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Repurposing niclosamide as a versatile antimicrobial surface coating against device-associated, hospital-acquired bacterial infections

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Abstract

Device-associated and hospital-acquired infections remain amongst the greatest challenges in regenerative medicine. Furthermore, the rapid emergence of antibiotic resistance and lack of new classes of antibiotics has made the treatment of these bacterial infections increasingly difficult. The repurposing of Food and Drug Administration (FDA) approved drugs for antimicrobial therapies is a powerful means of reducing the time and cost associated with drug discovery and development. In this work, niclosamide, a commercially available anthelmintic drug with recently identified antimicrobial properties, was found to prevent and combat existing biofilms of several relevant Gram-positive bacteria, namely strains of *Staphylococcus aureus*, including methicillin resistant *S. aureus* (MRSA), and *Staphylococcus epidermidis*, all common causes of hospital-acquired and device-associated infections. This anti-biofilm activity was demonstrated at niclosamide concentrations as low as 0.01 µg/mL. We then assessed niclosamide activity as an antibacterial coating, which could potentially be applied to medical device surfaces. We developed solvent cast niclosamide coatings on a variety of surfaces common amongst medical devices including glass, titanium, stainless steel, and aluminum. Niclosamide-coated surfaces exhibited potent *in vitro* activity against *S. aureus*, MRSA, and *S. epidermidis*. At niclosamide surface concentrations as low as 1.6×10^{-2} µg/mm², the coatings prevented attachment of these bacteria. The coatings also cleared bacteria inoculated suspensions at niclosamide surface concentrations of 3.1×10^{-2} µg/mm². Hemolysis was not observed at any of the antimicrobial coating concentrations tested. We report a facile, effective means of coating devices with niclosamide to both clear and prevent biofilm formation of common bacteria encountered in hospital-acquired and device-associated infections.

Keywords

antimicrobial coatings; niclosamide; device-associated infections; hospital-acquired infections; biofilms; *Staphylococcus aureus*; Gram-positive bacteria

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1. Introduction

Hospital-acquired infections are a major source of concern for patients in both developed and developing nations.^[1] These infections affect more than 700,000 patients annually in the United States alone and result in significant morbidity or mortality.^[2–4] Hospital-acquired infections often manifest as device-associated infections that are very difficult to treat due to potential biofilm formation on the device surface. Common pathogenic bacteria causing these infections include Gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis*. *S. aureus* is found asymptomatically on the skin but has emerged as the most prevalent pathogen in nosocomial infections, particularly surgical site infections, and is the largest source of skin and soft tissue infection in the United States.^[5–7] *S. epidermidis* is also part of the natural skin flora and has a low pathogenic potential in immunocompetent individuals.^[8] *S. epidermidis* has emerged as the most common cause of infection of indwelling medical devices, such as intravenous catheters.^[9] These infections primarily affect long-term hospitalized and critically ill patients and are usually introduced during device insertion when bacteria is transferred from the skin onto the device surface.^[6,10,11] Growing levels of antibiotic resistance to conventional antibiotics including β -lactams such as penicillin and its derivatives, which are commonly used to treat Gram-positive bacterial infections, have left only limited treatment options for these bacterial infections.^[12–15] In the United States, there are more than 2 million antibiotic-resistant bacterial infections annually, and a significant number of them are due to methicillin resistant *S. aureus* (MRSA).^[14] Therefore, there is a growing need for the discovery of new, effective antimicrobial agents.

Current approaches to antimicrobial drug discovery involve time-consuming *in vitro* screening for appropriate candidates using structure-activity relationship analysis followed by experimental testing *in vitro* and *in vivo* to establish efficacy and toxicity.^[16] This costly and lengthy process, as well as the short time frame of therapeutic relevance due to emerging resistance, has resulted in declining interest in antibiotic research and discovery.^[17] Furthermore, Food and Drug Administration (FDA) approval of new therapies is a similarly expensive and time-consuming process, which has led to a decline in FDA approval of new antibiotics over the last several decades.^[18] For these reasons, a significant interest in repurposing existing FDA approved drugs as antibiotics has emerged.^[18] Antimicrobial coating technologies based on promising repurposed FDA therapeutics have not yet been reported.

Salicylanilide derivatives, which have recently been shown to exhibit antimicrobial properties,^[19–21] are among the drugs of interest for repurposing. In particular, niclosamide, an FDA approved anthelmintic salicylanilide derivative that is widely used to treat tapeworm infection in humans, has been shown to possess antimicrobial activity,^[21] as well as anticancer^[22,23] and anti-diabetic activity.^[24] Niclosamide functions as a hydrogen ionophore, whose activity results in the uncoupling of mitochondrial oxidative phosphorylation from electron transfer, inhibiting adenosine triphosphate (ATP) production, leading to tapeworm death.^[25] Niclosamide was found to be highly effective against several Gram-positive bacteria *in vitro* including clinical isolates of MRSA, and it was also found to exhibit *in vivo* efficacy using a *Caenorhabditis elegans* infection model. The drug was found

to be bacteriostatic and displayed minimum inhibitory concentrations (MICs) far below that of vancomycin, which is commonly used in MRSA treatments.^[21] The absence of any reported resistance to niclosamide, along with its potent efficacy against Gram-positive bacteria, makes it a promising alternative to conventional antibiotics. Although niclosamide has shown promising antimicrobial properties, its potential for use in localized infection treatments as part of a medical device coating has not yet been explored. Localized treatment methods have the potential to prevent device-associated infections and lower susceptibility to resistance, and are therefore of particular interest for drugs like niclosamide to which bacteria have not yet developed resistance.

