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

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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 [$Ag(Ag_3O_4)_2NO_3$ or Ag_7NO_{11}], against other metal compounds with documented antimicrobial activity, including Ag_2SO_4, $AgNO_3$, silver sulfadiazine (AgSD), AgO, Ag_2O, and $CuSO_4$. Our findings reveal that Ag_7NO_{11} 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 Ag_7NO_{11} 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 ($AgNO_3$) (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 Ag^{1+} ion (7, 39). Briefly, Ag^{1+}, 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, Ag^{2+} and Ag^{3+}, 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, $Ag(Ag_3O_4)_2X$, the most stable of which is coordinated with nitrate, silver oxynitrate [$Ag(Ag_3O_4)_2NO_3$ or Ag_7NO_{11}], 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 Ag_7NO_{11}, AgSD, $AgNO_3$, silver sulfate (Ag_2SO_4), silver-(I,III) oxide (AgO), silver(I) oxide (Ag_2O), and copper sulfate ($CuSO_4$) 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). $CuSO_4$ 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 Ag_7NO_{11} eradicated planktonic and biofilm populations of the tested strains at lower concentrations than those of the other metal compounds tested. $AgNO_3$, the most soluble Ag salt, also had antimi-

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crobial 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 Ag_7NO_{11}, it was chosen as our comparator for testing of the efficacy of Ag_7NO_{11} in the proceeding experiments. Using the live/dead staining technique as well as crystal violet staining, we demonstrated that Ag_7NO_{11} reduced the biomass of the biofilm. In summary, our data demonstrate that Ag_7NO_{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 (Innovotech, Edmonton, AB, Canada). Briefly, following growth of the preculture 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). Ag_7NO_{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.l 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× objective. Images were processed by using Imaris ×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

Ag_7NO_{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.*

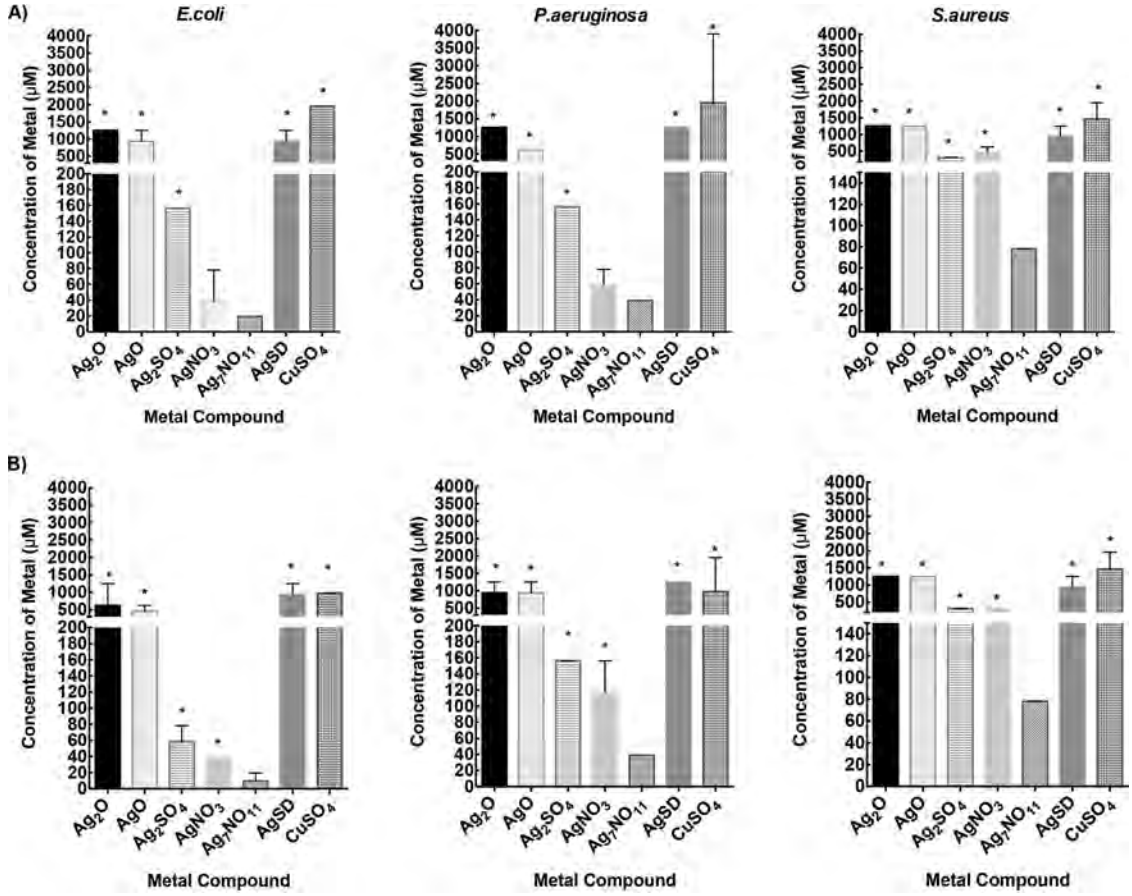

FIG 1 Ag_7NO_{11} prevents biofilm formation at lower concentrations than those of other metal compounds. The MBEC device was inoculated with *E. coli* (JM109), *P. aeruginosa* (PAO1), and *S. aureus* (ATCC 25923) concurrently with serial dilutions (2-fold) of Ag_2O, AgO, Ag_2SO_4, Ag_7NO_{11}, AgSD, and $CuSO_4$. 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 Ag_7NO_{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.

