

Silver Oxynitrate, an Unexplored Silver Compound with Antimicrobial and Antibiofilm Activity

1 uM Ag₂SO₄: 311.8
1 M = 311.8 gm/1000 gm
= 31.2 gm/100 gm = 31.2%

1 mM = .0312% = 312 ppm
1 uM = 0.312 ppm
100 uM = 31.2 ppm
1000 uM = 312 ppm

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Historically it has been accepted, and recent research has established, that silver (Ag) is an efficacious antimicrobial agent. A dwindling pipeline of new antibiotics, combined with an increase in the number of antibiotic-resistant infections, is bringing Ag to the fore as a therapeutic compound to treat infectious diseases. Currently, many formulations of Ag are being deployed for commercial and medical purposes, with various degrees of effectiveness at killing microbial cells. Here, we evaluated the antimicrobial and antibiofilm capacity of our lead compound, silver oxynitrate [Ag(Ag₃O₄)₂NO₃ or Ag₇NO₁₁], against other metal compounds with documented antimicrobial activity, including Ag₂SO₄, AgNO₃, silver sulfadiazine (AgSD), AgO, Ag₂O, and CuSO₄. Our findings reveal that Ag₇NO₁₁ eradicates biofilm and planktonic populations of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, uropathogenic *Escherichia coli* (UPEC), fluoroquinolone-resistant *Pseudomonas aeruginosa* (FQRP), and methicillin-resistant *Staphylococcus aureus* (MRSA) at lower concentrations than those of the other tested metal salts. Altogether, our results demonstrate that Ag₇NO₁₁ has an enhanced efficacy for the treatment of biofilm-forming pathogens.

History has demonstrated that silver (Ag) is an efficacious antimicrobial agent, finding utility as an antiquated preservative for food and water (1, 2). Currently, >100 Ag-containing medical devices have been approved for use by the FDA (<http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpmn/pmn.cfm>), and there is mounting evidence that Ag may be effective for preventing the spread of infectious disease (1, 3, 4). Although the toxicological profile of orally administered Ag remains to be resolved, topical application for the treatment of chronic wounds remains promising (5–8). Accordingly, Ag can address a timely public health issue, as chronic wounds represent a substantial financial and medical burden to the health care system. One of the hallmarks of a chronic wound is the presence of a biofilm, a factor that complicates wound healing and is hypothesized to be the fulcrum between the acute-to-chronic-wound transition (6, 9–15). Also, bacterial biofilms contaminate implanted medical devices (16) and exhibit high-level resistance to conventional antibiotics (17–23). New antibiofilm agents are thus desperately needed in medicine.

Recently, researchers have demonstrated that Ag formulations also have potential as antibiofilm agents; these formulations include silver sulfadiazine (AgSD) (24), Ag nanoparticles (AgNPs) (25, 26), and silver nitrate (AgNO₃) (27). Currently, the wound care industry is replete with Ag-based ointments and wound dressings with claimed efficacy at reducing bacterial bioburden (7, 28–32). However, only a few antibiofilm products exist. There are numerous reasons why this is so: (i) current standardized, antimicrobial testing methods focus on bacteria in their planktonic state and not as biofilms (33, 34); (ii) biofilms are characteristically more tolerant to metal poisoning (35–38); and (iii) the detailed mechanism of action for the toxicity of Ag to biofilms remains to be fully described (1).

The antimicrobial activity of Ag is intrinsically dependent on the formation of the Ag¹⁺ ion (7, 39). Briefly, Ag¹⁺, a Lewis soft acid, poisons the microbial cell by binding to reduced thiols (SH), impairing membrane function (1), and disrupting iron-sulfur clusters (40). That being said, the antimicrobial efficacy of higher oxidation states of Ag, Ag²⁺ and Ag³⁺, has not been

given adequate consideration, mainly due to instability in solution. A promising resolution to the instability of higher oxidation states of Ag is silver oxysalts, for example, Ag(Ag₃O₄)₂X, the most stable of which is coordinated with nitrate, silver oxynitrate [Ag(Ag₃O₄)₂NO₃ or Ag₇NO₁₁], where both the Ag(II) and Ag(III) oxidation states exist stably at room temperature (41, 42).

Here, we describe our observations from testing the antimicrobial and antibiofilm activities of various metal compounds. Namely, we tested Ag₇NO₁₁, AgSD, AgNO₃, silver sulfate (Ag₂SO₄), silver(I,III) oxide (AgO), silver(I) oxide (Ag₂O), and copper sulfate (CuSO₄) against 6 bacterial strains, *Pseudomonas aeruginosa* (strain PAO1), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (JM109), uropathogenic *Escherichia coli* (UPEC) (CFT703), fluoroquinolone-resistant *Pseudomonas aeruginosa* (FQRP), and methicillin-resistant *Staphylococcus aureus* (MRSA) (USA300), as well as two fungal strains, *Candida albicans* (ATCC 14053) and *Candida tropicalis* (99916). CuSO₄ was included in our analysis because copper (Cu) is another thiophilic metal with observed antimicrobial activity (1, 43, 44). Using the minimal biofilm eradication concentration (MBEC) assay, we found that Ag₇NO₁₁ eradicated planktonic and biofilm populations of the tested strains at lower concentrations than those of the other metal compounds tested. AgNO₃, the most soluble Ag salt, also had antimi-

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icrobial and antibiofilm activity at micromolar concentrations. Because AgNO_3 has high solubility, resulting in easily accessible Ag^+ ions, and it outperformed all other Ag salts with the exception of $\text{Ag}_7\text{NO}_{11}$, it was chosen as our comparator for testing of the efficacy of $\text{Ag}_7\text{NO}_{11}$ in the proceeding experiments. Using the live/dead staining technique as well as crystal violet staining, we demonstrated that $\text{Ag}_7\text{NO}_{11}$ reduced the biomass of the biofilm. In summary, our data demonstrate that $\text{Ag}_7\text{NO}_{11}$ has greater antimicrobial and antibiofilm activity than do the other tested metal compounds and may offer an alternative option for the treatment of chronic wounds infected by biofilm-forming microbes.

MATERIALS AND METHODS

Bacterial/fungal strains and media. Bacterial and fungal strains were stored in Microbank vials at -70°C according to the manufacturer's instructions (ProLab Diagnostics, Richmond Hill, ON, Canada). *Pseudomonas aeruginosa* (PAO1), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (JM109), *Candida albicans* (ATCC 14053), *Candida tropicalis* (99916), uropathogenic *Escherichia coli* (CFT703), fluoroquinolone-resistant *Pseudomonas aeruginosa*, and methicillin-resistant *Staphylococcus aureus* (USA300) were streak purified onto tryptic soy agar (TSA) (VWR International, Mississauga, ON, Canada) overnight at 37°C .

