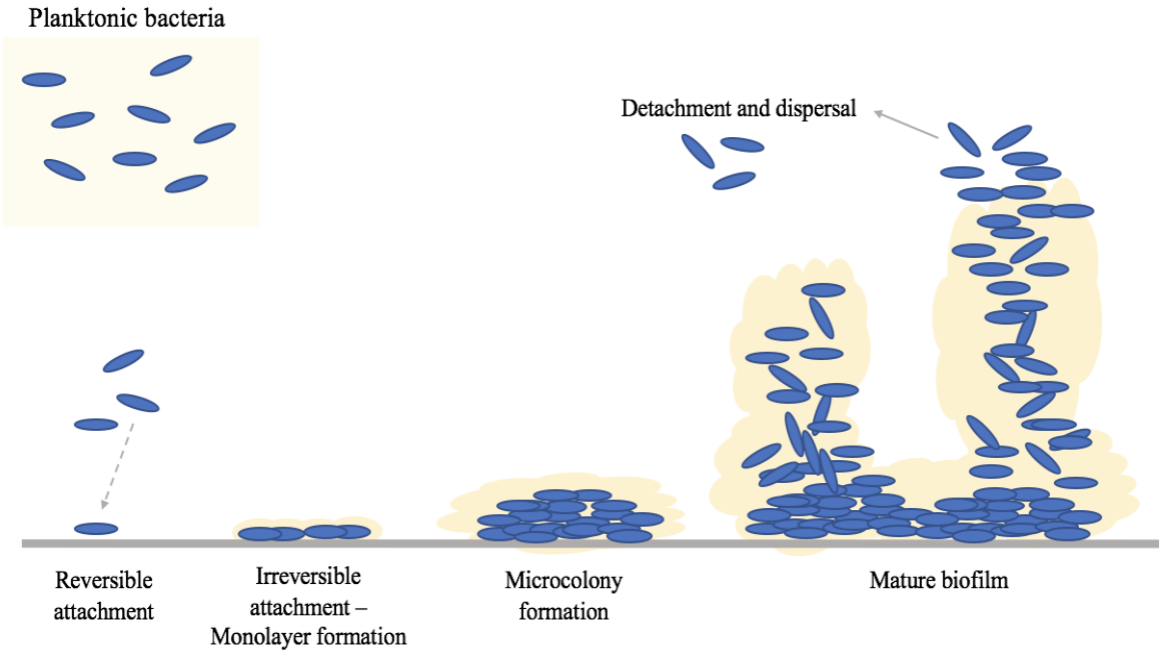



**Figure 1. Stages of Biofilm Formation.** Free-floating or planktonic cells irreversibly attach to a surface. This is followed by the formation of a monolayer and a matrix, resulting in irreversible attachment. Bacteria continue to attach forming layers and eventually a microcolony. After continued attachment, a mature biofilm forms a mushroom-like structure and cells begin to detach and disperse in order to attach to new surfaces (adapted from Vasudevan, 2014).

