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Next Science Wound Gel Technology, a Novel Agent That Inhibits Biofilm Development by Gram-Positive and Gram-Negative Wound Pathogens

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Loss of the skin barrier facilitates the colonization of underlying tissues with various bacteria, where they form biofilms that protect them from antibiotics and host responses. Such wounds then become chronically infected. Topical antimicrobials are a major component of chronic wound therapy, yet currently available topical antimicrobials vary in their effectiveness on biofilm-forming pathogens. In this study, we evaluated the efficacy of Next Science wound gel technology (NxtSc), a novel topical agent designed to kill planktonic bacteria, penetrate biofilms, and kill the bacteria within. *In vitro* quantitative analysis, using strains isolated from wounds, showed that NxtSc inhibited biofilm development by *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae* by inhibiting bacterial growth. The gel formulation NxtSc-G5, when applied to biofilms preformed by these pathogens, reduced the numbers of bacteria present by 7 to 8 $\log_{10}$ CFU/disc or CFU/g. *In vivo*, NxtSc-G5 prevented biofilm formation for 72 h when applied at the time of wounding and infection and eliminated biofilm infection when applied 24 h after wounding and infection. Storage of NxtSc-G5 at room temperature for 9 months did not diminish its efficacy. These results establish that NxtSc is efficacious *in vitro* and *in vivo* in preventing infection and biofilm development by different wound pathogens when applied immediately and in eliminating biofilm infection already established by these pathogens. This novel antimicrobial agent, which is nontoxic and has a usefully long shelf life, shows promise as an effective agent for the prevention and treatment of biofilm-related infections.

Infections in acute wounds due to trauma or surgery can lead to life-threatening sepsis, septic shock or multiple-organ dysfunction syndrome, and death (1, 2). In patients with burns, early excision therapy has reduced the rates of sepsis and mortality in the acute period following injury; infections, rather than burn shock or hypovolemia, are now responsible for 75% of deaths in this period (1, 2). Among patients with thermal injuries involving >15% of total body surface area, the rates of deaths resulting from infections reach 25% (1). Chronic wounds, such as pressure ulcers, venous leg ulcers, diabetic foot ulcers, and neuropathic ulcers, occur in geriatric patients, persons with perivascular disease, and persons with diabetes mellitus (3–5). Chronic wounds, which are stalled at the inflammatory phase of healing, begin as superficial ulcers and may become deep, sometimes extending to bone (3, 4, 6). Deep wounds are more difficult to treat than superficial wounds and may never heal completely (3, 4). Chronic deep infections of nontraumatic lower-limb ulcers lead to amputation in 60% of patients, with the majority being related to diabetes (3, 5). Thus, chronic wounds represent a serious problem, causing long-term pain and discomfort to patients and requiring costly treatment.

All wounds, especially chronic wounds, become colonized with bacteria arising from the patient's skin, respiratory tract, or gastrointestinal tract or with exogenous bacteria transferred from the environment or conveyed on the hands of health care workers (1, 2, 7). When the bacterial load reaches 10^5 CFU/g of tissue, the wound is considered infected; when levels are $\geq 10^6$ CFU/g or when ≥4 species are present, failure to heal may occur (6, 8). The formation of microbial biofilms within wounds has now been associated with this failure to heal (1, 6, 9). Compared with their planktonic counterparts, microorganisms within a biofilm are highly resistant to environmental stresses and host immune responses and are tolerant to antimicrobials (10–13). Most of these infections begin with colonization with Gram-positive cocci, followed by different genera of Gram-negative bacilli from the host or the environment; over time, the infections become polymicrobial, consisting of numerous species, including obligate anaerobes (1, 3, 4, 8). Once formed, the biofilm mechanically prevents wound contracture and epithelialization, dysregulates the host immune response by affecting intracellular signaling, and damages the tissue through a combination of chronic inflammation and microbial virulence factors (4, 6, 14–16).

While biofilm-infected wounds do not show signs of acute infection, the bioburden can be exponentially higher than 10^5 CFU/g (6). Previous studies have demonstrated the presence of bacterial biofilms within both acute and chronic wounds. Utilizing a murine model of thermal injury, Schaber et al. (17) demonstrated the presence of *Pseudomonas aeruginosa* biofilms within injured and infected tissues. Analysis of multiple biopsy specimens from numerous patients with burn wounds revealed the presence of bacterial biofilms within the ulcerated areas of the wounds (18). Additionally, James et al. (19) examined samples

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Pseudomonas aeruginosa		
PAO1	Prototroph; human isolate from wound	57
Xen5	*P. aeruginosa* ATCC 19660 carrying *P. luminescens luxACBDE* at single integration site on chromosome; mucoid isolate from human septicemia; tetracycline resistant	PerkinElmer
PA14	UCBPP-PA14; human isolate from burn wound	58
PA103	Human isolate	59
PACI-1	Burn wound isolate	36
PACI-57	Burn wound isolate	This study
PACI-75	Burn wound isolate	This study
Staphylococcus spp.		
AH133	*S. aureus* RN4220 carrying plasmid pCM11, in which gene for GFP[a] is constitutively expressed	60
Xen29	*S. aureus* 12600 (NCTC8532) carrying *P. luminescens luxACBDE* at single integration site on chromosome; pleural fluid isolate; kanamycin resistant	PerkinElmer
SACI-1	*S. aureus* from burn wound	37
SECI-1	*S. epidermidis* from burn wound	37
MRSA-201	Methicillin-resistant *S. aureus* isolate from blood	This study
Other genera		
ABCI-1	*Acinetobacter baumannii* from burn wound	37
KPCI-1	*Klebsiella pneumoniae* from burn wound	37
Plasmids		
pMRP9-1	pUCP18 carrying gene for enhanced GFP; carbenicillin resistant	61
pCM11	Plasmid stable in *S. aureus* that constitutively expresses GFP; erythromycin resistant	A. Horswill, personal communication

[a] GFP, green fluorescent protein.

from several chronic wounds, including venous leg ulcers, foot ulcers, and pressure ulcers, and noted the presence of polymicrobial microcolonies in more than 60% of the examined samples. Using peptide nucleic acid fluorescence *in situ* hybridization, Malic et al. (20) characterized the spatial distribution of biofilm-forming bacteria within tissue biopsy specimens obtained from human chronic skin wounds. Biofilms can become established within wounds by 5 h and mature by 10 h (6).

In nature, as well as at different infection sites, microorganisms can exist within a protective structure termed a biofilm, which consists of mushroom-like multicellular structures that are surrounded by an extracellular polysaccharide (EPS) matrix formed from bacterial and host products (6, 10). Biofilm-infected wounds are difficult to treat. Encasement of the bacterial pathogens within the EPS, combined with the presence of necrosis within the surrounding wound bed and decreased blood flow to the compromised areas, prevents host defense responses from reaching the infected tissues and eliminating the infection and protects the pathogens within from the effects of systemically administered antibiotics (21–23). Strategies to treat wound biofilms are multipronged, including debridement to remove the biofilm and necrotic tissue, wound dressing to control moisture in the wound bed and to protect granulating tissue from damage, and treatment of the wound bed with topical antimicrobials to prevent regrowth of the microorganisms (1, 6, 24–26). Systemic antimicrobials are added when the infection is deep and/or other infections are present (1–3, 6). Numerous *in vitro* and clinical studies have evaluated the ability of topical antimicrobials to eliminate biofilms formed by wound pathogens, with variable efficacies being reported (27–31). Thus, there is a need for additional agents that not only can kill planktonic bacteria but also can disrupt biofilms and kill the bacteria within them.

In this study, we describe Next Science wound gel technology (NxtSc), a novel topical antimicrobial agent designed to prevent bacterial growth and the formation of biofilms when applied to fresh wounds and to disrupt and to eliminate existing biofilms in infected wounds. NxtSc in aqueous solution (NxtSc-AS) and various formulations of NxtSc gel (NxtSc-G) were efficacious *in vitro*, and NxtSc-G5 was successful *in vivo* in preventing biofilm development in fresh wounds and in eliminating established biofilm infections caused by several different Gram-positive and Gram-negative pathogens.