Here, we report the development and characterization of highly versatile niclosamide-based antimicrobial device coatings. We used a solvent casting approach to develop niclosamide coatings on a variety of surfaces including medically relevant aluminum, stainless steel, and titanium. We demonstrated that these coatings act as transient local drug depots and not only prevent bacterial attachment, which is known to be the first step of biofilm formation,^[26] but are also capable of clearing bacterial suspensions of Gram-positive bacteria common in hospital-acquired infection. Additionally, these coatings demonstrated no hemolytic activity. These niclosamide coatings have the potential to effectively prevent and combat existing infections in a localized manner.

2. Materials and Methods

2.1 Materials

Bacto agar, cation-adjusted Mueller Hinton broth (CMHB), CMHB agar plates (with 5% sheep blood), tryptic soy broth (TSB), trypticase soy agar plates (w/5% sheep blood), blank susceptibility test disks, crystal violet, and 30 µg vancomycin susceptibility test disks were obtained from BD Biosciences (San Jose, CA). Ethanol (200 proof) was obtained from Pharmco-Aaper (Brookfield, CT). Round borosilicate glass cover slip substrates (12 mm diameter) and dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific (Waltham, MA). 18.2 Ω MilliQ water (EMD Millipore, Taunton, MA) was used in all experiments. Bovine red blood cells (10% v/v RBCs) were obtained from Innovative Research (Novi, MI). Aluminum (alloy 2024), titanium (Ti-6Al-4V, grade 5), and stainless steel (alloy 316L) substrates were obtained from OnlineMetals.com (Atlanta, GA). Niclosamide, gentamicin, vancomycin, bovine serum albumin (BSA), Dulbecco's phosphate buffered saline (PBS, 10×), D-glucose, acetone, and Triton-X-100 were purchased from Sigma-Aldrich (St. Louis, MO). *S. aureus* 25923 and *Streptococcus pyogenes* 19615 were obtained from ATCC (Manassas, VA). MRSA MW2,^[27] *S. epidermidis* 9142,^[28] *Escherichia coli* K-12,^[29] and *Pseudomonas aeruginosa* PA14^[30] were obtained from the Infectious Diseases research laboratories at Rhode Island Hospital.

2.2 Niclosamide Antibacterial Activity Characterization

Niclosamide antibacterial activity was first assessed using a modified Kirby-Bauer assay, which was adapted from established protocols.^[30] Bacteria at a concentration of 10⁸ colony forming units (CFU)/mL in the exponential growth phase was applied to CMHB (for *S. aureus* which was grown in 1× CMHB) or TSB (for MRSA, *S. epidermidis*, *S. pyogenes*, *E.*

coli, and *P. aeruginosa* which were grown in 1× TSB) agar plates. Blank susceptibility disks were loaded with niclosamide by exposing disks to a 1 mg/mL solution of niclosamide in DMSO at 20°C for two seconds. Positive controls were 30 µg vancomycin susceptibility disks (for *S. aureus*, MRSA, *S. epidermidis*, and *S. pyogenes*) or blank susceptibility disks similarly dipped in a 1 mg/mL solution of gentamicin in 1× PBS (for *E. coli* and *P. aeruginosa*) at 20°C for two seconds. Negative controls were blank disks dipped in DMSO (for *S. aureus*, MRSA, *S. epidermidis*, and *S. pyogenes*) or sterilized 1× PBS (for *E. coli* and *P. aeruginosa*). The disks were applied to the bacteria coated agar plates and incubated for 16–18 hours at 37°C. After incubation, the zone of inhibition surrounding the samples was examined.

The minimum inhibitory concentration (MIC) of niclosamide was determined using a microdilution assay adapted from established protocols.^[31] For the assay, solutions of niclosamide in 25% DMSO/75% 1× PBS, vancomycin in 1× PBS, and controls of 1× PBS and 25% DMSO/75% 1× PBS were serially diluted in the appropriate media (CMHB for *S. aureus* and TSB for MRSA and *S. epidermidis*) in a 96-well clear bottom plate. Bacteria at a concentration of 10⁵ CFU/mL in the exponential growth phase were added to each of the drug dilutions and positive controls, with no bacteria added to the negative controls. Plates were incubated for 16–18 hours at 37°C with constant shaking at 110 rpm. The absorbance (i.e. optical density) of each well was read at 600 nm using a BioTek Cytation 3 plate reader (Winooski, VT). Normalized bacteria density was calculated as follows using these absorbance values:

$$\text{Normalized bacteria density} = \frac{\text{sample absorbance} - \text{negative control absorbance}}{\text{positive control absorbance} - \text{negative control absorbance}}$$