CFT703 and USA300 were generous gifts from Joe J. Harrison (University of Calgary), while the FQRP isolate was a generous gift from Michael Parkins (University of Calgary). To simulate a wound environment, simulated wound fluid (SWF) (50% peptone water [0.85% NaCl, 0.1 g/liter peptone]–50% fetal calf serum [Invitrogen, Life Technologies]) (31, 45) was used as the growth medium and for susceptibility testing.

Biofilm formation. All biofilms used in this study were cultivated by using the Calgary Biofilm Device (CBD)/MBEC device as previously described (38, 46–50) and according to the manufacturer's guidelines (Invovotech, Edmonton, AB, Canada). Briefly, following growth of the pre-culture overnight, the colonies were suspended in SWF to match the density of a 1.0 McFarland standard. The cultures made with the optical standard were subsequently diluted 30 times in 150 μl of SWF, which served as the inoculum for the CBD. The biofilm was formed by placing the lid of the CBD, with 96 equivalent pegs, into a 96-well microtiter plate (Nunclon; VWR International) containing the inoculum. The CBD was then placed on a gyratory shaker operating at 150 rpm in a humidified incubator at 37°C for 4 h or 24 h. For mature biofilm experiments, the inoculated CBD was incubated for a period of 4 days (*P. aeruginosa*) or 6 days (*S. aureus* and *E. coli*). A period exceeding 4 days resulted in overgrowth of PAO1 in the MBEC device (personal observation).

Stock metal solutions. AgNO_3 , AgSD , Ag_2SO_4 , Ag_2O , AgO , and CuSO_4 were all obtained from Sigma-Aldrich (St. Louis, MO, USA). $\text{Ag}_7\text{NO}_{11}$ was obtained from Exciton Technologies Inc. (Edmonton, AB, Canada). All stock Ag solutions were made at equivalent molarities of Ag (up to 5 mM) in distilled and deionized water (ddH_2O). The CuSO_4 stock was made up to 2 M in ddH_2O . All metal solutions were stored in glass vials at 21°C for no longer than 1 week. Working solutions were made in SWF, from the stock metal solution, no more than 30 min prior to experimental use. From these solutions, serial dilutions (dilution factor of 2) were made in 96-well plates (challenge plate). The first row was reserved as a sterility control, and the second row was used as a growth control (0 mM metal salt). The range of concentrations tested for all Ag compounds was 0 to 1,250 μM , while CuSO_4 was tested at concentrations of 0 to 20,000 μM .

Biofilm and planktonic culture susceptibility testing. Two scenarios were tested: (i) the capacity of metal salts to prevent biofilm formation and (ii) the ability of metal salts to eradicate established biofilms. For the first scenario, the bacterial cultures were inoculated into the challenge plate in the presence of the metal salts and then subsequently placed onto a gyratory shaker at 150 rpm in a humidified incubator at 37°C for 4 h. The second scenario allowed for the establishment of a biofilm for 24 h, 4 days,

or 6 days on the pegged lid of the CBD. The pegged lid containing the established biofilm was then rinsed twice with 0.9% NaCl and subsequently placed into the 96-well microtiter plate containing 2-fold serial dilutions of the challenge metal (challenge plate). The plate was then incubated for 4 h, 24 h, or 48 h on a gyratory shaker at 150 rpm in a humidified incubator at 37°C .

To test the metal susceptibility of the planktonic and biofilm populations, the plates from the first and second scenarios were prepared by removing the pegged lid and rinsing it twice with 0.9% NaCl. The biofilms were disrupted from the pegs by sonication using a 250HT ultrasonic cleaner (VWR International), set at 60 Hz for 10 min, into 200 μl of tryptic soy broth (VWR International) containing universal neutralizer (UN) (0.05 g/liter histidine [Sigma, USA], 0.05 g/liter cysteine [Sigma, USA], and 0.1 g/liter reduced glutathione [Sigma, USA] in ddH_2O) and 0.1% Tween 20. The MBEC of the biofilm populations was ascertained by performing 8 dilutions (dilution factor of 10) of the disrupted biofilms in 0.9% NaCl. The numbers of viable cells from the biofilm were ascertained by spot plating the diluted sample onto TSA plates and subsequently inoculating the plates overnight at 37°C (36, 38, 51). Similarly, determination of the minimal bactericidal concentration (MBC) of the planktonic populations was carried out by serially diluting (8 times [10-fold dilutions]) the neutralized (UN, as described above) spent medium from the 96-well plate into 0.9% saline. Viable cell counts were taken following spot plating of the dilutions onto TSA plates incubated overnight at 37°C (36, 38, 51). The MBC and MBEC were determined by monitoring the concentration of metal compound at which there were no viable microbial colonies.

Confocal microscopy. Quantification of live and dead cells in the biofilm was performed by using a confocal microscope and the Live/Dead BacLight staining kit (Molecular Probes, Burlington, ON, Canada). Briefly, following metal exposure, cell viability staining of the bacteria was done by incubating the pegs containing the biofilms with Syto-9 (6.7 μM) and propidium iodide (PI) (40 μM) stains (provided in the BacLight kit) at 30°C for 30 min, as previously described (46). The pegs were rinsed with 0.9% saline twice and monitored by using a confocal laser scanning microscope (Leica DM IRE2 microscope) equipped with a $20\times$ objective. Images were processed by using Imaris $\times 64$ (Bitplane, USA). For PI, the excitation wavelength was set at 523 nm and the emission wavelength was set at 617 nm (red), while for Syto-9, the excitation wavelength was set at 488 nm and the emission wavelength was set at 498 nm (green). Quantification of the compiled confocal images was performed by using the Fiji software package (<http://fiji.sc/Fiji>).

Crystal violet assay. Crystal violet staining was used to quantify the amount of biomass in the biofilm. Following metal treatment, the pegged lids from the CBD were rinsed twice in 200 μl of 0.9% saline. By using a procedure similar to the one described previously by O'Toole (52), the biofilms were stained with 200 μl of a 0.1% crystal violet solution for 10 min. Following staining, the pegs were washed with 200 μl ddH_2O three times and blotted onto a paper towel to remove excess dye. The stain was left to set overnight at room temperature. Quantification of the biofilm was performed by solubilizing the crystal violet stain with 200 μl of 30% acetic acid for 15 min into a microtiter plate and reading the absorbance at 550 nm using acetic acid as the blank.