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 Ag_7NO_{11} were needed to prevent the formation of biofilms (Fig. 1A) and, with the exception of $AgNO_3$, to eliminate planktonic populations of our tested strains (Fig. 1B) following a 4-h incubation period.

Ag_7NO_{11} 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 Ag_2O, AgO, Ag_2SO_4, $AgNO_3$, Ag_7NO_{11}, AgSD, and $CuSO_4$ for 24 h (Fig. 2). A 4-h exposure of established biofilms to $AgNO_3$, Ag_7NO_{11}, and $CuSO_4$ 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 Ag_7NO_{11} 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 $AgNO_3$ 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 $AgNO_3$, $CuSO_4$, and Ag_7NO_{11} were required to reach the MBEC, with the exception of $CuSO_4$ in *E. coli* (Fig. 3). Nonetheless, Ag_7NO_{11} eradicated the mature biofilms of *S. aureus* and *E. coli* at lower concentrations than those of $AgNO_3$ and $CuSO_4$ (Fig. 3). Additionally, at the highest concentrations (of equivalent Ag) of $AgNO_3$ and Ag_7NO_{11} tested (1,250 μM), Ag_7NO_{11} significantly outperformed $AgNO_3$ and $CuSO_4$ at eradicating *C. albicans* and *C. tropicalis* biofilms (see Fig. S2 in the supplemental material).

Ag_7NO_{11} reduces the biomass of established biofilms. Since $AgNO_3$ and Ag_7NO_{11} 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 $AgNO_3$ and Ag_7NO_{11} (at equivalent Ag concentrations) on established biofilms of *E. coli*, *P. aeruginosa*, and *S. aureus* (Fig. 4; see also Fig. S3

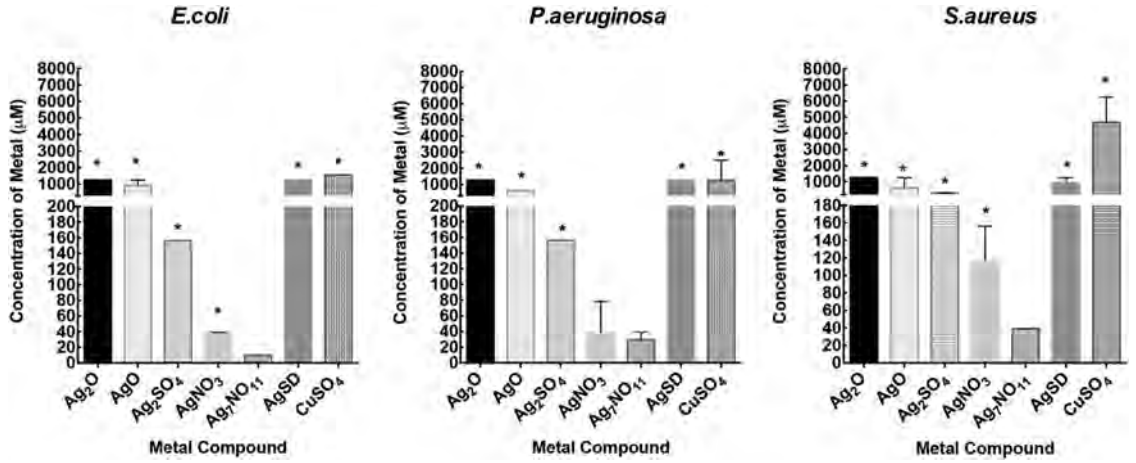

FIG 2 Ag_7NO_{11} eradicates established biofilms at lower concentrations than those of other metal compounds. The MBEC device was inoculated with *E. coli* (JM109), *P. aeruginosa* (PAO1), and *S. aureus* (ATCC 25923). Biofilms were established following a 24-h incubation. The established biofilms were then treated with serial dilutions (2-fold) of Ag_2O, AgO, Ag_2SO_4, Ag_7NO_{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 Ag_7NO_{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 Ag_7NO_{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 Ag_7NO_{11} (see Fig. S3 in the supplemental material). Following a 24-h exposure to $AgNO_3$ and Ag_7NO_{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 Ag_7NO_{11} (Fig. 4; see also Fig. S4 in the supplemental material). To confirm that Ag_7NO_{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 $AgNO_3$, Ag_7NO_{11}, and $CuSO_4$ (Fig. 5). We observed that Ag_7NO_{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, Ag_7NO_{11} outperformed only $CuSO_4$ for reducing the biomass of *S. aureus* biofilms (Fig. 5).

Ag_7NO_{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 Ag_7NO_{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 Ag_7NO_{11}, $AgNO_3$, and $CuSO_4$ (Fig. 6, 7, and 8, respectively). Our observations suggest that higher concentrations of $AgNO_3$, Ag_7NO_{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 Ag_7NO_{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 Ag_7NO_{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).