(1)

The MIC of the drug was expressed as the drug concentration range in which the normalized bacteria density showed a statistically significant transition from 0 to nonzero (>0), as described by Andrews et al.^[32]

2.2.1 Prevention of biofilm formation and matured biofilm disruption—*S.*

aureus, MRSA, and *S. epidermidis* were grown in TSB overnight. An aliquot of bacteria was inoculated in 5 mL of TSB with 1% glucose for 16 hours. The bacteria were collected through centrifugation at 4,000 rpm for 10 minutes and suspended in 5 mL of fresh TSB medium. The cells were diluted to a final optical density of 1.0 at 600 nm and then further diluted 1:40.

The ability of niclosamide to either inhibit biofilm formation or disrupt mature biofilms was then tested. The inhibition of biofilms was assessed by adding 200 µL of the diluted bacteria suspension to 96-well clear bottom plates and subsequently adding two fold serially diluted niclosamide from 4 to 0.01 µg/mL (n=3) and incubating at 37°C for 24 hours. Cells without drug were included as a control biofilm.

For mature biofilm disruption, the diluted bacteria suspension was added into the wells of a 96-well clear bottom plate for 24 hours at 37°C. The well plates were then washed with PBS to remove any unattached planktonic cells leaving behind attached biofilms. Two fold serially diluted niclosamide from 4 to 0.01 µg/mL (n=3) was added and the plates were incubated at 37°C for 24 hours.

For both the biofilm inhibition and disruption assays, after the 24 hours incubation with the drug, the plates were washed 3 times with 200 µL PBS to remove planktonic cells and then dried for 1 hour. Dried plates were stained with 200 µL of 0.02% crystal violet for 15 minutes and again washed 3 times with 200 µL of PBS to remove residual stain. Stained biofilms were solubilized by the addition of 200 µL of an 80:20 v/v mixture of ethyl alcohol:acetone for 15 minutes and the plates were read at 595 nm using a Molecular Devices Spectramax M2 plate reader (Sunnyvale, CA).

2.3 Niclosamide Coating Preparation

Niclosamide was dissolved in ethanol at concentrations ranging from 10 µg/mL to 100 µg/mL. Bare substrates (glass, titanium, aluminum, and stainless steel) were initially rinsed in ethanol and allowed to dry at 20°C, to clean the surfaces. The niclosamide solutions were drop cast on substrates to yield theoretical surface concentrations ranging from 3.1×10^{-3} to 3.1×10^{-2} µg/mm²; here, “theoretical” refers to the assumption that the substrates were coated evenly with niclosamide. Substrates were incubated at 37°C for five minutes to promote rapid ethanol evaporation and stored dry at 4°C prior to use in subsequent experiments.

2.4 Characterization of Niclosamide Coating Morphology

Static water contact angle measurements for niclosamide-coated glass substrates were recorded using a Ramé-Hart contact angle goniometer. Briefly, 3 µL water droplets were deposited on the surface and the contact angle was measured using DROPimage CA software (Ramé-Hart). Four separate measurements were performed on each sample. Morphology of the coatings on glass substrates was further examined using an Asylum MFP-3D origin atomic force microscope (AFM). Samples were evaluated over a 10 µm × 10 µm area at 512 pixels/line and 1 Hz. Root mean square (RMS) roughness of the surface was obtained using Asylum Research 13 and Gwyddion software. A JEOL JSM 845 scanning electron microscope (SEM) was used to examine surface topography and obtain micrographs of coatings on aluminum, stainless steel, and titanium substrates. All images were taken at a 10 kV accelerating voltage using an in-lens secondary electron detector. For both AFM and SEM imaging, at least 3 samples were measured per treatment group.

2.5 Bacteria Inhibition by Niclosamide Coatings

2.5.1. Bacteria Attachment Inhibition—The ability of niclosamide-coated glass substrates to inhibit the attachment of *S. aureus*, MRSA, *S. epidermidis*, *E. coli*, *S. pyogenes*, and *P. aeruginosa* was examined using a previously reported protocol.^[33] The range of niclosamide surface concentrations tested spanned from 3.1×10^{-3} to 3.1×10^{-2} µg/mm². Samples were incubated at 37°C in bacteria suspensions at a concentration of 10⁵ CFU/mL for 30 minutes (coating side up to maximize exposure to the bacteria suspension).

Controls of uncoated substrates were also incubated in bacteria suspensions. Following incubation, substrates and controls were rinsed briefly by submerging each substrate in 15 mL of sterile water for 5 seconds with gentle agitation, and then placed on blood agar plates (CMHB w/5% sheep's blood for *S. aureus*, and TSB w/5% sheep's blood for MRSA, *S. epidermidis*, *E. coli*, *S. pyogenes*, and *P. aeruginosa*). These agar plate media were selected to be identical to the broth used for that strain's suspension culture. The agar plates were incubated at 37°C for 16–18 hours. The presence of colonies after incubation, indicating bacteria attachment, was monitored using digital imaging.