Statistical analysis. The statistical significance of the results was determined by using nonparametric one-way analysis of variance (ANOVA) for the MBC and MBEC assays, two-way ANOVA for the crystal violet assays, or Student's *t* test for the data shown in Fig. S3 and S4 in the supplemental material. All experiments were performed, at minimum (stated otherwise in the figure legends), with two biological replicates and in duplicate.

RESULTS

$\text{Ag}_7\text{NO}_{11}$ prevents biofilm formation. In order to assess the capacity of various metal compounds to prevent biofilm formation in our selected pathogenic strains (*E. coli*, *P. aeruginosa*, and *S.*

need to convert uM to ppm

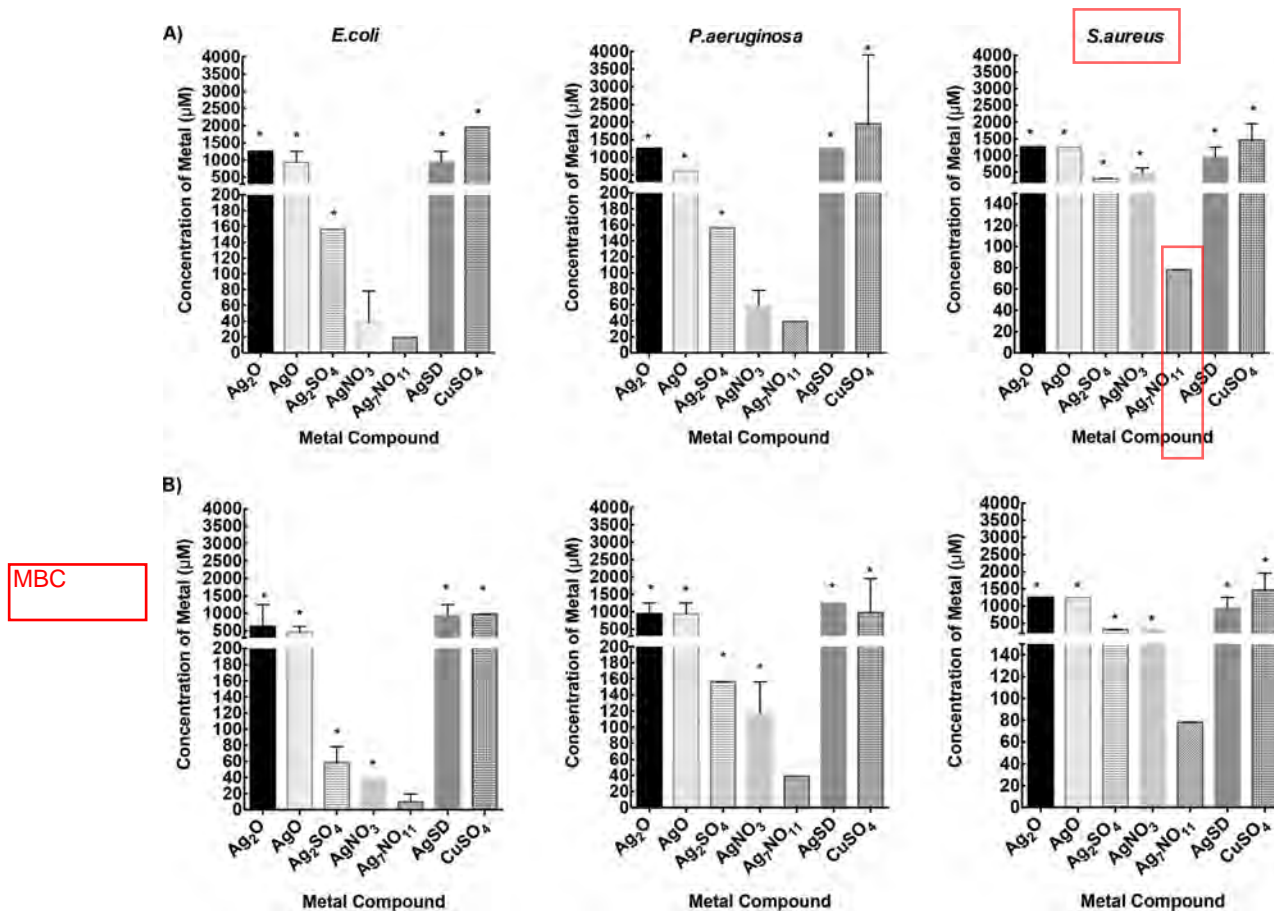


FIG 1 Ag₇NO₁₁ prevents biofilm formation at lower concentrations than those of other metal compounds. The MBEC device was inoculated with *E. coli* (JM109), *P. aeruginosa* (PAO1), and *S. aureus* (ATCC 25923) concurrently with serial dilutions (2-fold) of Ag₂O, AgO, Ag₂SO₄, Ag₇NO₁₁, AgSD, and CuSO₄. The MBEC (A) and MBC (B) were then determined after a 4-h incubation with the various metal compounds. Values are represented as medians and ranges ($n = 4$). * indicates a significant difference between Ag₇NO₁₁ and the indicated metal compound ($P \leq 0.05$). Note that all Ag stock solutions were prepared at equal molar concentrations of Ag molecules. Hence, concentrations found in this figure are reflective of the concentration of Ag and not the silver-containing compound itself.

aureus), we employed a MBEC assay (Fig. 1). Concurrently, the MBC for the metal compounds was also established. Compared to the other metal compounds tested, significantly low concentrations of Ag₇NO₁₁ were needed to prevent the formation of biofilms (Fig. 1A) and, with the exception of AgNO₃, to eliminate planktonic populations of our tested strains (Fig. 1B) following a 4-h incubation period.