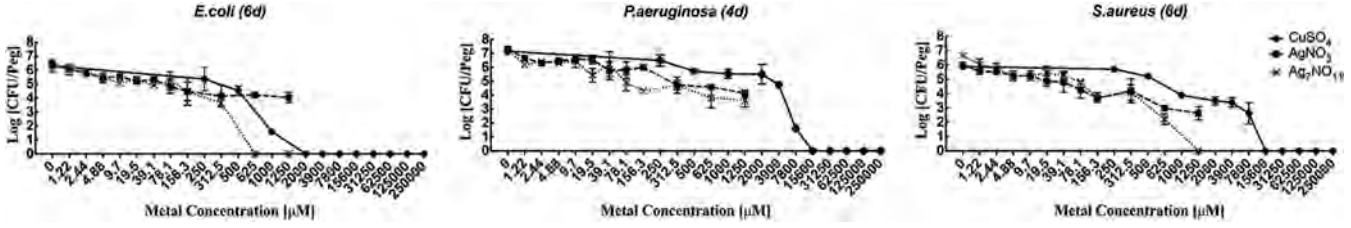

FIG 3 Ag_7NO_{11} is more efficacious for eradicating mature biofilms of *E. coli* and *S. aureus*. *E. coli* (JM109), *P. aeruginosa* (PAO1), and *S. aureus* (ATCC 25923) biofilms were cultivated by using the MBEC device. Established biofilms were formed following incubation periods of 4 days for *P. 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 Ag_7NO_{11} for 48 h. The MBEC was then determined for the various metal compounds. Values are represented as the means ± 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.

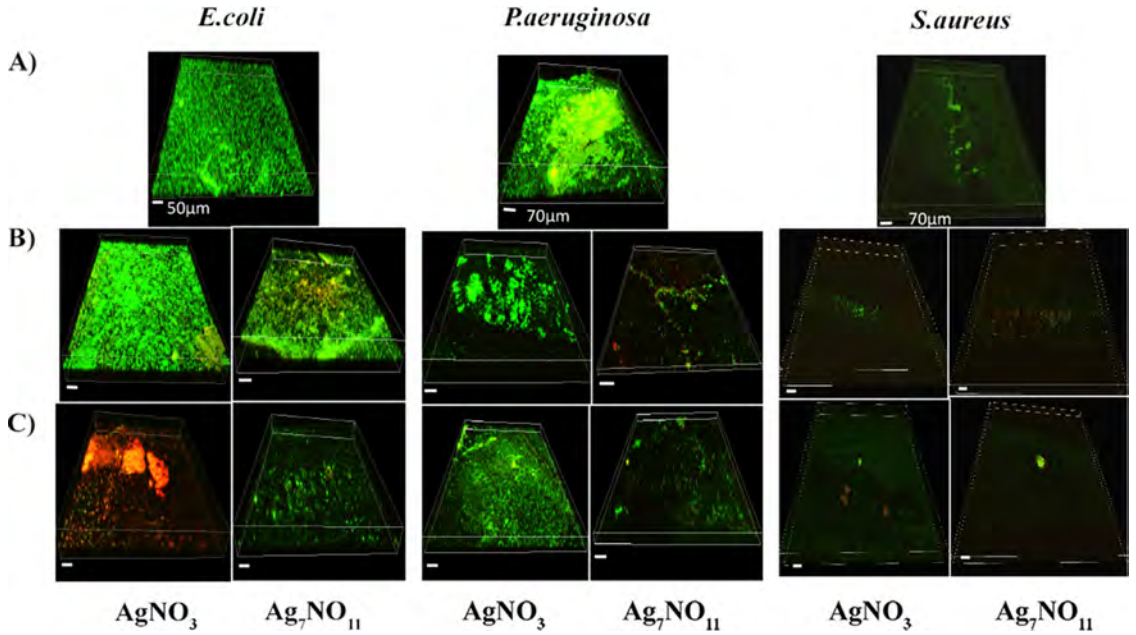

FIG 4 Ag_7NO_{11} reduces biofilm quantity. Biofilms of *E. coli* (JM109), *P. aeruginosa* (PAO1), and *S. aureus* (ATCC 25923) were established in the MBEC device for 24 h. The established biofilms were then exposed to 0 μM (A), 5 μM (B), and 12.5 μM (C) $AgNO_3$ or Ag_7NO_{11} (at equivalent Ag concentrations) 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 ×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 Ag_7NO_{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-

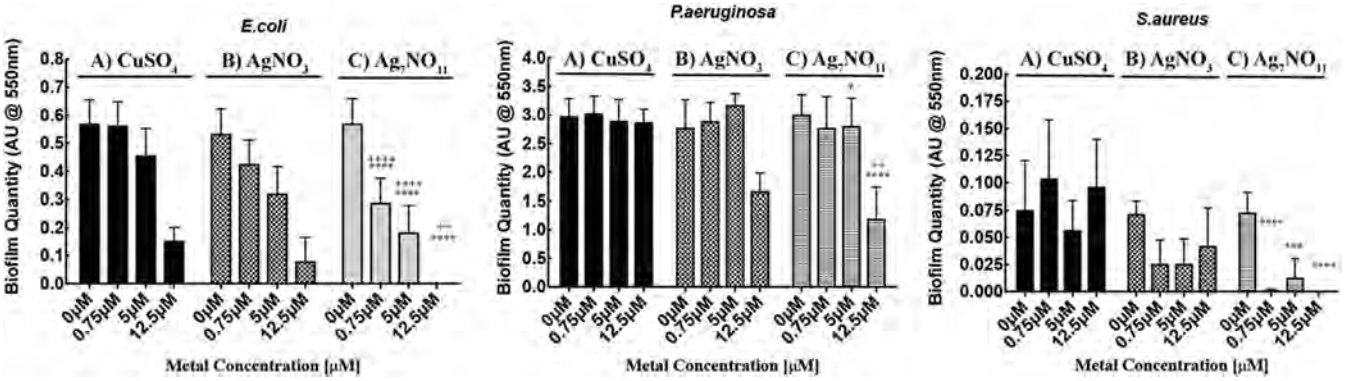

FIG 5 Ag_7NO_{11} reduces biofilm biomass. Biofilms of *E. coli* (JM109), *P. aeruginosa* (PAO1), and *S. aureus* (ATCC 25923) were established in the MBEC device for 24 h. The biofilms were then exposed to various concentrations of $CuSO_4$ (A), $AgNO_3$ (B), and Ag_7NO_{11} (C). Following this, crystal 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 ± standard deviations ($n = 15$). Asterisks indicate a significant difference between $CuSO_4$ and Ag_7NO_{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 Ag_7NO_{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.