MATERIALS AND METHODS

Bacterial strains and media. The strains and plasmids utilized in this study are listed in Table 1. Clinical isolates were obtained from burn patients in the Timothy J. Harnar Burn Center or the pediatric intensive care unit at the University Medical Center (Lubbock, TX), under a protocol approved by the institutional review board at the Texas Tech University Health Sciences Center. Bacterial strains were grown either in Luria-Bertani (LB) broth or on LB agar plates. Plasmids expressing green fluorescent protein (GFP) were introduced into *Staphylococcus aureus* AH133 (pCM11) and PAO1 (pMRP9-1) to allow visualization of biofilms *in vitro*. For live-animal imaging, *S. aureus* strain Xen29 and *P. aeruginosa* strain Xen5, which carry single stable chromosomal insertions of the *Photorhabdus luminescens luxACBDE* operon, were purchased from PerkinElmer (Boston, MA) (32, 33). Erythromycin at 10 μg/ml and carbenicillin at 300 μg/ml were used to maintain the plasmids in AH133 and PAO1, respectively; kanamycin at 30 μg/ml and tetracycline at 60 μg/ml were used with Xen29 and Xen5, respectively, to prevent contamination.

Next Science wound gel technology. NxtSc is a proprietary agent (M. Myntti, U.S. patent application PCT/US2012/059263) (Next Science,

Jacksonville, FL) that was designed with dual functions, i.e., to kill bacterial pathogens by removing proteins from bacterial membranes, leading to cell lysis, and to eliminate biofilms by destabilizing the exopolysaccharide matrix through the chelation of calcium. We received NxtSc in aqueous solution (NxtSc-AS) and or in gel formulations (NxtSc-G1 through NxtSc-G5) formulated in a water-soluble polyethylene glycol (PEG) base. Gel formulations contained 1.44 mg of a surfactant in 25% standardized buffer solution (3,522 mosmol). The formulations varied in the amounts of a second proprietary ingredient and in pH, with NxtSc-G1 and NxtSc-G3 at basic pH and the others at acidic pH. All products used in this study were prepared by Next Science (Jacksonville, FL) (34, 35).

Efficacy of NxtSc in killing planktonic bacteria. (i) Zone of inhibition assay. Strains were cultured overnight in LB broth. The cultures were diluted to a McFarland standard of 0.5 and spread evenly over the surface of 100-mm LB agar plates, to produce a confluent lawn of growth. Blank 6-mm discs (Becton, Dickinson, Franklin Lakes, NJ) were impregnated with 10 or 20 μl of phosphate-buffered saline (PBS) or NxtSc-AS (3.6 or 7.2 μg/ml NxtSc, respectively), allowed to dry, and then applied to the centers of the plates. Plates were incubated for 16 h at 37°C, and the zones of inhibition were measured.

(ii) Microtiter plate assay. Strains were cultured overnight in LB broth. A 1-ml aliquot of overnight culture was pelleted, washed, resuspended in LB broth, and inoculated in fresh LB broth to obtain a starting optical density at 600 nm (OD_{600}) of 0.05, with an inoculum of 10^2 to 10^3 CFU/ml. One milliliter of the inoculum was placed in each well of a sterile 96-well polystyrene plate (Corning Life Sciences, Lowell, MA). Either PBS (negative control) or NxtSc-AS, at concentrations of 3.6 μg/ml and 7.2 μg/ml, was added to the inoculated wells. The plates were incubated at 37°C for 16 h. Following incubation, the cell suspension in each well was serially diluted 10-fold in LB broth, and a 10-μl aliquot of each dilution was spotted on LB agar plates. The plates were incubated at 37°C for 24 h, and the number of colonies per spot was counted. The number of CFU/ml was determined using the formula CFU/ml = (CFU counted × dilution factor) × 100.

***In vitro* wound biofilm model.** The model was utilized as described previously, with slight modifications (36, 37). Briefly, five cellulose discs (Becton, Dickinson) were placed on the surface of freshly prepared LB agar plates and inoculated with approximately 1×10^2 CFU of each tested strain. Bacterial inocula were prepared as described above for the microtiter plate assay. The discs were then treated with 10 μl of solution containing 3.6 or 7. 2 μg of NxtSc-AS or with 10 μl of PBS, and the plates were incubated at 37°C for 24 h. Following incubation, the discs were rinsed lightly to remove any unattached bacteria, and the discs were vortex-mixed in 1 ml PBS to disperse the biofilm. Bacterial suspensions were serially diluted 10-fold in PBS, 10-μl aliquots of each dilution were plated on LB agar, and the number of CFU/disc was determined as described above, using the formula CFU/disc = (CFU counted × dilution factor) × 100.

To assess the efficacy of the NxtSc gel formulations in inhibiting biofilm development, the discs were inoculated with the tested strain and covered with 22-mm by 22-mm sections of gauze (cut from sterile, 2-inch by 2-inch, 100% cotton, 8-ply, gauze sponges [Kendall Curity; Covidien, Mansfield, MA] with sterile scissors and forceps) spread with 400 mg PEG or 400 mg NxtSc-G1, NxtSc-G2, NxtSc-G3, NxtSc-G4, or NxtSc-G5 containing 1.44 mg surfactant. After 24 h of incubation at 37°C, the gauze was removed and the number of CFU/disc was determined as described for the microtiter plate assay. To examine the ability of NxtSc-G1, NxtSc-G3, and NxtSc-G5 to disrupt established biofilms, the discs were inoculated with the tested strain as described above, and the LB agar plates were incubated at 37°C for 8 h to allow the development of partial biofilms. The discs were then covered with gauze spread with 400 mg PEG or 400 mg NxtSc-G1, NxtSc-G3, or NxtSc-G5 and were incubated at 37°C for 24 h. The gauze was removed and the CFU/disc was determined.

***In vitro* image analysis.** We used confocal laser scanning microscopy (CLSM) to visualize biofilms formed by AH133 or PAO1/pMRP9-1. This was done using an Olympus 1X7 Fluoview 300 confocal laser scanning microscope (Olympus America, Melville, NY). All images were obtained through a 203/0.40 Ph1 numerical-aperture objective, using a green helium laser (546 nm). Construction of the three-dimensional (3D) biofilm images was done using NIS Elements 2.2 software (Nikon Instruments, Melville, NY). All instrument settings were consistent for each individual experimental parameter tested.

Murine model of wound infection. This was done as described previously, with minor modifications (38). Briefly, adult female Swiss Webster mice were anesthetized and their backs were shaved. A circular, full-thickness, excision wound 15 mm in diameter was made in the shaved area. To test the inhibition of biofilm formation, the wound was covered with a 22-mm by 22-mm piece of sterile gauze spread with 400 mg of PEG or 400 mg of NxtSc-G5. The gauze was secured using transparent, semipermeable, polyurethane, OpSite dressing (Smith & Nephew, Andover, MA). Infection of the wound was initiated by injecting approximately 1×10^2 CFU of the tested strain into the wound bed. To evaluate the efficacy of NxtSc-G5 in disrupting preformed biofilms, the wound was covered with OpSite dressing and 10^2 CFU of the tested strain was injected into the wound under the dressing. At 24 h postinoculation, the dressing was removed, the infected wound was covered with a 22-mm by 22-mm piece of sterile gauze spread with 400 mg of PEG or 400 mg of NxtSc-G5, and fresh OpSite dressing was applied. The animals were then observed daily for 3 days after treatment. At 72 h after treatment, the mice were euthanized. The 15-mm wound bed was excised, weighed, resuspended in 1 ml of PBS, and homogenized. The homogenized materials were serially diluted 10-fold, and 10-μl aliquots of each dilution were spotted onto the LB agar plates. The numbers of CFU/g of tissue were determined using the formula CFU/g = (CFU counted × dilution factor × 100)/g of tissue. Animals were humanely treated in accordance with a protocol approved by the institutional animal care and use committee at the Texas Tech University Health Sciences Center (Lubbock, TX).