2.5.2. Growth Inhibition of Bacteria in Suspension—The ability of niclosamide-coated glass ($3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$) substrates to inhibit the growth of *S. aureus*, MRSA, and *S. epidermidis* in suspension was examined. The cover slips were placed in bacteria suspensions in the exponential growth phase at concentrations of 10^4 , 10^5 , or 10^6 CFU/mL. Positive controls of uncoated substrates in bacteria suspensions and negative controls of uncoated substrates in the relevant bacterial growth media were included. Samples and controls were incubated at 37°C for 16–18 hours with constant shaking at 110 rpm. Following incubation, the absorbance of the samples was measured at 600 nm. Normalized bacteria density was calculated using equation (1).

The ability of niclosamide coatings to inhibit the growth of *S. aureus* suspensions after repeated incubation with fresh bacteria inoculums was also assessed. Niclosamide-coated glass ($3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$ deposited on each side of the substrate) was incubated with *S. aureus* at 10^5 CFU/mL in the exponential growth phase for 30 minutes with constant shaking at 110 rpm. The substrates were then removed and briefly rinsed with sterile water and subsequently placed into fresh bacteria suspensions at 10^5 CFU/mL for 30 minutes. The process was repeated up to five times. Positive controls of uncoated substrates incubated in bacteria suspensions and negative controls of uncoated substrates incubated in CMHB were included. The absorbance of these bacteria suspensions and controls was measured at 600 nm following incubation at 37°C for 16–18 hours with constant shaking at 110 rpm and normalized bacteria density was calculated using equation (1). Similar experiments were conducted using vancomycin-coated cover glass. For these experiments, vancomycin was dissolved in water and drop cast on ethanol cleaned cover glass substrates to yield a surface concentration of $1.24 \times 10^{-1} \mu\text{g}/\text{mm}^2$ on each side of the substrate. The vancomycin-coated substrates were tested in the same way that niclosamide coatings were tested in serial *S. aureus* incubations and normalized bacteria density was obtained.

2.6 Measurement of Hemolytic Activity of Niclosamide Coatings

RBC hemolysis was examined to provide a preliminary assessment of niclosamide coating biocompatibility using previously reported protocols.^[33,34] Bovine RBCs were diluted in 1× PBS yielding a 5% v/v RBC suspension. The 5% RBC suspension was deposited on top of niclosamide-coated glass substrates (with a surface concentration range of $3.1 \times 10^{-3} \mu\text{g}/\text{mm}^2$ to $3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$) and incubated for one hour at 37°C with constant shaking at 110 rpm. Negative controls of the 5% RBC suspension and positive controls of the 5% RBC suspension with 1% v/v Triton-X-100 in 1× PBS deposited on uncoated glass substrates were included. Following incubation, the sample and control solutions were centrifuged at

1,000 rpm for 5 minutes. The supernatant was placed into a 96-well plate and the absorbance of each well was read at 540 nm using a BioTek Cytation 3 plate reader (Winooski, VT). The normalized hemolysis was calculated using equation (2):

$$\text{Normalized hemolysis} = \frac{\text{sample absorbance} - \text{negative control absorbance}}{\text{positive control absorbance} - \text{negative control absorbance}} \quad (2)$$

2.7 Statistical Analysis

All experiments were performed in triplicate at minimum. Measurements are reported as mean $\pm$ standard deviation. All statistical analyses were performed using one-way ANOVA with Tukey post hoc analysis. The p values are indicated where relevant.

3. Results and Discussion

3.1 Niclosamide Antimicrobial Activity

Prior to developing and evaluating niclosamide surface coatings, we evaluated free niclosamide activity against a variety of bacteria of interest in hospital-acquired infections. Niclosamide activity was recently examined against the prevalent ESKAPE pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* species).^[21] This work showed that niclosamide exhibits potent activity against only the Gram-positive members of the ESKAPE pathogens.^[21] Due to the prevalence of *S. epidermidis* in hospital-acquired infections,^[1] we sought to examine whether niclosamide is capable of inhibiting *S. epidermidis* growth. Figure 1 shows the results of niclosamide testing against *S. epidermidis* using a Kirby-Bauer disk diffusion method.^[31] We also included *S. aureus* and MRSA strains as bacteria controls that have previously demonstrated susceptibility to niclosamide. *S. pyogenes* was included as an additional Gram-positive strain that is a major cause of skin infection.^[5] Two common Gram-negative strains, *E. coli* and *P. aeruginosa*, were also tested. As expected, niclosamide inhibited the growth of *S. aureus* and MRSA, while demonstrating no inhibition against the Gram-negative bacteria strains. Niclosamide also demonstrated activity against *S. epidermidis* and *S. pyogenes*, indicating broad activity against a range of Gram-positive bacteria, not limited to only the Gram-positive members of the ESKAPE pathogens. Although the exact mechanism of action of niclosamide has yet to be determined, Rajmuthiah et al. have shown through Sytox staining (which penetrates only damaged cell membranes) that in contrast to the niclosamide analogue, oxyclozanide, niclosamide does not cause significant bacterial cell envelope damage.^[21] Gram-negative bacteria may have intrinsic resistance to niclosamide due to their functional and structural characteristics.^[32,36] Interestingly, despite the fact that niclosamide does not appear to inhibit *P. aeruginosa* growth, it has previously been observed to inhibit *P. aeruginosa* quorum sensing,^[37] a mechanism of antibiotic evasion in biofilms.