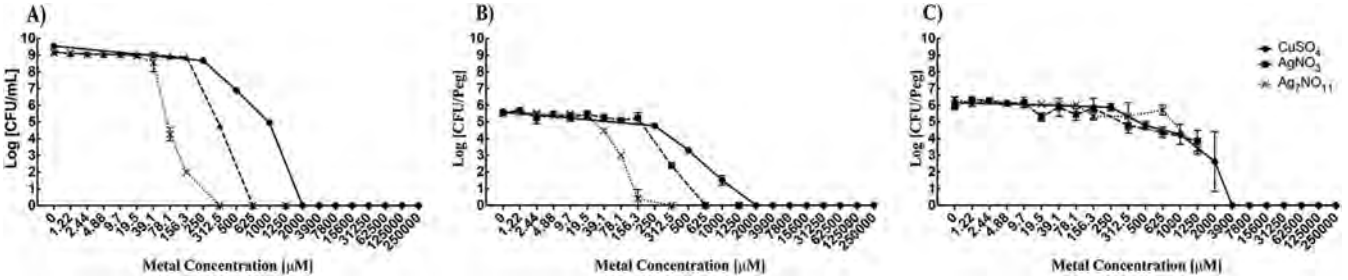

FIG 6 Ag_7NO_{11} has antimicrobial and antibiofilm activity against UPEC (CFT703). UPEC biofilms were cultivated by using the MBEC device. (A and B) The MBC for the planktonic population (A) and the MBEC for the biofilm population (B) were determined after a 4-h incubation in the presence of serial 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 ± 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 Ag_7NO_{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, Ag_7NO_{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, Ag_7NO_{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: $AgNO_3 > Ag_2SO_4 > AgO > Ag_2O$ (solubility product constant [Ksp] = 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 Ag_7NO_{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 Ag_7NO_{11} revealed that Ag_7NO_{11} has the capacity to reduce the biomass of *E. coli*, *P. aeruginosa*, and *S. aureus* biofilms. Both $AgNO_3$ and Ag_7NO_{11}

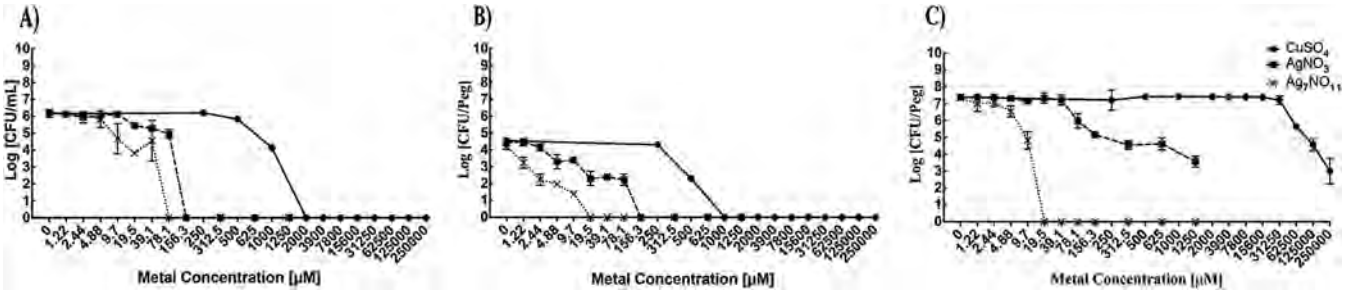

FIG 7 Ag_7NO_{11} has antimicrobial and antibiofilm activity against an FQRP isolate. FQRP biofilms were cultivated by using the MBEC device. (A and B) The MBC for the planktonic population (A) and the MBEC for the biofilm population (B) were determined after a 4-h incubation in the presence of serial 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 ± 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.