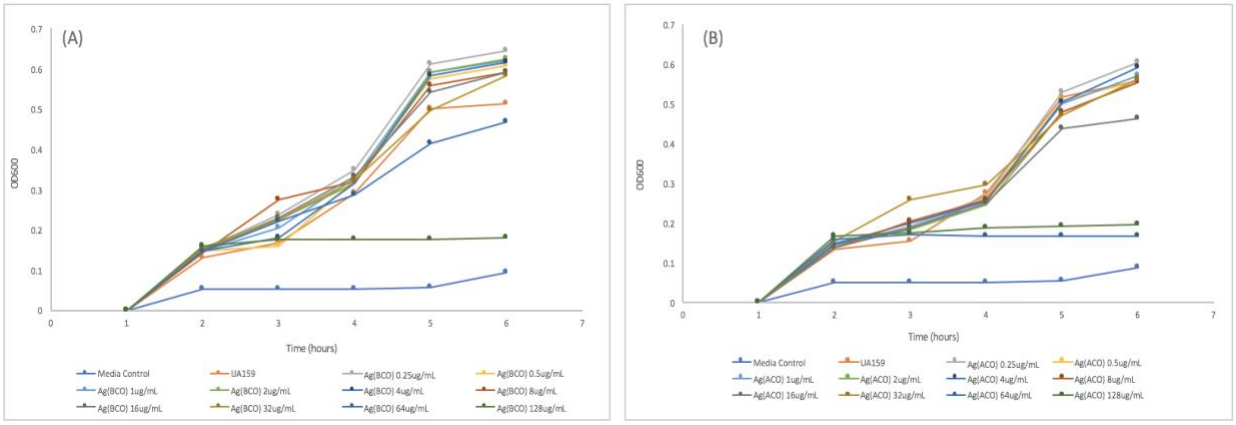



**Figure 2. Higher concentrations of both Ag(BCO) and Ag(ACO) demonstrated negative effects on *S. mutans* growth**. Growth curves were performed using 96-well microtiter plates that were inoculated with increasing concentrations of compounds (A) Ag(BCO) and (B) Ag(ACO) from 0.25 μg/mL to 128 μg/mL. Plates were inoculated and then incubated at 37ºC. Absorbance readings (OD600) were recorded approximately every 1.5 hours, except when left overnight, for a 25-hour time period. OD600 values were plotted against time to generate growth curves.

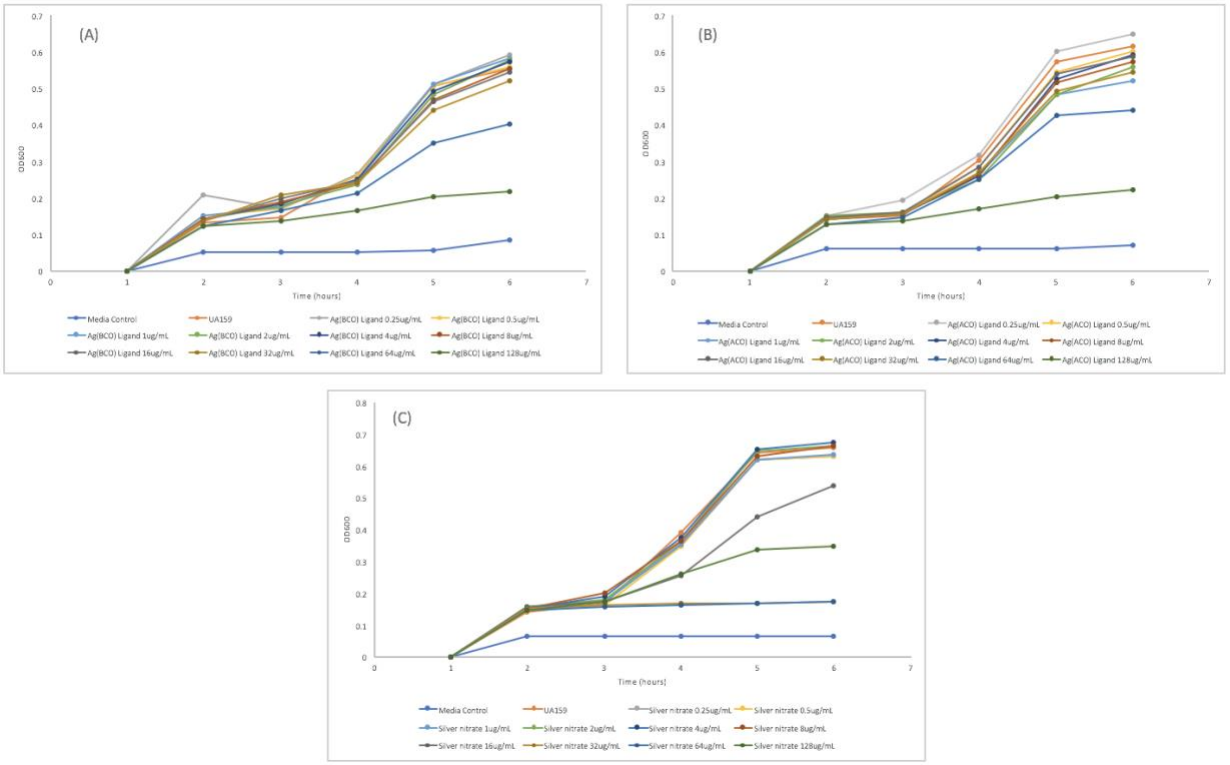
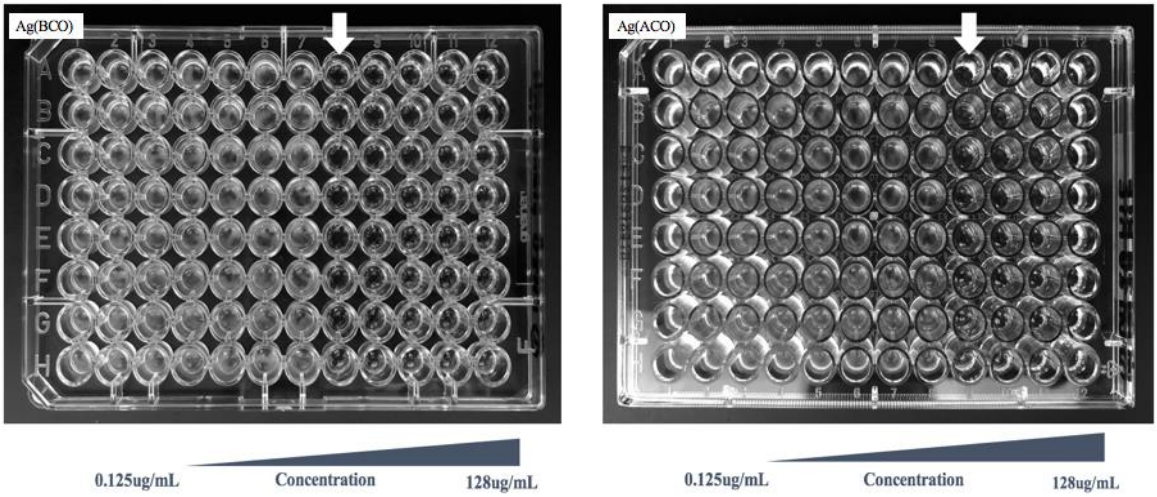