Ag₇NO₁₁ eradicates established biofilm populations. Our next aim was to assess the capacity of various metal compounds to eradicate an established biofilm. Biofilms from *E. coli*, *P. aeruginosa*, and *S. aureus* were established following a 24-h incubation period in the MBEC device. The established biofilms were then exposed to serial dilutions (2-fold) of Ag₂O, AgO, Ag₂SO₄, AgNO₃, Ag₇NO₁₁, AgSD, and CuSO₄ for 24 h (Fig. 2). A 4-h exposure of established biofilms to AgNO₃, Ag₇NO₁₁, and CuSO₄ was an insufficient time to eradicate them (see Fig. S1 in the supplemental material), with the exception of *E. coli* biofilms. Thus, a 24-h exposure to the metal compounds was evaluated as a potentially efficacious treatment period. Following a 24-h incubation with the various metal compounds, we observed that Ag₇NO₁₁ significantly reduced biofilm populations of *E. coli*, *P. aeruginosa*, and *S. aureus* at lower concentrations than those of the other

tested metal compounds, with the exception of AgNO₃ in *P. aeruginosa* (Fig. 2). When mature biofilms were established in the MBEC device, following a 4-day (*P. aeruginosa*) or 6-day (*E. coli* and *S. aureus*) period, higher concentrations of AgNO₃, CuSO₄, and Ag₇NO₁₁ were required to reach the MBEC, with the exception of CuSO₄ in *E. coli* (Fig. 3). Nonetheless, Ag₇NO₁₁ eradicated the mature biofilms of *S. aureus* and *E. coli* at lower concentrations than those of AgNO₃ and CuSO₄ (Fig. 3). Additionally, at the highest concentrations (of equivalent Ag) of AgNO₃ and Ag₇NO₁₁ tested (1,250 µM), Ag₇NO₁₁ significantly outperformed AgNO₃ and CuSO₄ at eradicating *C. albicans* and *C. tropicalis* biofilms (see Fig. S2 in the supplemental material).

Ag-NO₁₁ reduces the biomass of established biofilms. Since AgNO₃ and Ag₇NO₁₁ were most efficacious at eliminating planktonic populations, preventing biofilm formation, and eradicating established biofilms (Fig. 1 and 2), these two metal compounds were chosen as lead compounds to study the capacity of Ag to abolish established biofilms. We employed live/dead staining followed by confocal microscopy analysis to visualize the effects of 4-h and 24-h treatments with 5 µM and 12.5 µM AgNO₃ and Ag₇NO₁₁ (at equivalent Ag concentrations) on established biofilms of *E. coli*, *P. aeruginosa*, and *S. aureus* (Fig. 4; see also Fig. S3

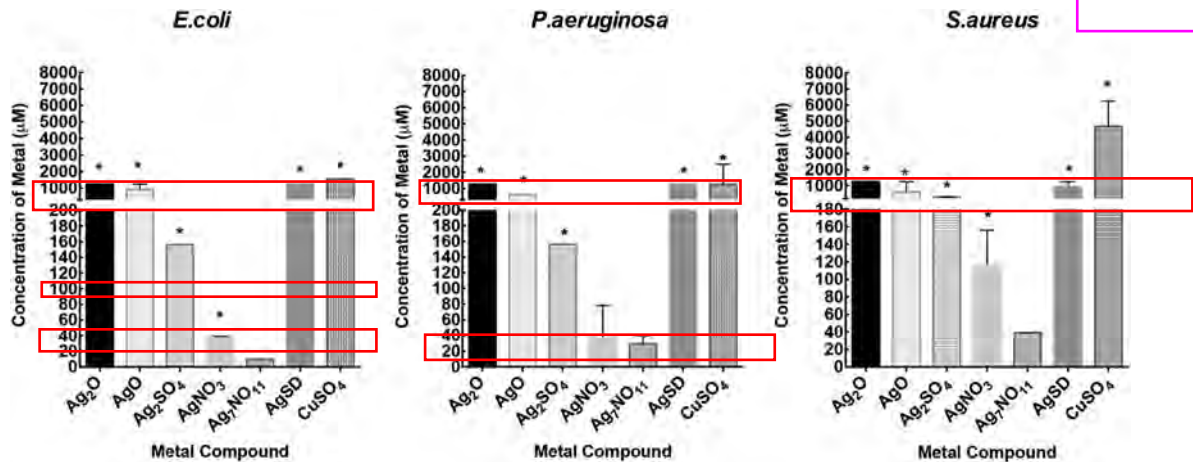


FIG 2 $\text{Ag}_7\text{NO}_{11}$ eradicates established biofilms at lower concentrations than those of other metal compounds. The MBEC device was inoculated with *E. coli* (JM109), *P. aeruginosa* (PAO1), and *S. aureus* (ATCC 25923). Biofilms were established following a 24-h incubation. The established biofilms were then treated with serial dilutions (2-fold) of Ag_2O , AgO , Ag_2SO_4 , $\text{Ag}_7\text{NO}_{11}$, AgSD , and CuSO_4 for 24 h. The MBEC was then determined for the various metal compounds. Values are represented as medians and ranges ($n = 4$). * indicates a significant difference between $\text{Ag}_7\text{NO}_{11}$ and the indicated metal compound ($P \leq 0.05$). Note that all Ag stock solutions were prepared at equal molar concentrations of Ag molecules. Hence, concentrations found in this figure are reflective of the concentration of Ag and not the silver-containing compound itself.

in the supplemental material). Although a 4-h exposure to AgNO_3 and $\text{Ag}_7\text{NO}_{11}$ was not adequate to eradicate the biofilm populations, an increase in the quantity of dead cells was observed in the biofilms exposed to 12.5 μM AgNO_3 and $\text{Ag}_7\text{NO}_{11}$ (see Fig. S3 in the supplemental material). Following a 24-h exposure to AgNO_3 and $\text{Ag}_7\text{NO}_{11}$, we observed an increase in the number of dead cells in the biofilms exposed to 12.5 μM AgNO_3 and a reduction in biofilm quantity in the biofilms exposed to $\text{Ag}_7\text{NO}_{11}$ (Fig. 4; see also Fig. S4 in the supplemental material). To confirm that $\text{Ag}_7\text{NO}_{11}$ bestowed the capacity to reduce the quantity of the biomass in the biofilms, we performed a crystal violet staining assay, according to methods described previously by O'Toole (52), on established *E. coli*, *P. aeruginosa*, and *S. aureus* biofilms following a 24-h exposure to AgNO_3 , $\text{Ag}_7\text{NO}_{11}$, and CuSO_4 (Fig. 5). We observed that $\text{Ag}_7\text{NO}_{11}$ had significantly greater efficacy than did AgNO_3 and CuSO_4 at reducing the quantity of biomass in *E. coli* and *P. aeruginosa* biofilms, although much higher concentrations were needed to reduce the *P. aeruginosa* biofilm biomass (Fig. 5). Meanwhile, $\text{Ag}_7\text{NO}_{11}$ outperformed only CuSO_4 for reducing the biomass of *S. aureus* biofilms (Fig. 5).