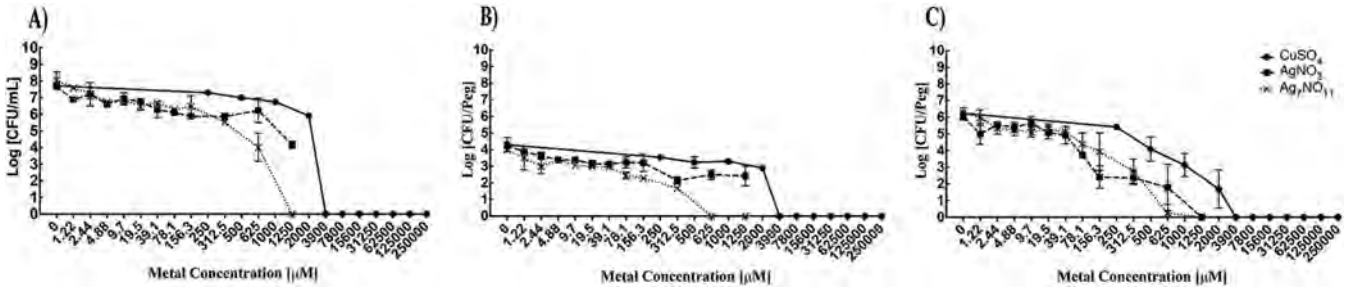

FIG 8 Ag_7NO_{11} has antimicrobial and antibiofilm activity against a MRSA strain (USA300). MRSA biofilms were cultivated by using the MBEC device. (A and B) The MBC for the planktonic population (A) and the MBEC for the biofilm population (B) were determined after a 4-h incubation in the presence of 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 ± 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 Ag_7NO_{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 Ag_7NO_{11} revealed that Ag_7NO_{11} reduces the amount of biofilm biomass significantly more than do $AgNO_3$ and $CuSO_4$ in *E. coli* and *P. aeruginosa*. Meanwhile, Ag_7NO_{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 Ag_7NO_{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, Ag_7NO_{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 Ag_7NO_{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 Ag_7NO_{11} was exceptionally efficacious in the low-micromolar range, tempting speculation that Ag_7NO_{11} can be used as an antimicrobial coating for endotracheal tubes. It is also noteworthy that Ag_7NO_{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, Ag_7NO_{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 Ag_7NO_{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 Ag_7NO_{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 Ag_7NO_{11} as a strategy for preventing and controlling infectious disease. Thus, Ag_7NO_{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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REFERENCES