Given the promising results seen in the agar disk diffusion assays, broth microdilution assays were utilized to quantify the MIC of niclosamide against *S. epidermidis*. Controls of *S. aureus* and MRSA were also included. As shown in Table 1 and Figure S1, the MIC range

of niclosamide against *S. epidermidis* was found to be lower than the MIC range of the drug against the *S. aureus* and MRSA strains tested, at 0.063 to 0.125 µg/mL compared to 0.156 to 0.313 µg/mL for *S. aureus* and MRSA. The MIC of niclosamide was compared to that of vancomycin, a common broad-spectrum antibiotic in the treatment of complicated and multi-drug-resistant Gram-positive bacterial infections.^[38] For the tested strains, the MIC of niclosamide was far lower than that of vancomycin (10 times lower for *S. epidermidis* and 4 times lower for *S. aureus* and MRSA). This improved activity compared to vancomycin has also been observed for other salicylanilide anthelmintic drugs. In addition to niclosamide, Rajamuthiah et al. studied oxyclozanide and found that this anthelmintic salicylanilide derivative exhibited MICs against MRSA and *E. faecium* of 0.5 to 2 µg/mL compared to a vancomycin MIC of 4 µg/mL.^[21] In a study by Macielag et al., the MIC of the anthelmintic drug, closantel, against *S. aureus*, *E. faecalis*, and *E. faecium* was found to be in the range 0.12–1 µg/mL, well below the MIC for vancomycin for the three strains.^[39,40] Rajmuthiah et al. also showed that closantel has extremely low MICs against vancomycin resistant *S. aureus* strain VRS1, of approximately 0.78 µg/mL.^[40] The low MICs of the salicylanilide anthelmintic drugs like niclosamide reduce the potential for toxicity and reduce potential costs associated with treating infections. Additionally, the efficacy of this class of drugs against a broad range of Gram-positive bacteria indicates their potential importance as antimicrobial agents, especially given the emergence of vancomycin resistance and the paucity of new classes of antibiotics for the treatment of Gram-positive bacterial infections.^[41,42]

Although a compound can be effective at inhibiting bacteria in a planktonic state, this antibacterial activity does not imply efficacy against biofilms. Thus, we investigated the inhibitory activity of niclosamide in both the prevention and inhibition of mature biofilms for the Gram-positive strains of interest, *S. aureus* 25923, MRSA MW2, and *S. epidermidis* 9142. We found that niclosamide was able to prevent the formation of biofilms for each of these three strains, therefore demonstrating a broad anti-biofilm activity against a range of staphylococcal biofilms (Figure 2a). Biofilm prevention for all of the tested strains was significant compared to the untreated controls starting at a low concentration of 0.01 µg/mL niclosamide for *S. aureus* ($p < 0.0001$), MRSA ($p < 0.0001$), and *S. epidermidis* ($p < 0.0001$).

Mature biofilms are often more resistant to antimicrobial compounds through several sophisticated mechanisms (e.g., slow penetration of drug into the biofilm matrix, quorum sensing, genetic exchange, and distinct phenotypes compared to planktonic cells).^[43,44] Antibiotic concentrations thousands of times greater than the MIC against planktonic bacteria have been shown to be ineffective against biofilms.^[44] Interestingly, we found that niclosamide continued to be effective at inhibiting *Staphylococcus* even against mature biofilms (Figure 2b) at concentrations comparable to the MICs against these strains. *S. epidermidis* biofilms exhibited the greatest susceptibility with inhibition at 0.01 µg/mL ($p < 0.0001$), followed by MRSA MW2, which demonstrated a reduction in biofilm at 0.125 µg/mL ($p < 0.001$). *S. aureus* 25923 experienced biofilm reduction beginning at 0.5 µg/mL ($p < 0.05$).