$\text{Ag}_7\text{NO}_{11}$ is more efficacious against select clinical isolates and antibiotic-resistant strains of *E. coli* (UPEC [CFT703]), *P.*

***aeruginosa* (FQRP), and *S. aureus* (MRSA [USA300]).** To demonstrate the potential of using $\text{Ag}_7\text{NO}_{11}$ as an antimicrobial beyond laboratory strains of bacterial pathogens, we performed MBC and MBEC assays on UPEC, FQRP, and MRSA planktonic and biofilm populations, respectively, with serial dilutions of $\text{Ag}_7\text{NO}_{11}$, AgNO_3 , and CuSO_4 (Fig. 6, 7, and 8, respectively). Our observations suggest that higher concentrations of AgNO_3 , $\text{Ag}_7\text{NO}_{11}$, and CuSO_4 are required to eradicate planktonic and biofilm populations of UPEC, FQRP, and MRSA (Fig. 6 to 8), with the one exception of $\text{Ag}_7\text{NO}_{11}$ in FQRP, which has a similar effectiveness at eradicating planktonic populations as well as preventing and eradicating biofilm populations as it does in the PAO1 strain (Fig. 7 and 1, respectively). Nonetheless, we observed that $\text{Ag}_7\text{NO}_{11}$ eliminated planktonic populations, prevented biofilm formation, and eradicated established biofilms of UPEC, FQRP, and MRSA at lower concentrations than those of AgNO_3 , and CuSO_4 , with the exception of established UPEC biofilms, which had no considerable log reduction values at the concentrations and treatment times tested (Fig. 6 to 8).

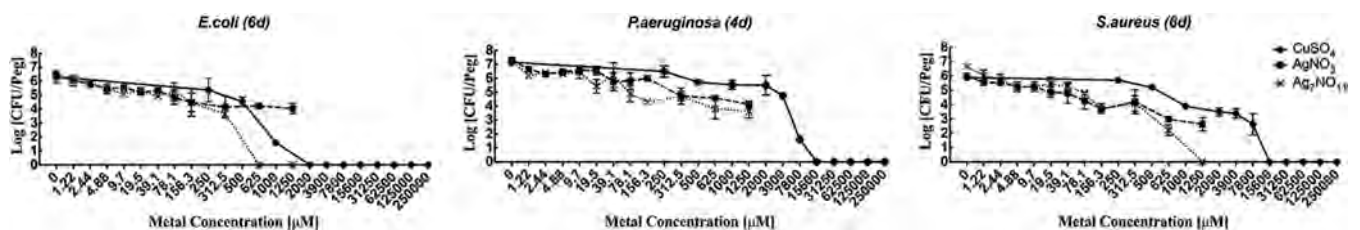


FIG 3 $\text{Ag}_7\text{NO}_{11}$ is more efficacious for eradicating mature biofilms of *E. coli* and *S. aureus*. *E. coli* (JM109), *P. aeruginosa* (PAO1), and *S. aureus* (ATCC 25923) biofilms were cultivated by using the MBEC device. Established biofilms were formed following incubation periods of 4 days for *P. aeruginosa* and 6 days for *E. coli* as well as *S. aureus*. The biofilms were then treated with various concentrations of CuSO_4 , AgNO_3 , and $\text{Ag}_7\text{NO}_{11}$ for 48 h. The MBEC was then determined for the various metal compounds. Values are represented as the means \pm standard deviations ($n = 3$). Note that all Ag stock solutions were prepared at equal molar concentrations of Ag molecules. Hence, concentrations found in this figure are reflective of the concentration of Ag and not the silver-containing compound itself.

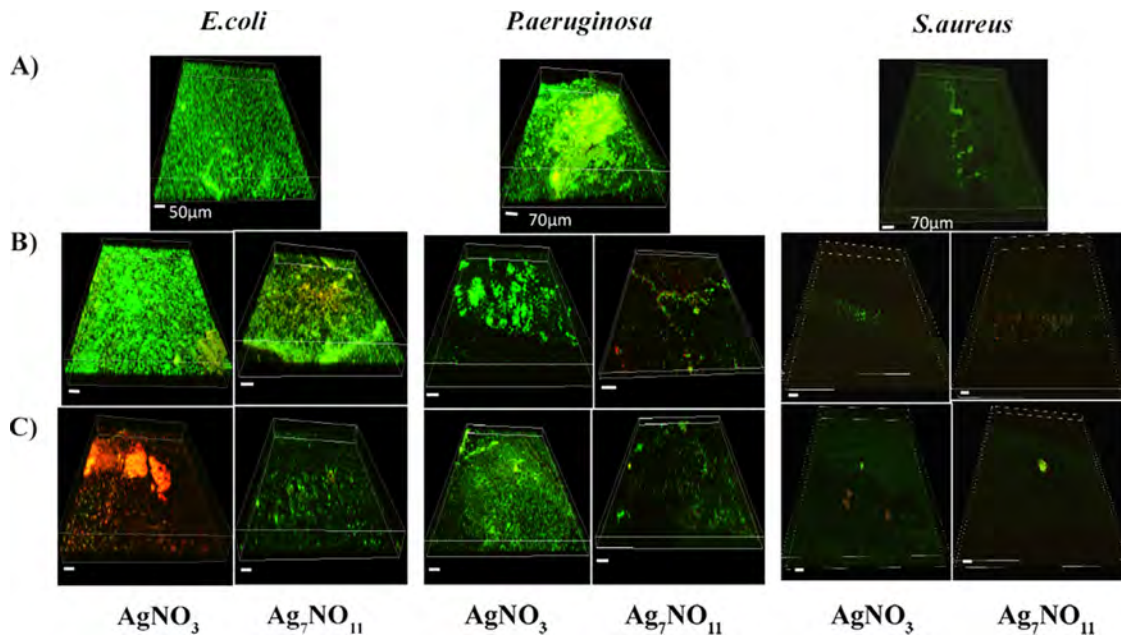


FIG 4 $\text{Ag}_7\text{NO}_{11}$ reduces biofilm quantity. Biofilms of *E. coli* (JM109), *P. aeruginosa* (PAO1), and *S. aureus* (ATCC 25923) were established in the MBEC device for 24 h. The established biofilms were then exposed to 0 μM (A), 5 μM (B), and 12.5 μM (C) AgNO_3 or $\text{Ag}_7\text{NO}_{11}$ (at equivalent Ag concentrations) for 24 h. Live/dead staining was then performed on the treated biofilms, and the biofilm was quantified by using a confocal microscope operating at a $\times 20$ magnification. Green (Syto-9 [excitation wavelength, 488 nm; emission wavelength, 498 nm]) indicates live cells. Red (propidium iodide [excitation wavelength, 523 nm; emission wavelength, 617 nm]) indicates dead cells. Note that all Ag stock solutions were prepared at equal molar concentrations of Ag molecules. Hence, concentrations found in this figure are reflective of the concentration of Ag and not the silver-containing compound itself.