1. **Lemire JA, Harrison JJ, Turner RJ.** 2013. Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nat Rev Microbiol **11:**371–384. http://dx.doi.org/10.1038/nrmicro3028.
2. **Alexander JW.** 2009. History of the medical use of silver. Surg Infect **10:**289–292. http://dx.doi.org/10.1089/sur.2008.9941.
3. **Mijnendonckx K, Leys N, Mahillon J, Silver S, Van Houdt R.** 2013. Antimicrobial silver: uses, toxicity and potential for resistance. Biometals **26:**609–621. http://dx.doi.org/10.1007/s10534-013-9645-z.
4. **Morones-Ramirez JR, Winkler JA, Spina CS, Collins JJ.** 2013. Silver enhances antibiotic activity against gram-negative bacteria. Sci Transl Med **5:**190ra181. http://dx.doi.org/10.1126/scitranslmed.3006276.
5. **Percival SL, Thomas J, Linton S, Okel T, Corum L, Slone W.** 2012. The antimicrobial efficacy of silver on antibiotic-resistant bacteria isolated from burn wounds. Int Wound J **9:**488–493. http://dx.doi.org/10.1111/j.1742-481X.2011.00903.x.
6. **Toy LW, Macera L.** 2011. Evidence-based review of silver dressing use on chronic wounds. J Am Acad Nurse Pract **23:**183–192. http://dx.doi.org/10.1111/j.1745-7599.2011.00600.x.
7. **Walker M, Parsons D.** 2014. The biological fate of silver ions following the use of silver-containing wound care products—a review. Int Wound J **11:**496–504. http://dx.doi.org/10.1111/j.1742-481X.2012.01115.x.
8. **Aziz Z, Abu SF, Chong NJ.** 2012. A systematic review of silver-containing dressings and topical silver agents (used with dressings) for burn wounds. Burns **38:**307–318. http://dx.doi.org/10.1016/j.burns.2011.09.020.
9. **Costerton JW, Stewart PS, Greenberg EP.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318–1322. http://dx.doi.org/10.1126/science.284.5418.1318.
10. **James GA, Swogger E, Wolcott R, Pulcini E, Secor P, Sestrich J, Costerton JW, Stewart PS.** 2008. Biofilms in chronic wounds. Wound Repair Regen **16:**37–44. http://dx.doi.org/10.1111/j.1524-475X.2007.00321.x.
11. **Bjarnsholt T, Kirketerp-Moller K, Jensen PO, Madsen KG, Phipps R, Krogfelt K, Hoiby N, Givskov M.** 2008. Why chronic wounds will not heal: a novel hypothesis. Wound Repair Regen **16:**2–10. http://dx.doi.org/10.1111/j.1524-475X.2007.00283.x.
12. **Wolcott RD, Rhoads DD, Bennett ME, Wolcott BM, Gogokhia L, Costerton JW, Dowd SE.** 2010. Chronic wounds and the medical biofilm paradigm. J Wound Care **19:**45–46, 48–50, 52–43. http://dx.doi.org/10.12968/jowc.2010.19.2.46966.
13. **Percival SL, Hill KE, Williams DW, Hooper SJ, Thomas DW, Costerton JW.** 2012. A review of the scientific evidence for biofilms in wounds. Wound Repair Regen **20:**647–657. http://dx.doi.org/10.1111/j.1524-475X.2012.00836.x.
14. **Valente PMDS, Deva A, Ngo Q, Vickery K.** The increased killing of biofilms in vitro by combining topical silver dressings with topical negative pressure in chronic wounds. Int Wound J, in press.
15. **Metcalf DG, Bowler PG.** Clinician perceptions of wound biofilm. Int Wound J, in press.
16. **Hoiby N, Ciofu O, Johansen HK, Song ZJ, Moser C, Jensen PO, Molin S, Givskov M, Tolker-Nielsen T, Bjarnsholt T.** 2011. The clinical impact of bacterial biofilms. Int J Oral Sci **3:**55–65. http://dx.doi.org/10.4248/IJOS11026.
17. **Mah TF, O'Toole GA.** 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol **9:**34–39. http://dx.doi.org/10.1016/S0966-842X(00)01913-2.
18. **Lopes SP, Ceri H, Azevedo NF, Pereira MO.** 2012. Antibiotic resistance of mixed biofilms in cystic fibrosis: impact of emerging microorganisms on treatment of infection. Int J Antimicrob Agents **40:**260–263. http://dx.doi.org/10.1016/j.ijantimicag.2012.04.020.
19. **Ito A, Taniuchi A, May T, Kawata K, Okabe S.** 2009. Increased antibiotic resistance of Escherichia coli in mature biofilms. Appl Environ Microbiol **75:**4093–4100. http://dx.doi.org/10.1128/AEM.02949-08.
20. **Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O.** 2010. Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents **35:**322–332. http://dx.doi.org/10.1016/j.ijantimicag.2009.12.011.
21. **Stewart PS, Costerton JW.** 2001. Antibiotic resistance of bacteria in biofilms. Lancet **358:**135–138. http://dx.doi.org/10.1016/S0140-6736(01)05321-1.
22. **Ceri H, Olson ME, Turner RJ.** 2010. Needed, new paradigms in antibiotic development. Expert Opin Pharmacother **11:**1233–1237. http://dx.doi.org/10.1517/14656561003724747.
23. **Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA.** 2003. A genetic basis for Pseudomonas aeruginosa biofilm antibiotic resistance. Nature **426:**306–310. http://dx.doi.org/10.1038/nature02122.
24. **Bjarnsholt T, Kirketerp-Moller K, Kristiansen S, Phipps R, Nielsen AK, Jensen PO, Hoiby N, Givskov M.** 2007. Silver against Pseudomonas aeruginosa biofilms. APMIS **115:**921–928. http://dx.doi.org/10.1111/j.1600-0463.2007.apm_646.x.
25. **Martinez-Gutierrez F, Boegli L, Agostinho A, Sanchez EM, Bach H, Ruiz F, James G.** 2013. Anti-biofilm activity of silver nanoparticles against different microorganisms. Biofouling **29:**651–660. http://dx.doi.org/10.1080/08927014.2013.794225.
26. **Fabrega J, Renshaw JC, Lead JR.** 2009. Interactions of silver nanoparticles with Pseudomonas putida biofilms. Environ Sci Technol **43:**9004–9009. http://dx.doi.org/10.1021/es901706j.
27. **Monteiro DR, Gorup LF, Silva S, Negri M, de Camargo ER, Oliveira R, Barbosa DB, Henriques M.** 2011. Silver colloidal nanoparticles: antifungal effect against adhered cells and biofilms of Candida albicans and Candida glabrata. Biofouling **27:**711–719. http://dx.doi.org/10.1080/08927014.2011.599101.
28. **Herron M, Agarwal A, Kierski PR, Calderon DF, Teixeira LB, Schurr MJ, Murphy CJ, Czuprynski CJ, McAnulty JF, Abbott NL.** 2014. Reduction in wound bioburden using a silver-loaded dissolvable microfilm construct. Adv Healthc Mater **3:**916–928. http://dx.doi.org/10.1002/adhm.201300537.
29. **Hooper SJ, Percival SL, Hill KE, Thomas DW, Hayes AJ, Williams DW.** 2012. The visualisation and speed of kill of wound isolates on a silver alginate dressing. Int Wound J **9:**633–642. http://dx.doi.org/10.1111/j.1742-481X.2012.00927.x.
30. **Leaper DJ.** 2006. Silver dressings: their role in wound management. Int Wound J **3:**282–294. http://dx.doi.org/10.1111/j.1742-481X.2006.00265.x.
31. **Jones SA, Bowler PG, Walker M, Parsons D.** 2004. Controlling wound bioburden with a novel silver-containing Hydrofiber dressing. Wound Repair Regen **12:**288–294. http://dx.doi.org/10.1111/j.1067-1927.2004.012304.x.
32. **Wilkinson LJ, White RJ, Chipman JK.** 2011. Silver and nanoparticles of silver in wound dressings: a review of efficacy and safety. J Wound Care **20:**543–549. http://dx.doi.org/10.12968/jowc.2011.20.11.543.
33. **Baker B, McKernan P, Marsik F.** 2014. Clinical and regulatory development of antibiofilm drugs: the need, the potential, and the challenges, p 469–486. *In* Rumbaugh KP, Ahmad I (ed), Antibiofilm agents, vol 8. Springer, Berlin, Germany.
34. **Beloin C, Renard S, Ghigo J-M, Lebeaux D.** 2014. Novel approaches to combat bacterial biofilms. Curr Opin Pharmacol **18:**61–68. http://dx.doi.org/10.1016/j.coph.2014.09.005.
35. **Chien C-C, Lin B-C, Wu C-H.** 2013. Biofilm formation and heavy metal resistance by an environmental Pseudomonas sp. Biochem Eng J **78:**132–137. http://dx.doi.org/10.1016/j.bej.2013.01.014.
36. **Harrison JJ, Ceri H, Turner RJ.** 2007. Multimetal resistance and tolerance in microbial biofilms. Nat Rev Microbiol **5:**928–938. http://dx.doi.org/10.1038/nrmicro1774.
37. **Harrison JJ, Turner RJ, Ceri H.** 2005. Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic Pseudomonas aeruginosa. Environ Microbiol **7:**981–994. http://dx.doi.org/10.1111/j.1462-2920.2005.00777.x.
38. **Harrison JJ, Rabiei M, Turner RJ, Badry EA, Sproule KM, Ceri H.** 2006. Metal resistance in Candida biofilms. FEMS Microbiol Ecol **55:**479–491. http://dx.doi.org/10.1111/j.1574-6941.2005.00045.x.
39. **Lansdown AB.** 2006. Silver in health care: antimicrobial effects and safety in use. Curr Probl Dermatol **33:**17–34. http://dx.doi.org/10.1159/000093928.
40. **Xu FF, Imlay JA.** 2012. Silver(I), mercury(II), cadmium(II), and zinc(II) target exposed enzymic iron-sulfur clusters when they toxify Escherichia coli. Appl Environ Microbiol **78:**3614–3621. http://dx.doi.org/10.1128/AEM.07368-11.