**Figure 3. Higher concentrations of Ag(BCO) ligand, Ag(ACO) ligand, and silver nitrate controls demonstrated negative effects on *S. mutans* growth**. Growth curves were performed using 96-well microtiter plates that were inoculated with increasing concentrations of (A) Ag(BCO) ligand, (B) Ag(ACO) ligand, and (C) silver nitrate from 0.25 µg/mL to 128 µg/mL. Plates were inoculated and then incubated at 37ºC. Absorbance readings (OD600) were recorded approximately every 1.5 hours, except when left overnight, for a 25-hour time period. OD600 values were plotted against time to generate growth curves.

(A)



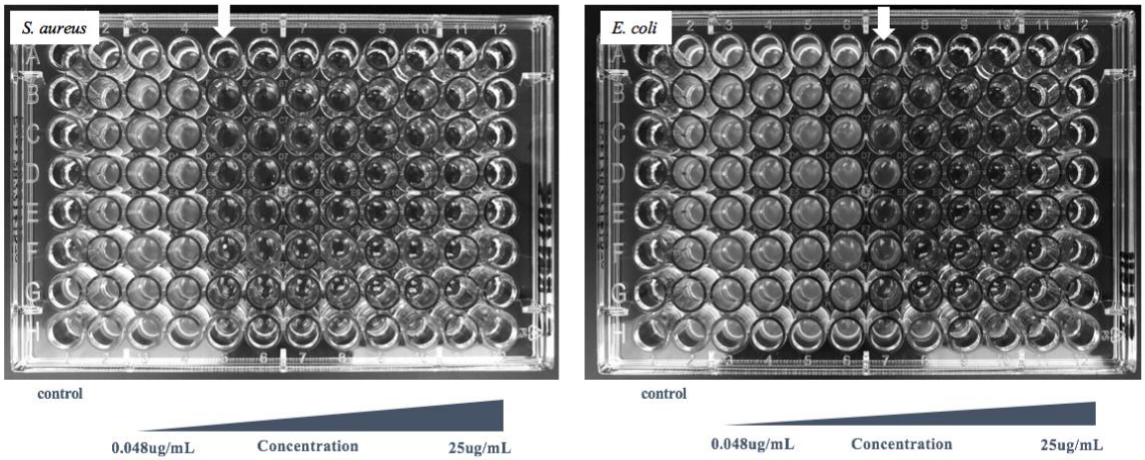

(B)