DISCUSSION

Biofilms represent an earnest concern in medicine, as they contribute to chronic infections, complicate wound healing, contaminate medical devices, and exhibit resistance to conventional antimicrobials (9, 16, 20, 21, 53–58). Ionic silver (Ag^+) has a demonstrated antimicrobial outcome at low concentrations (59–61; see reference 62 and references therein) and may offer an alternative option for the treatment of antibiotic-resistant infections. Here, we demonstrated how $\text{Ag}_7\text{NO}_{11}$, a high-oxidation-

state Ag compound, carries an enhanced antimicrobial and antibiofilm capacity. Additionally, our present study adds to the body of literature supporting the antimicrobial and antibiofilm potential of Ag.

In this study, we confirmed that Ag is effective at eliminating planktonic populations of *E. coli*, *P. aeruginosa*, and *S. aureus*. Additionally, we established that Ag is effective at preventing biofilm formation (Fig. 1) and eradicating established biofilms (Fig. 2). Indeed, we also observed that Cu is effective at killing plank-

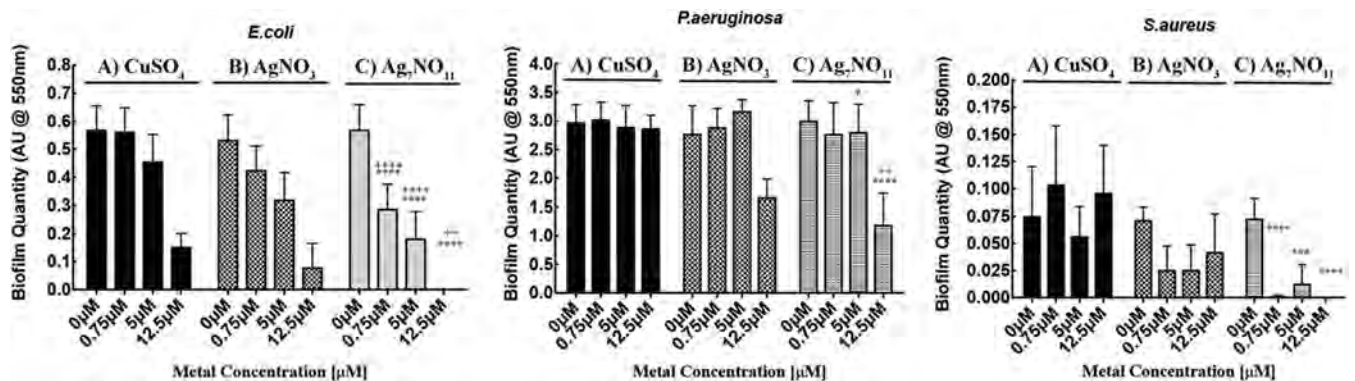


FIG 5 $\text{Ag}_7\text{NO}_{11}$ reduces biofilm biomass. Biofilms of *E. coli* (JM109), *P. aeruginosa* (PAO1), and *S. aureus* (ATCC 25923) were established in the MBEC device for 24 h. The biofilms were then exposed to various concentrations of CuSO_4 (A), AgNO_3 (B), and $\text{Ag}_7\text{NO}_{11}$ (C). Following this, crystal violet staining, according to the O'Toole method (52), was performed on the biofilms. The biomass was quantified by measuring the absorbance at 550 nm. Values are represented as the means \pm standard deviations ($n = 15$). Asterisks indicate a significant difference between CuSO_4 and $\text{Ag}_7\text{NO}_{11}$, where * indicates a P value of ≤ 0.05 , ** indicates a P value of ≤ 0.01 , *** indicates a P value of ≤ 0.001 , and **** indicates a P value of ≤ 0.0001 . Plus signs indicate a significant difference between AgNO_3 and $\text{Ag}_7\text{NO}_{11}$, where + indicates a P value of ≤ 0.05 , ++ indicates a P value of ≤ 0.01 , +++ indicates a P value of ≤ 0.001 , and ++++ indicates a P value of ≤ 0.0001 . Note that all Ag stock solutions were prepared at equal molar concentrations of Ag molecules. Hence, concentrations found in this figure are reflective of the concentration of Ag and not the silver-containing compound itself. AU, arbitrary units.

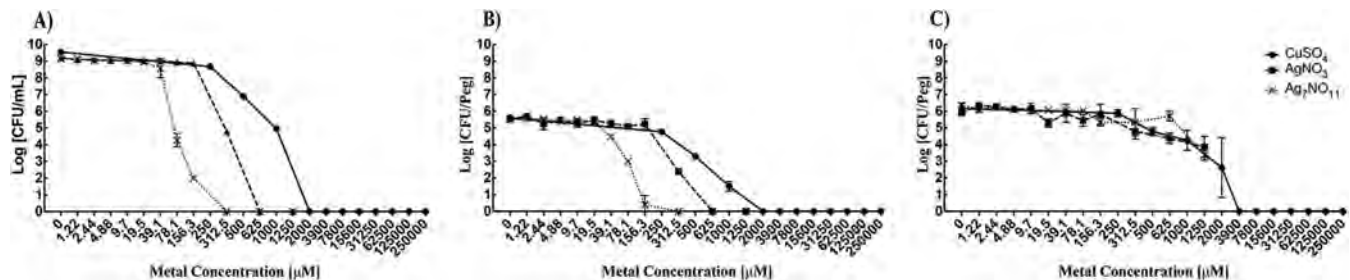


FIG 6 $\text{Ag}_7\text{NO}_{11}$ has antimicrobial and antibiofilm activity against UPEC (CFT703). UPEC biofilms were cultivated by using the MBEC device. (A and B) The MBC for the planktonic population (A) and the MBEC for the biofilm population (B) were determined after a 4-h incubation in the presence of serial dilutions (2-fold) of various metal compounds. (C) Established biofilms were cultivated in the MBEC device for 24 h. The MBEC was then determined following a 24-h treatment with serial dilutions (2-fold) of various metal compounds. Values are represented as the means \pm standard deviations ($n = 4$). Note that all Ag stock solutions were prepared at equal molar concentrations of Ag molecules. Hence, concentrations found in this figure are reflective of the concentration of Ag and not the silver-containing compound itself.

tonic and biofilm populations of laboratory pathogens albeit at millimolar concentrations. Mechanistically, both Cu and Ag are thiophilic metals that have similar targets in the microbial cell (1). One key distinction between these two metals is that Cu is an essential metal, while Ag is nonessential and has no known function in bacterial cells (1). However, it is important to recognize that the precise manner in which Ag poisons microbial cells remains to be fully elaborated.