41. **McMillan JA.** 1962. Higher oxidation states of silver. Chem Rev **62:**65–80. http://dx.doi.org/10.1021/cr60215a004.
42. **Djokić SS.** 2004. Deposition of silver oxysalts and their antimicrobial properties. J Electrochem Soc **151:**C359–C364. http://dx.doi.org/10.1149/1.1723498.
43. **Ruparelia JP, Chatterjee AK, Duttagupta SP, Mukherji S.** 2008. Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomater **4:**707–716. http://dx.doi.org/10.1016/j.actbio.2007.11.006.
44. **Grass G, Rensing C, Solioz M.** 2011. Metallic copper as an antimicrobial surface. Appl Environ Microbiol **77:**1541–1547. http://dx.doi.org/10.1128/AEM.02766-10.
45. **Said J, Dodoo CC, Walker M, Parsons D, Stapleton P, Beezer AE, Gaisford S.** 2014. An in vitro test of the efficacy of silver-containing wound dressings against Staphylococcus aureus and Pseudomonas aeruginosa in simulated wound fluid. Int J Pharm **462:**123–128. http://dx.doi.org/10.1016/j.ijpharm.2013.12.037.
46. **Harrison JJ, Ceri H, Yerly J, Stremick CA, Hu Y, Martinuzzi R, Turner RJ.** 2006. The use of microscopy and three-dimensional visualization to evaluate the structure of microbial biofilms cultivated in the Calgary Biofilm Device. Biol Proced Online **8:**194–215. http://dx.doi.org/10.1251/bpo127.
47. **Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A.** 1999. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol **37:**1771–1776.
48. **Olson ME, Ceri H, Morck DW, Buret AG, Read RR.** 2002. Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res **66:**86–92.
49. **Harrison JJ, Ceri H, Roper NJ, Badry EA, Sproule KM, Turner RJ.** 2005. Persister cells mediate tolerance to metal oxyanions in Escherichia coli. Microbiology **151:**3181–3195. http://dx.doi.org/10.1099/mic.0.27794-0.
50. **Harrison JJ, Turner RJ, Ceri H.** 2005. High-throughput metal susceptibility testing of microbial biofilms. BMC Microbiol **5:**53. http://dx.doi.org/10.1186/1471-2180-5-53.
51. **Harrison JJ, Ceri H, Stremick CA, Turner RJ.** 2004. Biofilm susceptibility to metal toxicity. Environ Microbiol **6:**1220–1227. http://dx.doi.org/10.1111/j.1462-2920.2004.00656.x.
52. **O'Toole GA.** 2011. Microtiter dish biofilm formation assay. J Vis Exp **2011:**2437. http://dx.doi.org/10.3791/2437.
53. **Donlan RM, Costerton JW.** 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev **15:**167–193. http://dx.doi.org/10.1128/CMR.15.2.167-193.2002.
54. **Marsh PD, Moter A, Devine DA.** 2011. Dental plaque biofilms: communities, conflict and control. Periodontol 2000 **55:**16–35. http://dx.doi.org/10.1111/j.1600-0757.2009.00339.x.
55. **Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME.** 2011. Staphylococcus aureus biofilms. Virulence **2:**445–459. http://dx.doi.org/10.4161/viru.2.5.17724.
56. **Gurjala AN, Geringer MR, Seth AK, Hong SJ, Smeltzer MS, Galiano RD, Leung KP, Mustoe TA.** 2011. Development of a novel, highly quantitative in vivo model for the study of biofilm-impaired cutaneous wound healing. Wound Repair Regen **19:**400–410. http://dx.doi.org/10.1111/j.1524-475X.2011.00690.x.
57. **Guo S, Dipietro LA.** 2010. Factors affecting wound healing. J Dent Res **89:**219–229. http://dx.doi.org/10.1177/0022034509359125.
58. **Roy S, Elgharably H, Sinha M, Ganesh K, Chaney S, Mann E, Miller C, Khanna S, Bergdall VK, Powell HM, Cook CH, Gordillo GM, Wozniak DJ, Sen CK.** 2014. Mixed-species biofilm compromises wound healing by disrupting epidermal barrier function. J Pathol **233:**331–343. http://dx.doi.org/10.1002/path.4360.
59. **Kim JS, Kuk E, Yu KN, Kim JH, Park SJ, Lee HJ, Kim SH, Park YK, Park YH, Hwang CY, Kim YK, Lee YS, Jeong DH, Cho MH.** 2007. Antimicrobial effects of silver nanoparticles. Nanomedicine **3:**95–101. http://dx.doi.org/10.1016/j.nano.2006.12.001.
60. **Spacciapoli P, Buxton D, Rothstein D, Friden P.** 2001. Antimicrobial activity of silver nitrate against periodontal pathogens. J Periodontal Res **36:**108–113. http://dx.doi.org/10.1034/j.1600-0765.2001.360207.x.
61. **Holt KB, Bard AJ.** 2005. Interaction of silver(I) ions with the respiratory chain of Escherichia coli: an electrochemical and scanning electrochemical microscopy study of the antimicrobial mechanism of micromolar Ag+. Biochemistry **44:**13214–13223. http://dx.doi.org/10.1021/bi0508542.
62. **Chernousova S, Epple M.** 2013. Silver as antibacterial agent: ion, nanoparticle, and metal. Angew Chem Int Ed Engl **52:**1636–1653. http://dx.doi.org/10.1002/anie.201205923.