***In vivo* imaging.** Mice infected with either *S. aureus* Xen29 or *P. aeruginosa* Xen5 (Table 1) were observed at 24, 48, and 72 h after treatment, using live imaging. The mice were lightly anesthetized, and the infected wounds were observed using an IVIS Lumina XR system with Living Image software (PerkinElmer). Upon recovery from anesthesia, the mice were returned to their cages. Imaging experiments were conducted at the Texas Tech University Health Sciences Center Image Analysis Core Facility (Lubbock, TX).

***In vivo* safety analyses.** The ISO intracutaneous reactivity test was conducted in albino rabbits in accordance with ISO procedures (39, 40). The dermal limit study, which provides information on acute dermal toxicity and systemic toxicity, was performed in rats using abraded dermal application of 2,000 mg/kg NxtSc, according to ISO procedures (39, 41, 42). The ISO guinea pig maximization sensitization test was used to determine the allergenic potential or sensitizing capacity of NxtSc (39, 40). These experiments were conducted at a commercial testing laboratory (WuXi AppTec, St. Paul, MN). Animals were humanely treated in accordance with protocols approved by the institutional animal care and use committee for the company.

Statistical analyses. Analyses were done using GraphPad InStat 3.06 (GraphPad Software, San Diego, CA). Analysis of variance (ANOVA) with Dunnett's multiple-comparison test was used to determine significant differences between the control and several treatments applied to the same strain. Unpaired two-tailed *t* tests were used to compare an individual treatment with its control. Data were transformed using the formula $y = \log(y)$ before statistical analyses were performed.

RESULTS

NxtSc-AS inhibits biofilm development by Gram-positive and Gram-negative wound pathogens. Initially, we conducted titration experiments to determine the concentrations of NxtSc-AS that inhibit and/or significantly reduce the planktonic growth of two major wound pathogens, i.e., *S. aureus* and *P. aeruginosa*

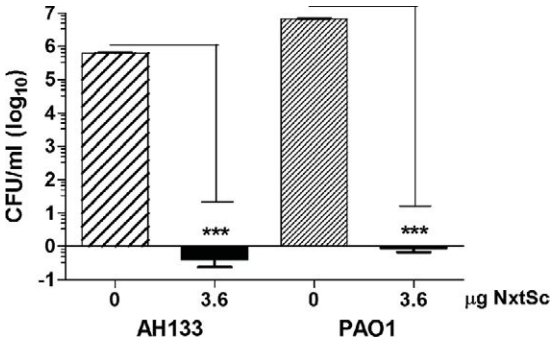


FIG 1 NxtSc-AS inhibits *S. aureus* and *P. aeruginosa* growth. One-milliliter aliquots of overnight cultures of *S. aureus* AH133 and *P. aeruginosa* PAO1 were pelleted, washed, resuspended in LB broth, and inoculated into fresh LB broth to obtain a starting inoculum of 10^2 to 10^3 CFU/ml. One milliliter of the inoculum was placed into each well of a sterile 96-well polystyrene plate, and PBS (0) or NxtSc-AS (3.6 μg/ml) was added to each well. The plates were incubated at 37°C for 16 h. Cell suspensions from each well were serially diluted 10-fold in LB broth, and 10 μl of each dilution was spotted on LB agar to determine the number of CFU/ml. Values represent the means ± standard errors (SE) of 3 independent experiments. ***, $P < 0.001$.

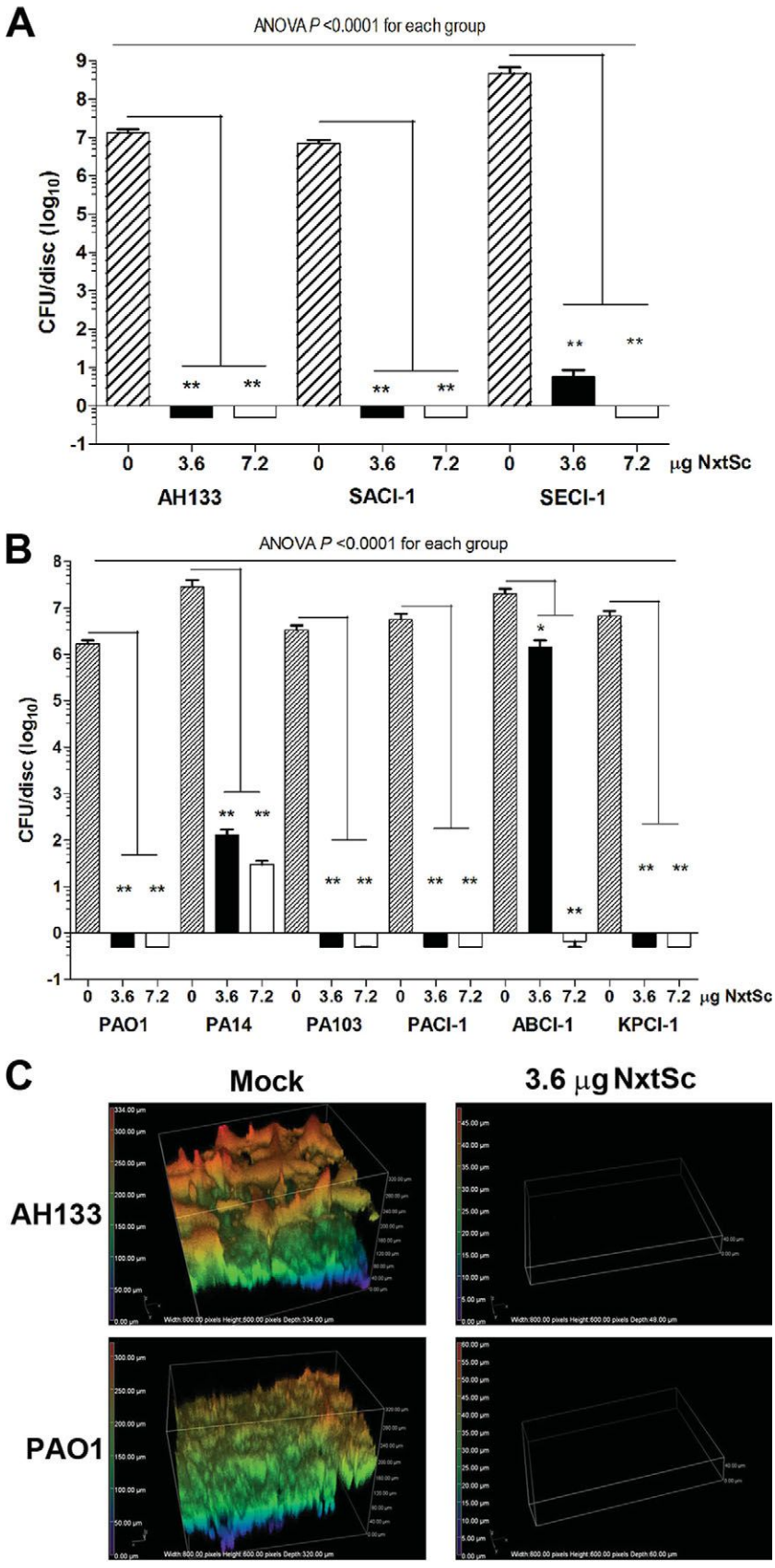
(strains AH133 and PAO1, respectively). Using the zone of inhibition assay, NxtSc-AS at concentrations of 3.6 and 7.2 μg/ml produced zones of inhibition with each strain (data not shown). We confirmed these results by determining the numbers of CFU in a microtiter plate assay using 3.6 μg/ml NxtSc-AS. NxtSc-AS significantly reduced the growth of AH133 from approximately 10^6 CFU to 10^0 ($\log_{10}$) CFU and that of PAO1 from 10^7 CFU to 10^0 CFU (Fig. 1).