One novel highlight of this study was that it demonstrated, for the first time, that $\text{Ag}_7\text{NO}_{11}$, a source of higher oxidation states of Ag, had a MBC and a MBEC that were much lower (2-fold or lower) than those of the other metal compounds tested. The advantage of having lower MBCs and MBECs is that less of the compound would be required for the desired therapeutic effect. Interestingly, $\text{Ag}_7\text{NO}_{11}$ was not significantly better than AgNO_3 at preventing *E. coli* and *P. aeruginosa* biofilm formation or at eradicating established *P. aeruginosa* biofilms (Fig. 1A and 2). However, $\text{Ag}_7\text{NO}_{11}$ was significantly better than AgNO_3 at eradicating planktonic populations of all tested bacterial strains (Fig. 1B). This observation is not surprising, as biofilms are notoriously more difficult to eliminate than are their planktonic counterparts (36, 51, 63, 64), an observation confirmed by this study (Fig. 1 and 2; see also Fig. S1 in the supplemental material). In terms of the MBC and MBEC of the Ag salts, the trend in efficacy appeared to follow the molar solubility of the salts in water: $\text{AgNO}_3 > \text{Ag}_2\text{SO}_4 > \text{AgO} > \text{Ag}_2\text{O}$ (solubility product constant [K_{sp}] = 51.6 M, 1.4×10^{-5} M, 4.8×10^{-8} M,

and 3.6×10^{-11} M, respectively). However, we noted strain-specific sensitivities. For example, *E. coli* planktonic populations were more sensitive to Ag_2SO_4 than were *P. aeruginosa* and *S. aureus*. We were surprised at the poor efficacy of AgSD, the silver-sulfonamide synergistic biocidal compound, as it is part of the current standard of care for the treatment of burn wounds (65–67). In some cases, CuSO_4 outperformed AgSD as a bactericidal compound (Fig. 1B and 2). Not surprisingly, much higher concentrations of and much longer treatment periods with Ag and Cu were needed to reduce the number of viable cells in mature biofilms (4 days for *P. aeruginosa* or 6 days for *E. coli* and *S. aureus*) (Fig. 3). The enhanced metal resistance of mature biofilms has been reported previously (68). However, making comparisons across studies is difficult due to differing methodologies used for growing the biofilms. Regardless, this study demonstrated that $\text{Ag}_7\text{NO}_{11}$ outperformed AgNO_3 and CuSO_4 for eradicating mature *E. coli* and *S. aureus* biofilms.

A universally recognized challenge in treating biofilm-associated infections is reducing the biomass of the biofilm. Without a reduction of the biofilm biomass, many antimicrobials will kill the outermost bacteria in the biofilm but will fail to penetrate and kill the innermost bacterial cells, resulting in recurring or persistent infections (9, 69–71). Live/dead staining following a 24-h exposure of established biofilms to AgNO_3 and $\text{Ag}_7\text{NO}_{11}$ revealed that $\text{Ag}_7\text{NO}_{11}$ has the capacity to reduce the biomass of *E. coli*, *P. aeruginosa*, and *S. aureus* biofilms. Both AgNO_3 and $\text{Ag}_7\text{NO}_{11}$

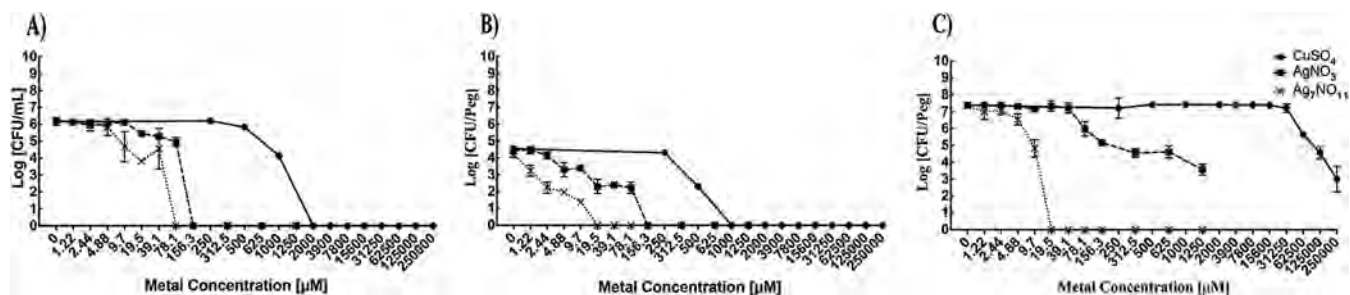


FIG 7 $\text{Ag}_7\text{NO}_{11}$ has antimicrobial and antibiofilm activity against an FQRP isolate. FQRP biofilms were cultivated by using the MBEC device. (A and B) The MBC for the planktonic population (A) and the MBEC for the biofilm population (B) were determined after a 4-h incubation in the presence of serial dilutions (2-fold) of various metal compounds. (C) Established biofilms were cultivated in the MBEC device for 24 h. The MBEC was then determined following a 24-h treatment with serial dilutions (2-fold) of various metal compounds. Values are represented as the means \pm standard deviations ($n = 4$). Note that all Ag stock solutions were prepared at equal molar concentrations of Ag molecules. Hence, concentrations found in this figure are reflective of the concentration of Ag and not the silver-containing compound itself.