3.2 Deposition and Morphological Characterization of Niclosamide Coatings

Motivated by niclosamide's broad activity against Gram-positive bacteria, particularly biofilms which are often responsible for hospital-acquired infections, we proceeded to develop niclosamide coatings for future use on medical device and hospital surfaces for localized treatment and prevention of bacterial infection. A facile solvent casting approach was implemented to deposit coatings of varying concentrations on these surfaces as shown in Figure 3; niclosamide solubilized in ethanol at various concentrations was drop cast over the intended substrate and allowed to dry to form the coating. AFM was used to examine the surface morphology of niclosamide coatings on glass in comparison to uncoated surfaces. Figure 4 shows the highly heterogeneous niclosamide coatings on glass surfaces. The surfaces contain relatively smooth regions interspersed with large niclosamide aggregates. Based on these micrographs, it is clear that a uniform surface coating is not present; however, we will refer to an average niclosamide surface concentration (μg niclosamide/ mm^2) in our discussion, not to imply uniform coverage but to accurately represent the overall mass of niclosamide deposited on a given substrate area. At a larger niclosamide surface concentration of $3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$, more aggregates appear for a given substrate area, as compared to the lower concentration coating ($3.1 \times 10^{-3} \mu\text{g}/\text{mm}^2$). The largest aggregates in the more concentrated coating had an average height of $324.6 \pm 15.5 \text{ nm}$ versus $181.1 \pm 21.8 \text{ nm}$ for the less concentrated coatings. These large aggregates likely form due to the high niclosamide hydrophobicity (octanol:water coefficient, logP, of 4.48 at pH 7.0)^[45], leading to phase separation as the coating dries and a patchy rather than uniform surface coating on the hydrophilic glass. Water contact angle measurements of the surfaces further supported the non-uniform nature of the coating, with values comparable to uncoated glass controls (approximately 56°) as shown in Table S1.

The practical applicability of the coatings to medically relevant substrates was also investigated. Coatings were deposited via solvent casting on aluminum, stainless steel, and titanium. Aluminum is often used in hospital equipment including bed frames, while stainless steel is commonly used in surgical tools, and titanium is a major component of indwelling medical implants.^[46] Figure 5 shows SEM images of niclosamide-coated ($3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$) and uncoated metals. As with coatings on glass, the niclosamide coatings on these medically relevant metals appear heterogeneous, with clear differences between the non-coated and coated samples. In general, the underlying features of the substrates are visible even with the coating, indicating a patchy surface coverage.

3.3 Inhibition of Bacterial Attachment by Niclosamide Coatings

Biofilm formation is the root cause of many chronic bacterial infections.^[26] The Gram-positive bacterial strains against which niclosamide has demonstrated activity are all capable of biofilm formation.^[5] The first step of biofilm formation is the attachment of bacteria on a surface.^[46] Inhibiting bacterial attachment can consequently prevent the formation of a biofilm. We examined the ability of our niclosamide coatings to inhibit bacterial attachment using strains of *S. epidermidis*, *S. aureus*, and MRSA. Figure 6 shows representative results for niclosamide-coated glass at niclosamide concentrations ranging from $3.1 \times 10^{-3} \mu\text{g}/\text{mm}^2$ to $3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$ that were incubated with the respective Gram-positive bacteria and subsequently plated (coating side down) on blood agar to examine bacteria attachment. For

the *S. aureus*, MRSA, and *S. epidermidis* strains examined, niclosamide coatings completely inhibited bacterial attachment at concentrations above $1.6 \times 10^{-2} \mu\text{g}/\text{mm}^2$, indicated by the lack of any bacteria colonies visible under or surrounding the cover glass coatings at these concentrations. There was minimal attachment at a niclosamide concentration of $7.7 \times 10^{-3} \mu\text{g}/\text{mm}^2$ for these strains, especially for *S. epidermidis*, as seen by comparing the relative number of colonies visible surrounding and under the cover glass coating at this concentration to the dense bacteria colonies under and surrounding the uncoated controls. Niclosamide's lower MIC against *S. epidermidis* may explain its greater efficacy in inhibiting *S. epidermidis* attachment at a concentration of $7.7 \times 10^{-3} \mu\text{g}/\text{mm}^2$ as compared to *S. aureus* and MRSA.

Bacteria attachment is a complex process that is governed by multiple interactions between the bacteria cell and the substrate surface. These interactions can be greatly influenced by the properties of the surface such as hydrophobicity and surface topography.^[45] To examine whether prevention of bacterial attachment by niclosamide coatings was a result of surface characteristics of the coating, such as roughness, in addition to or rather than niclosamide antimicrobial activity, we examined whether these coatings at a surface concentration of $3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$ were capable of inhibiting Gram-negative bacterial attachment. As shown in Figure 7, niclosamide did not inhibit *E. coli* or *P. aeruginosa* attachment, indicating that specific antimicrobial activity of the drug against Gram-positive bacteria is required to prevent attachment of bacteria to the surface.

At all surface concentrations examined, if all of the adsorbed niclosamide were to dissolve and enter the surrounding bacterial suspension during the incubation of the coated substrates with bacteria, the media would contain a concentration of niclosamide above the MIC range against *S. epidermidis* (the same is true for all but the lowest coating concentration for the other Gram-positive species tested). This finding suggests that the mechanism of attachment inhibition is a localized growth inhibition of the bacteria exposed to the niclosamide. However, attachment of the bacteria was not inhibited at all surface concentrations, indicating that not all of the niclosamide was released from the surface, leading to lower than MIC niclosamide concentrations in solution for the lower niclosamide coating concentrations.