We then tested the ability of NxtSc-AS to inhibit biofilm formation by AH133 (carrying pCM11) and PAO1/pMRP9-1 by using our previously described *in vitro* wound biofilm model (36, 37). Each strain contains a plasmid that carries the gene encoding green fluorescent protein, to allow visualization of the biofilms (Table 1). To mimic the infection of a freshly debrided wound, we inoculated the cellulose discs with 1×10^2 CFU/disc. Discs were then treated with PBS or NxtSc-AS at 3.6 or 7.2 μg/disc. Quantitative analysis of the untreated control discs revealed the development of mature AH133 and PAO1/pMRP9-1 biofilms; after 24 h of incubation at 37°C, the levels of AH133 and PAO1/pMRP9-1 were increased to 10^7 CFU/disc and 10^6 CFU/disc, respectively (Fig. 2A and B). However, after incubation under the same conditions, NxtSc-AS-treated discs contained only 0.05×10^{-1} CFU/disc of AH133 or PAO1/pMRP9-1 (Fig. 2A and B). Visualization of the biofilms with CLSM and 3D reconstruction of the biofilms confirmed these results. While both AH133 and PAO1/pMRP9-1 produced mature well-developed biofilms on the untreated discs, no biofilm structures were detected on discs treated with 3.6 μg/disc of NxtSc-AS (Fig. 2C). To determine whether the effects of NxtSc-AS extended to other wound pathogens, we examined the effects of 3.6 μg/disc on biofilm development by two Gram-positive wound isolates, i.e., *S. aureus* SACI-1 and *Staphylococcus epidermidis* SECI-1, and three Gram-negative wound isolates, i.e., *P. aeruginosa* PACI-1, *Acinetobacter baumannii* ABCI-1, and *Klebsiella pneumoniae* KPCI-1 (Table 1). We also assessed the effects of NxtSc-AS on biofilm development by the *P. aeruginosa* strains PA103 and PA14, which were isolated from wounds (Table 1). Similar to its effects on AH133 and PAO1/pMRP9-1, NxtSc-AS at 3.6 μg/disc prevented biofilm development by SACI-1, PA103, PACI-1, and KPCI-1, with reductions in levels from 10^6 to 10^7 ($\log_{10}$) CFU/disc to 0.05×10^{-1} ($\log_{10}$) CFU/disc (Fig. 2A and B). While 3.6 μg NxtSc-AS/disc reduced SECI-1 biofilm from 10^9 CFU/disc to $<10^1$ ($\log_{10}$) CFU/disc and PA14 biofilm from $>10^7$ CFU/disc to 10^2 ($\log_{10}$) CFU/disc, it reduced the CFU/disc value for ABCI-1 by only 1 log unit (Fig. 2A and B). However, 7.2 μg NxtSc-AS/disc reduced SECI-1 and ABCI-1 to 0.05×10^{-1} ($\log_{10}$) CFU/disc and PA14 to 5×10^1 ($\log_{10}$) CFU/disc (Fig. 2A and B). These results suggest that, at 3.6 or 7.2 μg/disc, NxtSc-AS significantly interferes with biofilm development by several different wound pathogens.

NxtSc in gel formulations inhibits biofilm development by Gram-positive and Gram-negative wound pathogens. In wounds (especially burn wounds), necrotic tissue and the avascular eschar reduce the delivery of immune cells and systemic antibiotics, thereby enhancing the susceptibility of the wound to different bacterial infections (21–23). Topical application of an agent in an ointment or a gel provides direct contact between the antimicrobial agents and the wound pathogens. Additionally, the ointment or gel extends the time of contact between the pathogens and the agent. Furthermore, the agent may be released from the ointment or gel and reach the deeper layers of the infected connective tissue over an extended period. Therefore, we formulated the NxtSc in water-soluble PEG, which is inert, nonvolatile, and innocuous and does not hydrolyze or deteriorate.

We recently modified the *in vitro* wound biofilm model to utilize it in assessing the antibiofilm effects of different antimicrobial ointments, including commercially available antibiotic ointments (36). Briefly, after inoculating the discs with the tested pathogen, we apply sterile gauze spread with the agent to the disc. After 24 h of incubation, the gauze is removed and the biofilm is processed as described for the original model. We first tested the effectiveness of several different formulations of NxtSc in PEG (NxtSc gels NxtSc-G1 through NxtSc-G5) in preventing biofilm development by the wound pathogens, beginning with AH133 and PAO1/pMRP9-1. These gel formulations contain 1.44 mg of surfactant in 400 mg PEG but vary in the relative amounts of a second proprietary ingredient and the pH. With the exception of NxtSc-G2, all formulations inhibited the development of AH133 biofilm, reducing the levels from 10^7 CFU/disc to 0.05×10^{-1} ($\log_{10}$) CFU/disc (Fig. 3A). These results were confirmed by CLSM (Fig. 3C). We obtained similar results when we examined the effects on biofilm development by SACI-1 (data not shown). With the exception of NxtSc-G4, all formulations inhibited the development of PAO1/pMRP9-1 biofilm, reducing the levels from 5×10^7 CFU/disc to 0.05×10^{-1} ($\log_{10}$) CFU/disc, as confirmed by visualization (Fig. 3B and C). We observed similar results when we examined the biofilm developed by PACI-1 (data not shown). These results suggest that the NxtSc-G1, NxtSc-G3, and NxtSc-G5 formulations are effective at inhibiting biofilm development by Gram-positive and Gram-negative wound pathogens.

NxtSc in gel formulations eliminates established biofilms formed by either *S. aureus* or *P. aeruginosa*. We then examined the possibility that NxtSc-G1, NxtSc-G3, and/or NxtSc-G5 would reduce the bacterial bioburden within infected wounds by disrupting already established biofilms. We first established 8-h partial biofilms for AH133 and PAO1/pMRP9-1. We then treated the biofilms with PEG or one of the indicated NxtSc gel formulations and incubated the discs for 24 h. The PEG-treated partial biofilms produced well-developed mature biofilms that efficiently covered


A
ANOVA P <0.0001 for each group
CFU/disc (log10)
0 3.6 7.2 0 3.6 7.2 0 3.6 7.2 µg NxtSc
AH133
SACI-1
SECI-1
B
ANOVA P <0.0001 for each group
CFU/disc (log10)
0 3.6 7.2 0 3.6 7.2 0 3.6 7.2 0 3.6 7.2 0 3.6 7.2 0 3.6 7.2 µg NxtSc
PAO1
PA14
PA103
PACI-1
ABCI-1
KPCI-1
C
Mock
3.6 µg NxtSc
AH133
PAO1