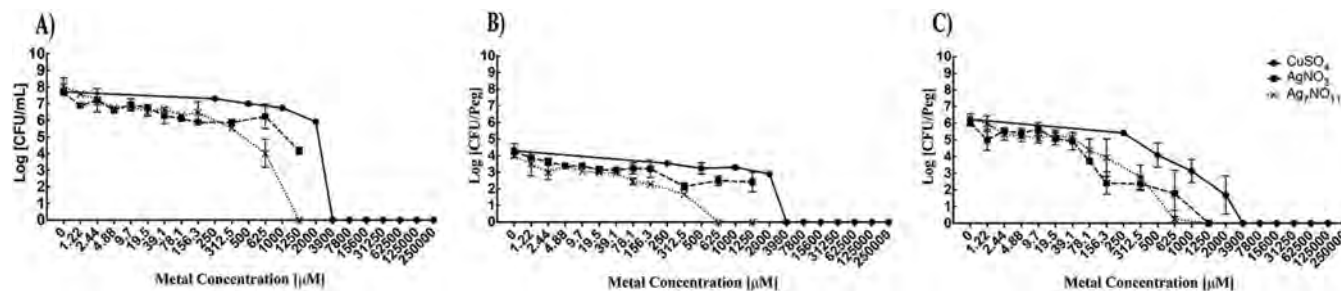


FIG 8 $\text{Ag}_7\text{NO}_{11}$ has antimicrobial and antibiofilm activity against a MRSA strain (USA300). MRSA biofilms were cultivated by using the MBEC device. (A and B) The MBC for the planktonic population (A) and the MBEC for the biofilm population (B) were determined after a 4-h incubation in the presence of serial dilutions (2-fold) of various metal compounds. (C) Established biofilms were cultivated in the MBEC device for 24 h. The MBEC was then determined following a 24-h treatment with serial dilutions (2-fold) of various metal compounds. Values are represented as the means \pm standard deviations ($n = 4$). Note that all Ag stock solutions were prepared at equal molar concentrations of Ag molecules. Hence, concentrations found in this figure are reflective of the concentration of Ag and not the silver-containing compound itself.

increased the amount of dead cells within the biofilm, but $\text{Ag}_7\text{NO}_{11}$ was superior for reducing the biomass of the biofilm (Fig. 4; see also Fig. S4 in the supplemental material). Monitoring the quantity of biomass in established biofilms of *E. coli*, *P. aeruginosa*, and *S. aureus* following a 24-h exposure to CuSO_4 , AgNO_3 , and $\text{Ag}_7\text{NO}_{11}$ revealed that $\text{Ag}_7\text{NO}_{11}$ reduces the amount of biofilm biomass significantly more than do AgNO_3 and CuSO_4 in *E. coli* and *P. aeruginosa*. Meanwhile, $\text{Ag}_7\text{NO}_{11}$ was significantly better than only CuSO_4 in reducing the biomass of *S. aureus* biofilms (Fig. 4). Interestingly, while the biomass-reducing effects of the metal compounds on *E. coli* biofilms were concentration dependent, this was not the case with *P. aeruginosa* or *S. aureus*. This phenomenon raises interesting insights into the potential necessity to target therapies toward particular infections. However, no conclusions should be drawn from these limited observations.

Our overarching goal was to demonstrate the universal antimicrobial and antibiofilm potential of $\text{Ag}_7\text{NO}_{11}$. Accordingly, we performed MBC and MBEC assays on three clinically relevant pathogenic strains, uropathogenic *E. coli*, fluoroquinolone-resistant *P. aeruginosa*, and methicillin-resistant *S. aureus* (Fig. 6 to 8), as well as MBEC assays on two laboratory strains of fungi, *C. albicans* and *C. tropicalis* (see Fig. S2 in the supplemental material). Again, $\text{Ag}_7\text{NO}_{11}$ had a low MBC against planktonic populations, prevented biofilm formation, and eradicated established biofilms at lower concentrations than those of AgNO_3 and CuSO_4 . Certainly, Ag has been explored as a strategy to control infection by uropathogens (72) as well as to combat and control antibiotic-resistant pathogens, including MRSA (67, 73–76). The results from this study demonstrated variable sensitivities of the planktonic and biofilm populations to CuSO_4 , AgNO_3 , and $\text{Ag}_7\text{NO}_{11}$. In general, all of the planktonic and biofilm populations of the clinically relevant strains were capable of tolerating higher concentrations of the tested metals than those tolerated by their laboratory counterparts (Fig. 1, 2, and 6 to 8). The single exception to this trend was observed with FQRP, where $\text{Ag}_7\text{NO}_{11}$ was exceptionally efficacious in the low-micromolar range, tempting speculation that $\text{Ag}_7\text{NO}_{11}$ can be used as an antimicrobial coating for endotracheal tubes. It is also noteworthy that $\text{Ag}_7\text{NO}_{11}$ eliminated planktonic populations, prevented biofilm formation, and eradicated established biofilms at lower concentrations than those of AgNO_3 and CuSO_4 , with the exception of established UPEC biofilms, which could not be reduced to a significant level by Ag

under the conditions tested, again confirming the challenge involved in eradicating an established biofilm. Although only limited analyses were performed in this study, we observed that *Candida* sp. biofilms are also difficult to eradicate (see Fig. S4 in the supplemental material). The enhanced tolerance of *Candida* sp. has been observed previously by our research group (38, 77, 78). However, $\text{Ag}_7\text{NO}_{11}$ still has a MBEC in the millimolar range, much within a potentially therapeutic range. This is ever important, as *Candida* species are biofilm-forming microorganisms with the potential to be infectious and can cause deleterious health outcomes (79–82).

To conclude, we have demonstrated here that thiophilic metals, such as Cu and Ag, are efficacious antimicrobial and antibiofilm agents. Furthermore, we have demonstrated that $\text{Ag}_7\text{NO}_{11}$, a compound that delivers higher oxidation states of Ag, has a greater antimicrobial and antibiofilm capacity, at equal concentrations of Ag, than do AgNO_3 , Ag_2SO_4 , AgO , Ag_2O , and CuSO_4 . Additionally, we demonstrated that $\text{Ag}_7\text{NO}_{11}$ reduces the biomass of biofilms, an important strategy for combating biofilm-associated infections.

Recent realizations have focused on biofilms as the causative agent of many infectious diseases, but we are at a loss for adequate therapies to eradicate them. This includes chronic wounds, where many wound care products exist but very few have an antibiofilm capacity. Fortunately, wound care products offer the greatest potential for implementing metal-based antimicrobials, due to decreased internalization of the therapeutic by the patient. This is the venue for which we envision the best utility for $\text{Ag}_7\text{NO}_{11}$ as a strategy for preventing and controlling infectious disease. Thus, $\text{Ag}_7\text{NO}_{11}$ will add to our arsenal for combating infection, an arsenal that is desperately needed in the face of increasing numbers of antibiotic-resistant infections and a dwindling pipeline of new antibiotics.

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