3.4 Evaluation of Bacterial Clearance by Niclosamide Coatings

We hypothesized that the niclosamide coatings may serve as local drug delivery depots, which in the appropriate time frame, are capable of clearing existing bacterial infections. Thus, we evaluated the potential of the niclosamide coatings to clear bacteria in suspension at varying bacteria concentrations, as shown in Figure 8. Bacteria concentrations of 10^4 , 10^5 , and 10^6 CFU/mL were examined as 10^5 CFU/gram tissue is typically considered to be enough bacteria to inhibit normal wound healing in infected wounds.^[34] Again, Gram-positive *S. aureus*, MRSA, and *S. epidermidis* were examined. A set niclosamide surface concentration of $3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$ was used for the coatings based on the results of the attachment inhibition study, which showed that this concentration worked very well to inhibit bacterial attachment for all Gram-positive strains tested, and likely yielded above MIC values of niclosamide in the surrounding bacteria solution. For *S. aureus*, the

niclosamide coatings completely cleared bacterial suspensions when incubated with bacteria at concentrations of 10^4 and 10^5 CFU/mL. Although we found niclosamide to exhibit a lower MIC against *S. epidermidis* as compared to the *S. aureus* and MRSA strains tested, niclosamide coatings were unable to clear *S. epidermidis* concentrations above 10^4 CFU/mL. For MRSA, the coatings were unable to clear bacterial suspensions at any of the tested bacteria concentrations.

Although the exact mechanism of antimicrobial action of niclosamide has not yet been examined, from our microdilution assays (Table 1 and Figure S1), we have seen that free niclosamide in solution is able to inhibit growth of *S. aureus*, MRSA, and *S. epidermidis* at concentrations that were lower than the niclosamide concentration that would be obtained if all of the niclosamide was released from the surface, namely 0.7 µg/mL. As with the attachment inhibition studies, the results of these coating clearance studies indicate that the hydrophobic niclosamide is not completely released into solution from the surface of the coated substrates, leading to a lack of MRSA and *S. epidermidis* inhibition at a bacteria concentration of 10^5 CFU/mL (i.e., the concentration of bacteria tested in microdilution studies).

We also examined the ability of a single niclosamide coating to clear fresh *S. aureus* (10^5 CFU/mL) suspensions over repeated incubations, which is considered a measure of coating stability. Niclosamide-coated substrates (3.1×10^{-2} µg/mm²) were incubated with a bacteria suspension for 30 minutes followed by a brief rinse and introduction to a fresh bacteria solution; this process was repeated multiple times. As shown in Figure 9a, the coatings were considerably stable for three successive incubations with bacteria, acting rapidly to inhibit *S. aureus* growth during these relatively short incubations, and exhibiting a large increase in normalized bacteria density from 0.15 ± 0.03 to 0.85 ± 0.07 between the third and fourth incubation (suggesting that this is where coating stability is lost). Similarly, we examined the efficacy of vancomycin coatings developed using a similar solvent casting approach upon repeated exposure to fresh *S. aureus* suspensions. A vancomycin surface concentration four times that of the niclosamide surface concentration was utilized for these experiments (1.2×10^{-1} µg/mm²) to account for the vancomycin MIC against *S. aureus* being four times the MIC of niclosamide against *S. aureus* (as shown in Table 1). As shown in Figure 9b, the vancomycin coatings were able to clear the bacteria during the first incubation. However, a sharp increase in normalized bacteria density from approximately 0 to 0.64 ± 0.17 was observed between the first and second incubations, indicating incomplete bacterial clearance following the first incubation. These results suggest that the coatings formulated from the hydrophilic vancomycin likely fully dissolve during the first bacterial exposure, proving less stable than the niclosamide coatings. To further improve their stability, the niclosamide coatings could be integrated with a more durable polymer layer, such as a perfluorinated antifouling polymer like Nafion.^[47]

3.5 Assessment of Hemolysis in the Presence of Niclosamide Coatings

The niclosamide coatings developed in this work may be useful in a variety of antimicrobial applications, particularly the prevention and treatment of hospital-acquired infections, due to their demonstrated activity against common Gram-positive strains implicated in causing

hospital-acquired infections. A preliminary evaluation of biocompatibility of the niclosamide coatings was performed in order to confirm their usefulness in potential *in vivo* applications, by examining the hemolytic activity of the coatings using previously reported methods.^[34,35] As shown in Figure S2, even at the highest tested niclosamide coating surface concentration of $3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$, no hemolysis was observed. These results agree with previously reported findings on the lack of hemolytic activity by free niclosamide.^[21] Due to the lack of hemolysis observed, the niclosamide surface coatings developed in this work are highly promising for further development for future *in vivo* applications.