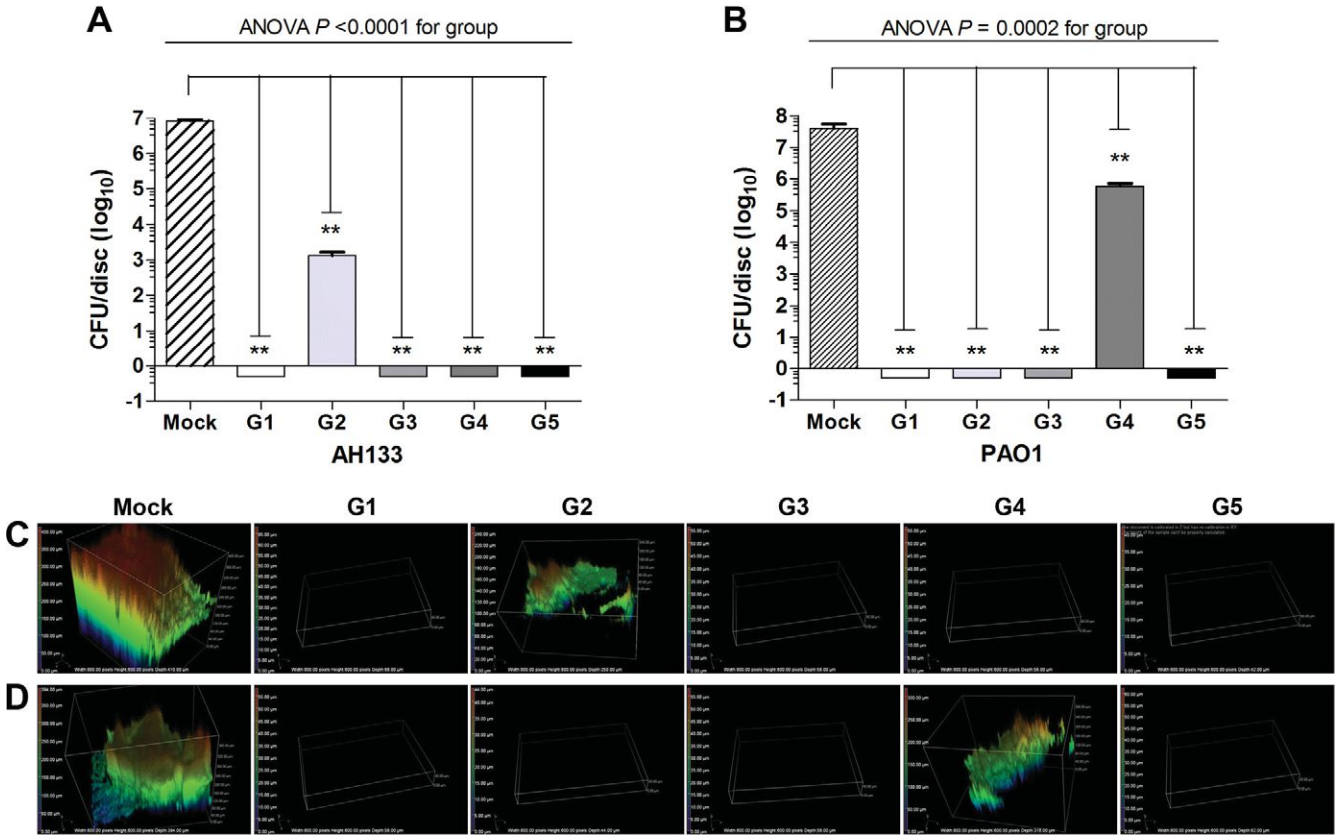


FIG 3 NxtSc gels inhibit *S. aureus* and *P. aeruginosa* biofilm formation. (A and B) Discs were inoculated with 10^2 CFU AH133 (A) or PAO1/pMRP9-1 (B), and the discs were covered with 22-mm by 22-mm pieces of gauze spread with 400 mg PEG (Mock) or 400 mg of one of five different formulations of NxtSc gel (NxtSc-G1 through NxtSc-G5), containing 1.44 mg of a surfactant in a standardized buffer but varying in the amounts of a second proprietary ingredient and in pH. The plates were incubated as described for Fig. 2, and the CFU/disc values were determined by the colony count assay. (C and D) The microscopic structure of biofilms formed by AH133 (C) and PAO1/pMRP9-1 (D) grown as described for panels A and B was examined by CLSM. Images show 3D reconstructions of biofilm architecture using Nis-Elements software. Red, orange, and yellow indicate areas of increased cell density; green, blue, and purple indicate areas of decreased cell density. Values in the graphs represent the means ± SE of 3 independent experiments. **, $P < 0.01$. ANOVA *P* value summaries are indicated over each group. Images shown are representative of 3 separate experiments.

the surfaces of the discs (Fig. 4). As seen in the inhibition experiments, all tested formulations significantly reduced AH133 biofilms (Fig. 4A). To our surprise, however, only NxtSc-G5 significantly reduced PAO1/pMRP9-1 biofilm (Fig. 4B). We confirmed the effectiveness of NxtSc-G5 by determining its effects on biofilms formed by additional *P. aeruginosa* and *S. aureus* clinical isolates. The *P. aeruginosa* isolates PACI-57 and PACI-75 were obtained from infected burn wounds, while the methicillin-resistant *S. aureus* strain MRSA-201 was isolated from blood (Table 1). NxtSc-G5 eliminated biofilms formed by all isolates (Fig. 5). These results were confirmed by CLSM and 3D reconstruction of the biofilms (Fig. 4C and D). These results suggest that NxtSc-G5 is the most effective among the tested gel formulations in inhibiting as well as eliminating biofilm developed by Gram-positive and Gram-negative wound pathogens. Therefore, further experiments were conducted using NxtSc-G5 only.

To examine the shelf stability of NxtSc-G5, we stored it in a sterile container for 9 months at room temperature and utilized it in the biofilm assays. After 9 months of storage, NxtSc-G5 was as effective as freshly prepared NxtSc-G5 in inhibiting the development of biofilms formed by AH133 and PAO1/pMRP9-1 as well as eliminating established biofilms formed by these microorganisms (data not shown).

NxtSc-G5 inhibits the growth of *S. aureus* and *P. aeruginosa* in infected wounds. Using the murine model of wound biofilm

FIG 2 NxtSc-AS inhibits biofilm formation by Gram-positive and Gram-negative bacteria. (A) Blank 6-mm cellulose discs were placed on LB agar plates and inoculated with 10^2 CFU of *S. aureus* strains AH133 (expressing GFP) or SACI-1 or *S. epidermidis* strain SECI-1. Discs were then treated with PBS (0) or NxtSc-AS (3.6 or 7.2 μg/disc), and the plates were incubated at 37°C for 24 h. Discs were removed from the plates, rinsed gently with PBS to remove unattached bacteria, and vortex-mixed in 1 ml PBS to release attached (biofilm) bacteria. The CFU/disc values were determined by the colony count assay. (B) Discs on LB agar plates were inoculated with 10^2 CFU of *P. aeruginosa* strains PAO1/pMRP9-1 (expressing GFP), PA14, PA103, and PACI-1, *A. baumannii* ABCI-1, or *K. pneumoniae* KPCI-1 and then were treated and incubated as in panel A. The CFU/disc were determined by the colony count assay. (C) The microscopic structure of biofilms formed by AH133 and PAO1 grown as described for panels A and B and treated with PBS (Mock) or NxtSc-AS (3.6 μg NxtSc) was examined by CLSM. Images show 3D reconstructions of biofilm architecture using Nis-Elements software. Red, orange, and yellow indicate areas of increased cell density; green, blue, and purple indicate areas of decreased cell density. Values in the graphs represent the means ± SE of 3 independent experiments. **, $P < 0.01$; *, $P < 0.05$. ANOVA *P* value summaries are indicated over each group. The images shown are representative of 3 separate experiments.