63. **Drenkard E.** 2003. Antimicrobial resistance of Pseudomonas aeruginosa biofilms. Microbes Infect **5:**1213–1219. http://dx.doi.org/10.1016/j.micinf.2003.08.009.
64. **Anderson GG, O'Toole GA.** 2008. Innate and induced resistance mechanisms of bacterial biofilms. Curr Top Microbiol Immunol **322:**85–105. http://dx.doi.org/10.1007/978-3-540-75418-3_5.
65. **Atiyeh BS, Costagliola M, Hayek SN, Dibo SA.** 2007. Effect of silver on burn wound infection control and healing: review of the literature. Burns **33:**139–148. http://dx.doi.org/10.1016/j.burns.2006.06.010.
66. **Fuller FW.** 2009. The side effects of silver sulfadiazine. J Burn Care Res **30:**464–470. http://dx.doi.org/10.1097/BCR.0b013e3181a28c9b.
67. **Ulkur E, Oncul O, Karagoz H, Yeniz E, Celikoz B.** 2005. Comparison of silver-coated dressing (Acticoat), chlorhexidine acetate 0.5% (Bactigrass), and fusidic acid 2% (Fucidin) for topical antibacterial effect in methicillin-resistant staphylococci-contaminated, full-skin thickness rat burn wounds. Burns **31:**874–877. http://dx.doi.org/10.1016/j.burns.2005.05.002.
68. **Teitzel GM, Parsek MR.** 2003. Heavy metal resistance of biofilm and planktonic Pseudomonas aeruginosa. Appl Environ Microbiol **69:**2313–2320. http://dx.doi.org/10.1128/AEM.69.4.2313-2320.2003.
69. **Hall-Stoodley L, Stoodley P.** 2009. Evolving concepts in biofilm infections. Cell Microbiol **11:**1034–1043. http://dx.doi.org/10.1111/j.1462-5822.2009.01323.x.
70. **Fux CA, Costerton JW, Stewart PS, Stoodley P.** 2005. Survival strategies of infectious biofilms. Trends Microbiol **13:**34–40. http://dx.doi.org/10.1016/j.tim.2004.11.010.
71. **Stewart PS.** 2002. Mechanisms of antibiotic resistance in bacterial biofilms. Int J Med Microbiol **292:**107–113. http://dx.doi.org/10.1078/1438-4221-00196.
72. **Desai DG, Liao KS, Cevallos ME, Trautner BW.** 2010. Silver or nitrofurazone impregnation of urinary catheters has a minimal effect on uropathogen adherence. J Urol **184:**2565–2571. http://dx.doi.org/10.1016/j.juro.2010.07.036.
73. **Wright JB, Lam K, Burrell RE.** 1998. Wound management in an era of increasing bacterial antibiotic resistance: a role for topical silver treatment. Am J Infect Control **26:**572–577. http://dx.doi.org/10.1053/ic.1998.v26.a93527.
74. **Nanda A, Saravanan M.** 2009. Biosynthesis of silver nanoparticles from Staphylococcus aureus and its antimicrobial activity against MRSA and MRSE. Nanomedicine **5:**452–456. http://dx.doi.org/10.1016/j.nano.2009.01.012.
75. **Strohal R, Schelling M, Takacs M, Jurecka W, Gruber U, Offner F.** 2005. Nanocrystalline silver dressings as an efficient anti-MRSA barrier: a new solution to an increasing problem. J Hosp Infect **60:**226–230. http://dx.doi.org/10.1016/j.jhin.2005.04.001.
76. **Percival SL, Bowler PG, Dolman J.** 2007. Antimicrobial activity of silver-containing dressings on wound microorganisms using an in vitro biofilm model. Int Wound J **4:**186–191. http://dx.doi.org/10.1111/j.1742-481X.2007.00296.x.
77. **Harrison JJ, Turner RJ, Ceri H.** 2007. A subpopulation of Candida albicans and Candida tropicalis biofilm cells are highly tolerant to chelating agents. FEMS Microbiol Lett **272:**172–181. http://dx.doi.org/10.1111/j.1574-6968.2007.00745.x.
78. **Harrison JJ, Ceri H, Yerly J, Rabiei M, Hu Y, Martinuzzi R, Turner RJ.** 2007. Metal ions may suppress or enhance cellular differentiation in Candida albicans and Candida tropicalis biofilms. Appl Environ Microbiol **73:**4940–4949. http://dx.doi.org/10.1128/AEM.02711-06.
79. **Al-Fattani MA, Douglas LJ.** 2006. Biofilm matrix of Candida albicans and Candida tropicalis: chemical composition and role in drug resistance. J Med Microbiol **55:**999–1008. http://dx.doi.org/10.1099/jmm.0.46569-0.
80. **Kothavade RJ, Kura MM, Valand AG, Panthaki MH.** 2010. Candida tropicalis: its prevalence, pathogenicity and increasing resistance to fluconazole. J Med Microbiol **59:**873–880. http://dx.doi.org/10.1099/jmm.0.013227-0.
81. **Douglas LJ.** 2003. Candida biofilms and their role in infection. Trends Microbiol **11:**30–36. http://dx.doi.org/10.1016/S0966-842X(02)00002-1.
82. **Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL.** 2005. Candida biofilms: an update. Eukaryot Cell **4:**633–638. http://dx.doi.org/10.1128/EC.4.4.633-638.2005.