| Treatment | Visible Growth |
|---|---|
| BHI + UA159 Control | + |
| 0.125ug/mL Ag(BCO) | + |
| 0.25ug/mL Ag(BCO) | + |
| 0.5ug/mL Ag(BCO) | + |
| 1ug/mL Ag(BCO) | + |
| 2ug/mL Ag(BCO) | + |
| 4ug/mL Ag(BCO) | + |
| 8ug/mL Ag(BCO) | - |
| 16ug/mL Ag(BCO) | - |
| 32ug/mL Ag(BCO) | - |
| 64ug/mL Ag(BCO) | - |
| 128ug/mL Ag(BCO) | - |

| Treatment | Visible Growth |
|---|---|
| BHI + UA159 Control | + |
| 0.125ug/mL Ag(ACO) | + |
| 0.25ug/mL Ag(ACO) | + |
| 0.5ug/mL Ag(ACO) | + |
| 1ug/mL Ag(ACO) | + |
| 2ug/mL Ag(ACO) | + |
| 4ug/mL Ag(ACO) | + |
| 8ug/mL Ag(ACO) | + |
| 16ug/mL Ag(ACO) | - |
| 32ug/mL Ag(ACO) | - |
| 64ug/mL Ag(ACO) | - |
| 128ug/mL Ag(ACO) | - |

**Figure 4. Minimum inhibitory concentration assays demonstrate that Ag(ACO) inhibits planktonic *S. mutans* growth at 8 ug/mL and Ag(BCO) inhibits planktonic growth at 16 ug/mL.** (A) Microtiter plates were inoculated with increasing concentrations of Ag(BCO) and Ag(ACO) from 0.125 µg/mL to 128 µg/mL and one column with bacteria alone to serve as a growth control. Plates were incubated at 37ºC overnight and turbidity of the wells was observed and recorded to determine the MIC of each compound. Columns containing the minimum inhibitory concentrations are marked with arrows. (B) Visible growth was observed and recorded for each concentration, with + indicating growth and - indicating no growth.

(A)




(B)

| Treatment | Visible Growth |
|---|---|
| LB only | - |
| LB + *S. aureus* | + |
| 0.048ug/mL Amp + *S. aureus* | + |
| 0.098ug/mL Amp + *S. aureus* | + |
| 0.195ug/mL Amp + S. aureus | - |
| 0.391ug/mL Amp + *S. aureus* | - |
| 0.781ug/mL Amp + *S. aureus* | - |
| 1.5625g/mL Amp + *S. aureus* | - |
| 3.125ug/mL Amp + *S. aureus* | - |
| 6.25ug/mL Amp + *S. aureus* | - |
| 12.5ug/mL Amp + *S. aureus* | - |
| 25ug/mL Amp + *S. aureus* | - |

| Treatment | Visible Growth |
|---|---|
| LB only | - |
| LB + *E. coli* | + |
| 0.048ug/mL Amp + *E. coli* | + |
| 0.098ug/mL Amp + *E. coli* | + |
| 0.195ug/mL Amp + *E. coli* | + |
| 0.391ug/mL Amp + *E. coli* | + |
| 0.781ug/mL Amp + *E. coli* | + |
| 1.5625g/mL Amp + *E. coli* | - |
| 3.125ug/mL Amp + *E. coli* | - |
| 6.25ug/mL Amp + *E. coli* | - |
| 12.5ug/mL Amp + *E. coli* | - |
| 25ug/mL Amp + *E. coli* | - |

**Figure 5. Minimum inhibitory concentration assays demonstrate that ampicillin inhibits planktonic growth of ATCC *S. aureus* and *E. coli* strains at 0.195 µg/mL and 1.5625 µg/mL, respectively.** (A) Microtiter plates were inoculated with increasing concentrations of ampicillin from 0.048 µg/mL to 25 µg/mL. Plates were also inoculated with *S. aureus* and *E. coli* with columns 1 and 2 on each plate serving as media only and bacteria only controls, respectively. Plates were incubated at 37ºC overnight and turbidity of the wells was observed and recorded to determine the MIC of each compound. Columns containing the minimum inhibitory concentrations are marked with arrows. (B) Visible growth was observed and recorded for each concentration, with + indicating growth and - indicating no growth.