4. Conclusions

In this study, the recently repurposed FDA-approved small molecule anthelmintic therapeutic, niclosamide, was incorporated into device coatings to prevent and treat bacterial infections. Niclosamide activity against a variety of Gram-positive bacterial strains often encountered in hospital-acquired and device-associated infections was assessed. This included activity against *S. epidermidis*, which has not previously been examined for its susceptibility to niclosamide. We observed that niclosamide is able to inhibit the growth of *S. epidermidis* at extremely low concentrations, below the MIC of vancomycin, while confirming that niclosamide MICs against *S. aureus* and MRSA fall below that of vancomycin.^[21] Further, we found that niclosamide can also prevent formation of and reduce mature staphylococcal biofilms at concentrations near the MIC against these bacteria. These findings are significant, supporting the use of niclosamide to form coatings on devices that often serve as platforms for bacterial cell attachment and initiation sites for biofilm formation. We successfully coated niclosamide on a variety of medically relevant substrates, finding that the highly heterogeneous surface coatings were able to prevent Gram-positive bacterial attachment at low surface concentrations of $1.6 \times 10^{-2} \mu\text{g}/\text{mm}^2$, and clear existing bacterial growth. The chemical properties of niclosamide enable its direct adsorption to a surface as a heterogeneous surface coating, which can serve to prevent bacterial attachment while simultaneously acting as a local drug delivery depot for drug release to inhibit bacterial growth. Unlike coatings formulated from more hydrophilic therapeutics, niclosamide coatings also display improved stability throughout repeated bacterial exposures.

This niclosamide coating has the potential to be utilized on implant and bandage surfaces where a short-term local supply of antimicrobial is desired or reapplication of the material is possible. The potent activity and potential for local application are exciting in the fight against drug resistant bacteria. This work is the first demonstration of the potential of niclosamide, a newly identified antimicrobial, to be used as an antimicrobial device coating that can both prevent biofilm formation and clear existing infections. By combatting device-associated infection, one of the greatest challenges in regenerative medicine, these coatings have the potential to greatly increase the success of therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Antimicrobial activity of niclosamide against Gram-positive bacteria. Niclosamide (N); negative control containing no drug (-); positive control (+) is vancomycin (for Gram-positive bacteria strains, *S. aureus* 25923, MRSA MW2, *S. epidermidis* 9142, and *S. pyogenes* 19615) or gentamicin (for Gram-negative bacteria strains, *E. coli* K-12 and *P. aeruginosa* PA14). Representative results for each bacterial strain are shown (n = 3).

Figure 2.

Prevention and disruption of *Staphylococcus* biofilms by niclosamide. Serial concentrations of niclosamide were incubated with *Staphylococcus* strains to test (2a) biofilm prevention and (2b) reduction of mature biofilms. Absorbance of crystal violet staining of biofilms was measured at 595 nm. Data are shown as mean $\pm$ standard deviation; significance was calculated using one-way ANOVA with Tukey post hoc analysis (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

Figure 3. Schematic of niclosamide solvent casting approach used to deposit coatings of various average niclosamide surface concentrations.

Figure 4.

Niclosamide coating morphology measured by atomic force microscopy (AFM). AFM images of uncoated, $3.1 \times 10^{-3} \mu\text{g}/\text{mm}^2$, and $3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$ niclosamide-coated glass substrates. Representative images are shown and the RMS roughness of each sample is shown as mean $\pm$ standard deviation ($n = 3$).

Figure 5. Niclosamide coatings on medically relevant metal substrates. Scanning electron microscopy images of uncoated and $3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$ niclosamide-coated stainless steel, aluminum, and titanium substrates. Representative images of each sample are shown ($n = 3$).

Figure 6.

Gram-positive bacterial attachment inhibition by niclosamide-coated glass substrates.

Inhibition of attachment depends on niclosamide coating concentration (controls are uncoated substrates). Representative images are shown ($n = 3$). Note, cover glass is 12 mm in diameter.

Figure 7. Gram-negative bacterial attachment on niclosamide-coated glass substrates. Controls are uncoated substrates. Representative images are shown (n = 3). Note, cover glass is 12 mm in diameter.

Figure 8.

Bacteria growth in the presence of niclosamide coatings. Normalized bacteria density following *S. aureus* 25923, MRSA MW2, and *S. epidermidis* 9142 exposure to niclosamide coatings on glass substrates at a concentration of $3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$. Data are shown as mean $\pm$ standard deviation; significance was calculated using one-way ANOVA with Tukey post hoc analysis (***) ($p < 0.0001$).

Figure 9.

Normalized bacteria density following repeated incubations of (9a) niclosamide coatings and (9b) vancomycin coatings with fresh *S. aureus* 25923. A niclosamide concentration of $3.1 \times 10^{-2} \mu\text{g}/\text{mm}^2$ and a vancomycin concentration of $1.2 \times 10^{-1} \mu\text{g}/\text{mm}^2$ was utilized with 10^5 CFU/mL per each incubation. Data are shown as mean $\pm$ standard deviation; significance was calculated using one-way ANOVA with Tukey post hoc analysis (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

Table 1

Minimum Inhibitory Concentration of Comparable Antibiotics

Strain	Niclosamide MIC (µg/ml)	Vancomycin MIC (µg/ml)
<i>S. epidermidis</i> 9142	0.063 – 0.125	0.625 – 1.250
MRSA MW2	0.156 – 0.313	0.625 – 1.250
<i>S. aureus</i> 25923	0.156 – 0.313	0.625 – 1.250