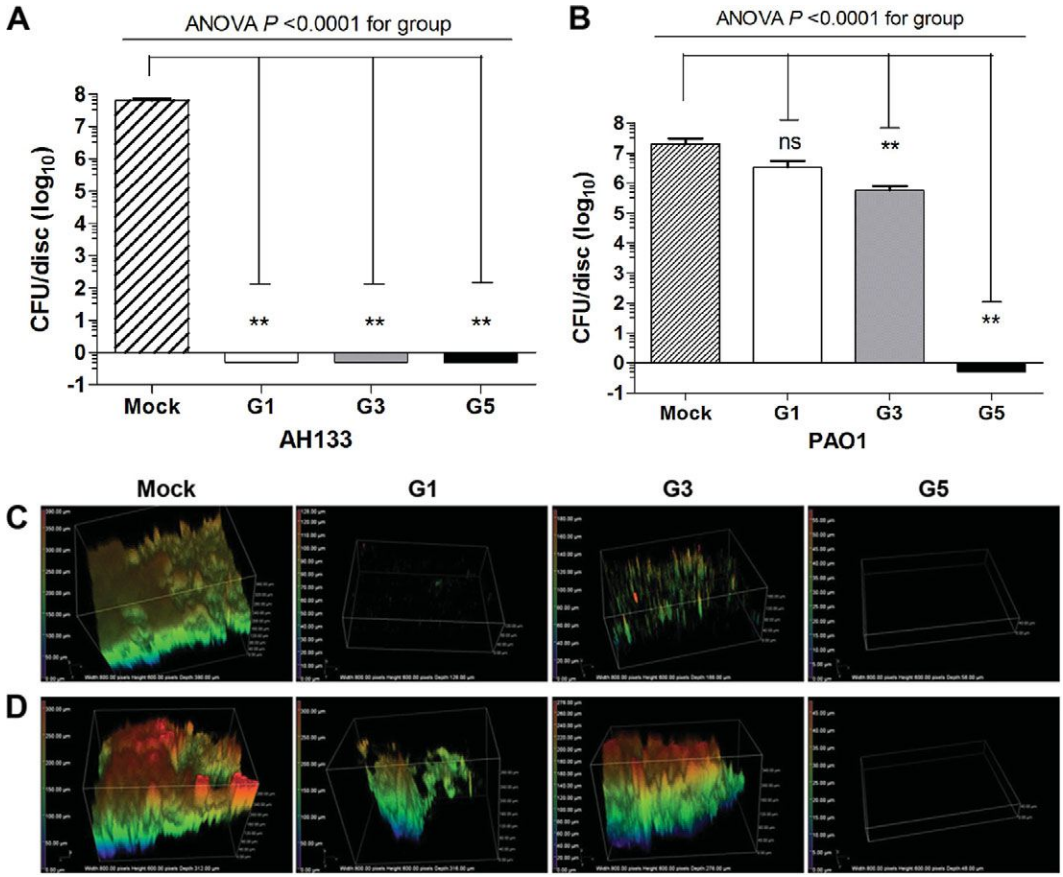


FIG 4 NxtSc-G5 eliminates partial biofilms formed by *S. aureus* and *P. aeruginosa*. (A and B) Four sets of discs were inoculated with 10^2 CFU of AH133 (A) or PAO1/pMRP9-1 (B) and incubated for 8 h to allow partial biofilm development. One set of discs was treated with 400 mg PEG (Mock), and the other three sets were treated with 400 mg NxtSc-G1, NxtSc-G3, or NxtSc-G5. The treated discs were incubated for 24 h, and CFU/disc values were determined by the colony count assay. (C and D) The microscopic structure of biofilms formed by AH133 (C) and PAO1/pMRP9-1 (D) grown as described for panels A and B was examined by CLSM. Images show 3D reconstructions of biofilm architecture using Nis-Elements software. Red, orange, and yellow indicate areas of increased cell density; green, blue, and purple indicate areas of decreased cell density. Values in the graphs represent the means ± SE of 3 independent experiments. **, $P < 0.01$; ns, not statistically significant. ANOVA *P* value summaries are indicated over each group. Images shown are representative of 3 separate experiments.

infection, we examined the effectiveness of NxtSc-G5 in inhibiting the development of *S. aureus* or *P. aeruginosa* biofilms *in vivo*. To monitor the development of the biofilms through live image analysis, we utilized *S. aureus* strain Xen29 and *P. aeruginosa* strain Xen5, which contain a single stable copy of the *P. luminescens luxABCDE* operon in their chromosomes (32, 43). Therefore, when they are metabolically active, the strains emit luminescence, which can be detected and measured. Using the IVIS Lumina XR system with Living Image software, the development of biofilms and the efficacy of antimicrobial agents can be measured over extended periods.

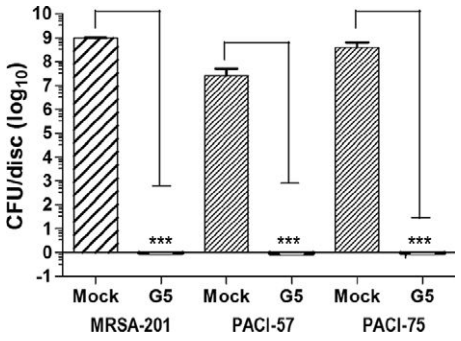


FIG 5 NxtSc-G5 eliminates biofilms formed by additional *P. aeruginosa* and methicillin-resistant *S. aureus* clinical isolates. Biofilms were developed and analyzed as described for Fig. 4. PACI-57 (PACI-3) and PACI-75 (PACI-4) were isolated from burn wounds. MRSA-201 was isolated from blood. Values represent the means ± SE of 3 independent experiments. ***, $P < 0.001$.

The wound biofilm model described in detail in Materials and Methods was used to determine the efficacy of NxtSc-G5 in preventing biofilm formation by Xen29 and Xen5. The wounds were covered with gauze spread evenly with 400 mg of PEG or NxtSc-G5, and OpSite dressing was applied over the gauze. We then inoculated 200 to 250 CFU of Xen29 or Xen5 between the gauze and the wound. The mice were briefly anesthetized, and the extent of biofilm formation was visualized at 24, 48, and 72 h after treatment. Both Xen29 and Xen5 colonized and grew within the PEG-treated wounds (Fig. 6). However, neither Xen29 nor Xen5 grew within the infected wounds treated with NxtSc-G5 (Fig. 6), which suggests that the agent inhibited successful colonization and biofilm development by either microorganism within the infected wounds.

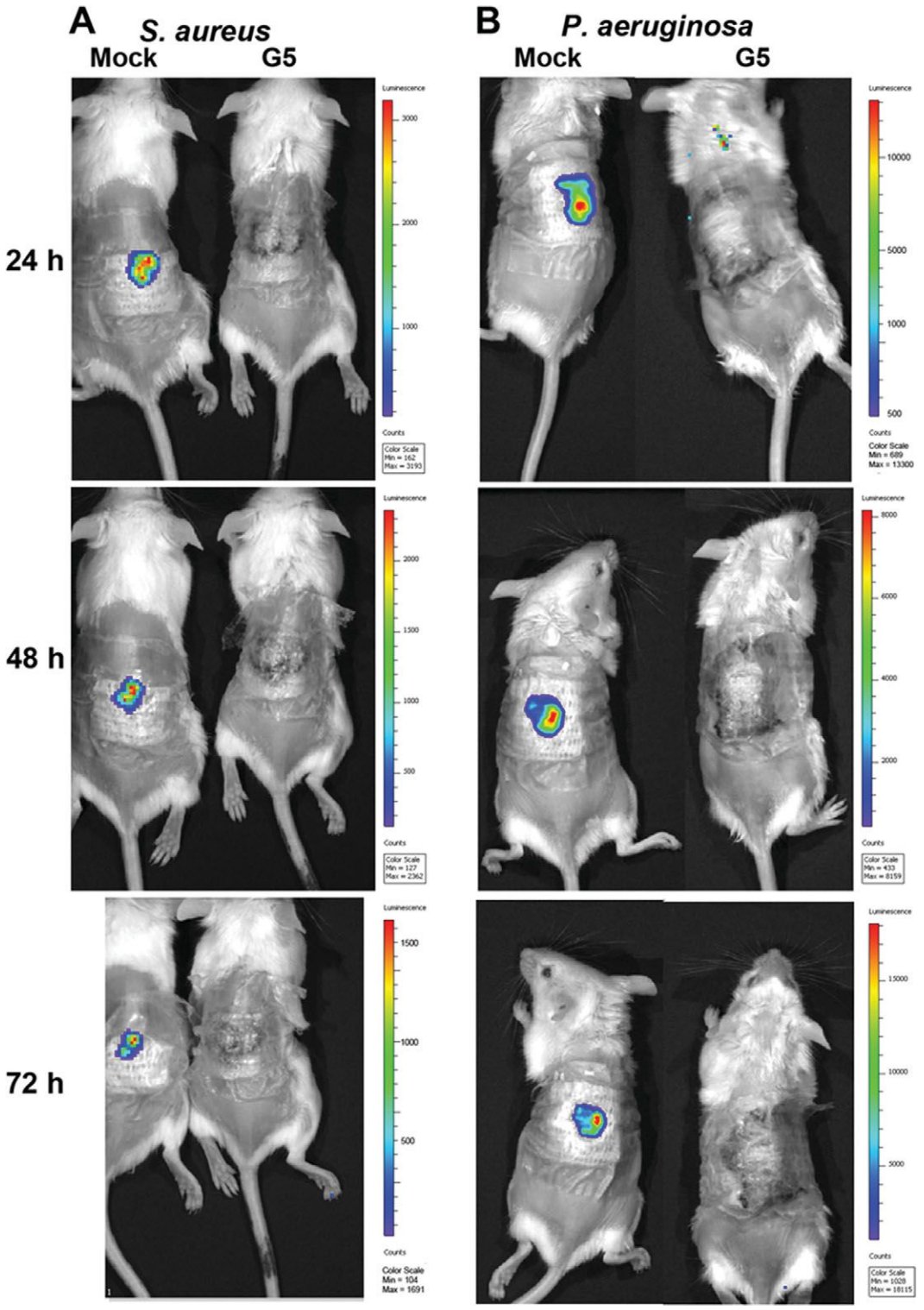


FIG 6 NxtSc-G5 inhibits wound infections with *S. aureus* and *P. aeruginosa in vivo*. A 15-mm, circular, full-thickness, excision wound was made on the backs of Swiss Webster mice. The wounds were covered with 22-mm by 22-mm pieces of gauze spread with PEG (Mock) or 400 mg of NxtSc-G5 and then were covered with OpSite dressing. The wounds were inoculated with 200 to 250 CFU of *S. aureus* Xen5 (A) or *P. aeruginosa* Xen29 (B) between the gauze and the wound bed. The animals were examined with *in vivo* live imaging at 24, 48, and 72 h after treatment. Images shown are representative of 3 separate experiments.