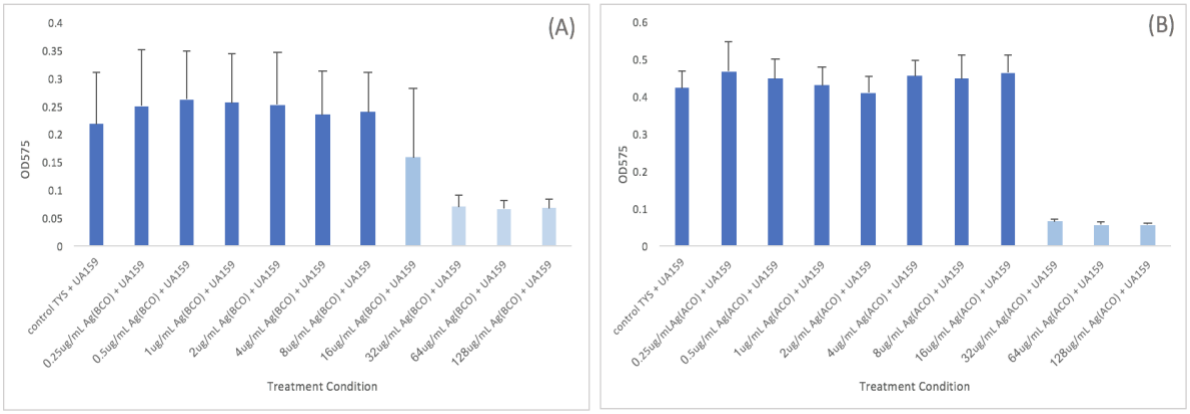



**Figure 6. Minimum biofilm inhibitory concentration assays demonstrate that Ag(BCO) decreases *S. mutans* biofilm growth at 16 ug/mL and Ag(ACO) decreases *S. mutans* biofilm growth at 32 ug/mL.** Microtiter plates were inoculated with increasing concentrations of (A) Ag(BCO) and (B) Ag(ACO) from 0.25 µg/mL to 128 µg/mL and one column with bacteria alone to serve as a growth control. Plates were incubated at 37ºC overnight, supernatant was removed, wells were washed with distilled water, and plates were left to dry. Biofilms were stained with 0.1% crystal violet then washed again with distilled water. Crystal violet was solubilized with 30% acetic acid and then transferred to new microtiter plates. Absorbance was measured with a microplate reader at 575 nm and graphs displaying average absorbance values for all samples and trials were generated to determine the MBIC of each compound. This data is the average of three total experiments performed for each compound.

**Table 1. Percent increase or decrease in *S. mutans* biofilms when grown in the presence of Ag(BCO) and Ag(ACO).** Compound concentration indicates the concentration of Ag(BCO) or Ag(ACO) added prior to biofilm cultivation, respectively. Percent increase or decrease in biofilm growth was determined in comparison to the positive growth control of UA159 alone. An increase in biofilm growth is indicated by + and - indicates a decrease in biofilm growth.

| Compound Concentration | Ag(BCO) | Ag(ACO) |
|---|---|---|
| 0.25 ug/mL | + 14.32% | + 10.37% |
| 0.5 ug/mL | + 19.95% | + 5.76% |
| 1 ug/mL | + 17.41% | + 1.6% |
| 2 ug/mL | + 15.8% | - 3.18% |
| 4 ug/mL | + 7.41% | + 7.64% |
| 8 ug/mL | + 9.89% | + 5.75% |
| 16 ug/mL | - 27.6% | + 9.75% |
| 32 ug/mL | - 68.1% | - 84.31% |
| 64 ug/mL | - 69.69% | - 84.42% |
| 128 ug/mL | - 69.48% | - 86.7% |