NxtSc-G5 eliminates Gram-positive and Gram-negative organisms from infected wounds. We determined whether NxtSc-G5 would eliminate Xen29 and Xen5 from the infected wounds, again using the murine model of wound infection as described above, with the following exception. Once generated, each wound was covered with sterile gauze and OpSite dressing, and the particular strain was injected between the gauze and the wound. After 24 h, the wound was visualized to confirm the establishment of the infection. Once the infection was confirmed, the gauze was removed and replaced with gauze spread with 400 mg PEG or NxtSc-G5, and the mice were monitored every 24 h for 72 h after treatment. As shown in Fig. 7, PEG-treated wounds maintained infections with Xen29 and Xen5. However, treatment with NxtSc-G5 eliminated infections with either microorganism by 48 h after treatment (Fig. 7).

To explore the efficacy of NxtSc-G5 further, we repeated the same experiments using SECI-1, ABCI-1, and KPCI-1. Since none of these isolates carries the *luxABCDE* operon, we collected the infected/treated tissues 48 h after treatment, homogenized them, and determined the number of CFU/g within the tissues. All three strains grew well within the wounds, reaching levels of approximately 10^5 to 10^7 ($\log_{10}$) CFU/g in untreated wounds (Fig. 8). Similar to the results observed in our *in vitro* experiments, NxtSc-G5 reduced levels to approximately 0.05×10^{-1} ($\log_{10}$) CFU/g (Fig. 8). These results suggest that NxtSc-G5 is a potential antimicrobial agent that eliminates established Gram-positive and Gram-negative infections from wounds.

NxtSc has no potential adverse effects. Evaluation of potential adverse effects caused by NxtSc-AS and NxtSc-G formulations was performed at a commercial facility (WuXi AppTec, St. Paul, MN), in accordance with ISO standards and following IACUC-approved protocols. The dermal limit study, using abraded dermal application of 2,000 mg/kg NxtSc-AS, was done in rats (39, 41, 42). There was no apparent acute systemic toxicity and very slight initial dermal irritation potential. In the intracutaneous reactive test in albino rabbits (39, 40), the differences in the mean test and control scores of the dermal observations were less than 1.0, indicating that no intracutaneous reactivity was observed with NxtSc-AS. Finally, in the guinea pig maximization sensitization test (39, 40), NxtSc in gel formulation did not elicit a sensitization response. These results suggest that NxtSc, whether in solution or in gel, would be unlikely to produce adverse effects when used clinically.

DISCUSSION

In infected wounds, whether acute or chronic, pathogenic microorganisms form biofilms, within which they are more resistant to the host immune response as well as antibiotics. Numerous topical antimicrobial agents have been developed; they vary in their ability to eliminate planktonic microorganisms and/or to penetrate biofilms. In this study, we evaluated the efficacy of NxtSc, a novel wound antimicrobial agent. The key features of NxtSc are its broad spectrum, its ability to eliminate already established biofilms *in vitro* and *in vivo*, and its stable shelf life of at least 9 months. NxtSc-AS was very effective in inhibiting biofilm development by Gram-negative and Gram-positive wound pathogens *in vitro* (Fig. 2), reducing CFU values by 6 to 8 log CFU/disc to $<10^0$ ($\log_{10}$) CFU/disc. The NxtSc gel formulations NxtSc-G1 to NxtSc-G5 also inhibited biofilm development by *S. aureus* and *P. aeruginosa*, with 4 of the 5 products reducing CFU values for each microorganism by a margin similar to that seen with NxtSc-AS (Fig. 3). While NxtSc-G1, NxtSc-G3, and NxtSc-G5 eliminated preformed *S. aureus* biofilms *in vitro*, only NxtSc-G5 eliminated preformed *P. aeruginosa* biofilms (Fig. 4 and 5). This clearly indicates that NxtSc-G5 is capable of penetrating the EPS layer and killing the bacteria within the biofilm. Additionally, in the murine model of wound infection, NxtSc-G5 prevented infection by *S. aureus* and *P. aeruginosa* and eliminated these pathogens, as well as *S. epidermidis*, *A. baumannii*, and *K. pneumoniae*, from wounds infected for 24 h prior to treatment (Fig. 6, 7, and 8). This feature is critical since many wounds are already colonized and carrying high bioburdens when the patients present for treatment. Finally, NxtSc-G5 stored for 9 months at room temperatures was as efficacious as freshly prepared NxtSc-G5 in inhibiting and eliminating biofilms (data not shown). Perhaps more importantly, NxtSc-G5 maintained its antimicrobial effect *in vivo* for up to 5 days after its application to *S. aureus*- or *P. aeruginosa*-infected wounds (Fig. 6 and 7; data not shown).

Silver-based products have been widely used as effective antimicrobial agents in treating infected burn wounds and in preventing bacterial infections of leg ulcers, diabetic foot ulcers, and pressure ulcers (44–47). However, based on data from meta-analyses, the effectiveness of these silver-based products varies widely (45, 48, 49). Recent studies described products designed to either penetrate the biofilm or prolong the effects of the silver ions. Using the drop-flow model to examine biofilm development by *P. aeruginosa* treated with Acticoat absorbent (noncrystalline silver), Aquacel Ag, or Tegaderm Ag, Ammons et al. (50) obtained log reductions in viability within *P. aeruginosa* biofilms of 1.3, 1.6, and 0.17, respectively. When lactoferrin/xylitol wound hydrogel was combined with these products, the log reductions in viability increased to 4.0, 3.0, and 1.4, respectively. In comparison, NxtSc-G5 alone reduced the viability of 5 different wound pathogens by 6 to 8 $\log_{10}$ CFU/disc *in vitro* and eliminated infections with these organisms *in vivo*. Furthermore, the effectiveness of silver dressings depends on the release of silver ions from the dressing and thus gradually decreases over time (29, 51), whereas NxtSc-G5 was stable *in vivo* for up to 5 days. Therefore, NxtSc-G5 will likely maintain its effectiveness, although clinical studies are essential to corroborate our findings.

Exopolysaccharide is the key element in maintaining the structure of the biofilm and protecting the bacteria within the biofilm from the effects of different antibiotics. Previous studies described several compounds that have the potential to destabilize the structures, damage the biofilm, or facilitate the penetration of antibiotics, including dispersin B, DNase I, and α-amylase (52–54). Dispersin B hydrolyzes the poly-*N*-acetylglucosamine surface polysaccharide within the biofilm matrix of many bacteria, including *S. aureus* (53), while DNase I degrades extracellular DNA, which serves as a matrix adhesion molecule in biofilms produced by most bacteria (54). Similar to the problem with silver, the effectiveness of these two compounds varied. For example, dispersin B detached preformed *S. epidermidis* biofilms but not *S. aureus* biofilms (53). DNase I, on the other hand, detached preformed *S. aureus* biofilms but not *S. epidermidis* biofilms (53). Similarly, α-amylase, an enzyme found in human pancreatic juice and saliva, rapidly detached *S. aureus* biofilms and dissociated *S. aureus* aggregates in liquid culture but had no effect on *S. epidermidis* biofilms (52). Compared with the reported performance of these compounds, NxtSc-G5 appears to be efficacious in penetrating