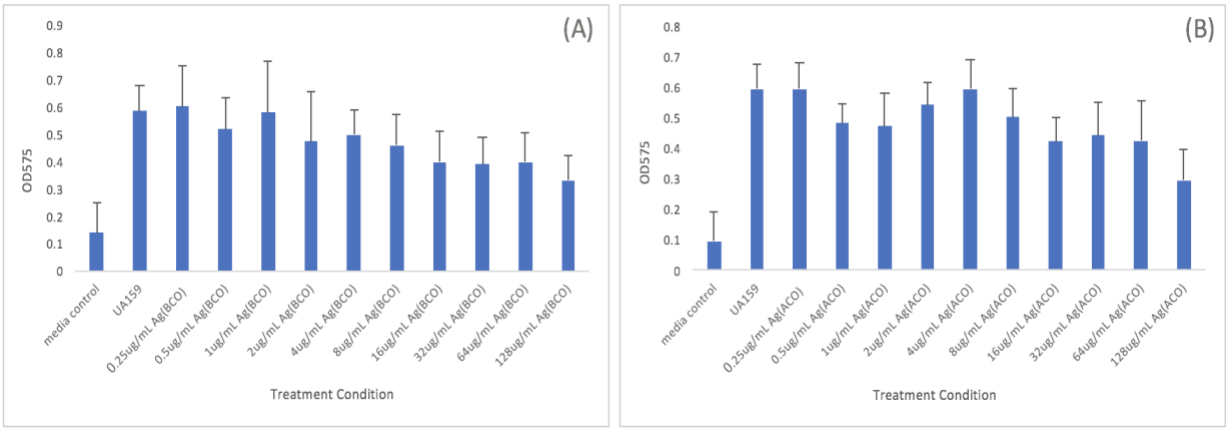



**Figure 7. Biofilm disruption assays demonstrate that higher concentrations of Ag(BCO) and Ag(ACO) disrupt biofilm growth, with a significant reduction around 16 to 32 µg/mL.** Microtiter plates were inoculated with *S. mutans* UA159 and incubated overnight at 37ºC to allow for biofilm growth. Fresh media containing increasing concentrations of (A) Ag(BCO) and (B) Ag(ACO) from 0.25 µg/mL to 128 µg/mL was added and plates were incubated at 37ºC for another 24 hours. After incubation, supernatant was removed, wells were washed with distilled water, and plates were left to dry. Biofilms were stained with 0.1% crystal violet then washed again with distilled water. Crystal violet was solubilized with 30% acetic acid and then transferred to new microtiter plates. Absorbance was measured with a microplate reader at 575 nm and graphs displaying average absorbance values for all samples and trials were generated. This data is the average of three total experiments performed for each compound.

**Table 2. Percent increase or decrease in *S. mutans* biofilm robustness when disrupted with Ag(BCO) and Ag(ACO).** Compound concentration indicates the concentration of Ag(BCO) or Ag(ACO) added after 24 hours of biofilm growth, respectively. Percent increase and decrease in biofilm growth was determined in comparison to the positive growth control of UA159 alone**.** An increase in biofilm robustness is indicated by + and - indicates a decrease in biofilm robustness.

| Compound Concentration | Ag(BCO) | Ag(ACO) |
|---|---|---|
| 0.25 ug/mL | + 2.55% | - 0.04% |
| 0.5 ug/mL | - 11.71% | - 18.89% |
| 1 ug/mL | - 1.39% | - 20.58% |
| 2 ug/mL | - 19.16% | - 8.49% |
| 4 ug/mL | - 15.14% | + 0.07% |
| 8 ug/mL | - 21.75% | - 15.47% |
| 16 ug/mL | - 31.86% | - 28.99% |
| 32 ug/mL | - 33.55% | - 25.57% |
| 64 ug/mL | - 32.26% | - 28.59% |
| 128 ug/mL | - 43.72% | - 50.18% |