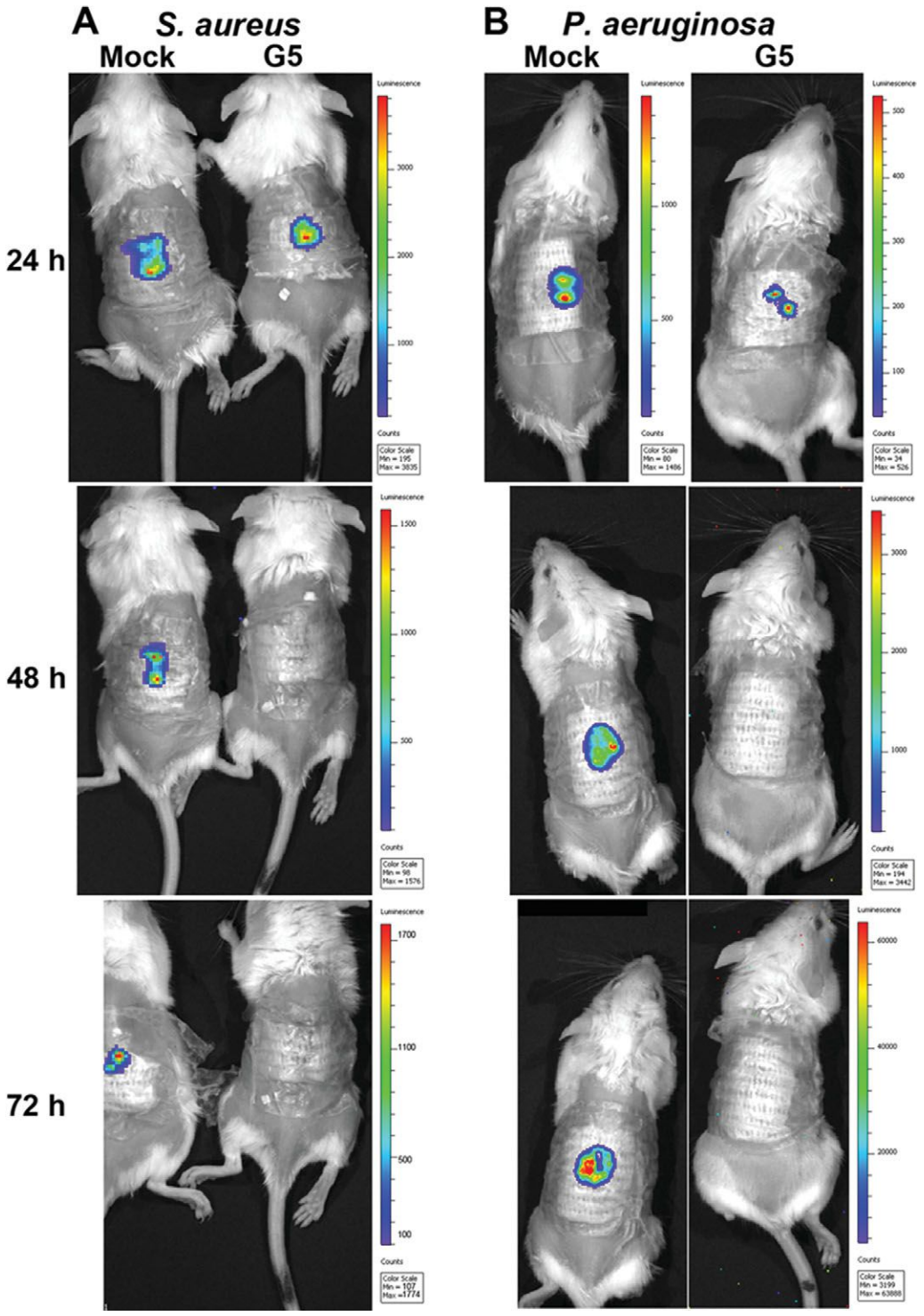


FIG 7 NxtSc-G5 eliminates wound infections with *S. aureus* and *P. aeruginosa in vivo*. A 15-mm, circular, full-thickness, excision wound was made on the backs of Swiss Webster mice. The wounds were covered with 22-mm by 22-mm pieces of sterile gauze and OpSite and were inoculated with 200 to 250 CFU Xen5 (A) or Xen29 (B) between the gauze and the wound bed. Animals were left untreated for 24 h, and then the wounds were treated with PEG (Mock) or 400 mg of NxtSc-G5. The animals were examined with *in vivo* live imaging at 24, 48, and 72 h after treatment. Images shown are representative of 3 separate experiments.

biofilms formed by *S. aureus* and *P. aeruginosa in vitro* and *in vivo* and eliminating the microorganisms within the biofilms. NxtSc-G5 also eliminated *S. epidermidis*, *A. baumannii*, and *K. pneumoniae* from wounds infected for 24 h prior to treatment.

Unlike silver and destabilizing compounds, the efficacy of different antibiotic ointments in reducing wound bioburdens or inhibiting bacterial biofilms has not been extensively analyzed. We recently utilized the *in vitro* wound biofilm model described in

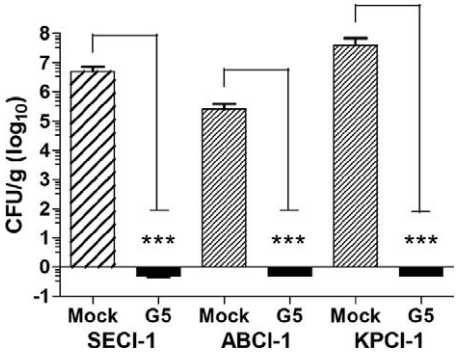


FIG 8 NxtSc-G5 reduces wound infections with *S. epidermidis*, *A. baumannii*, and *K. pneumoniae in vivo*. A 15-mm, circular, full-thickness, excision wound was made on the backs of Swiss Webster mice. The wounds were covered with 22-mm by 22-mm pieces of sterile gauze and OpSite and were inoculated with 10^2 CFU of SECI-1, ABCI-1, or KPCI-1 between the gauze and the wound bed. Animals were left untreated for 24 h, and then the wounds were treated with PEG (Mock) or 400 mg of NxtSc-G5. At 48 hours after treatment, animals were euthanized and the wounds were excised and homogenized in PBS. CFU/g of tissue values were determined by the colony count assay. Values represent the means ± SE of 3 independent experiments. ***, $P < 0.001$.

this study to compare the effects of gentamicin sulfate, mupirocin, and triple-antibiotic (bacitracin, neomycin, and polymixin B) ointments on biofilm development by *P. aeruginosa* and *S. aureus* wound isolates (36). Biofilms were developed for 24 h, treated with the tested antibiotic ointments, and examined for biofilm eradication. Compared with control biofilms, and with the exception of one *P. aeruginosa* wound isolate that was resistant to gentamicin, all ointments reduced biofilms formed by *S. aureus* and *P. aeruginosa* strains by no more than 0.3 to 2.0 log_{10} CFU/disc (36). Using the murine model of contaminated crush wounds, Erdur et al. (55) compared the prophylactic effects of topical mupirocin and nitrofurazone. Neither mupirocin nor nitrofurazone significantly reduced the levels of *Streptococcus pyogenes* within the wound (55); nitrofurazone but not mupirocin reduced the *S. aureus* CFU within the crush wound (55). Compared with the results of these studies, NxtSc-AS and NxtSc-G5 produced consistent and more-significant reductions (5 to 7 log_{10} CFU/disc or CFU/g) in biofilm formation by diverse wound pathogens *in vitro* and *in vivo*.

A critical issue to be considered in the development of any new antimicrobial agent is the emergence of resistant mutants. We conducted preliminary experiments to detect the presence of *P. aeruginosa* or *S. aureus* mutants resistant to NxtSc-G5. In one set of experiments, we developed and treated *P. aeruginosa* and *S. aureus* biofilms with NxtSc-G5 as described for the *in vitro* wound biofilm model. We dispersed the treated biofilms by vortex-mixing the discs in PBS, and we spotted aliquots of the undiluted suspensions on LB agar plates. We incubated the plates at 37°C for 48 h, sealed them to prevent drying, and incubated them at room temperature for 2 weeks. We did not detect a single colony on any of the plates (data not shown). We also examined the possible development of NxtSc-G5-resistant *P. aeruginosa* or *S. aureus* mutants within the infected wounds. Using the murine model of wound infection, we treated *P. aeruginosa*- or *S. aureus*-infected wounds with NxtSc-G5, as described above. We homogenized the infected tissues in PBS and spotted undiluted aliquots of the homogenates on LB agar plates. After 48 h of incubation at 37°C and 2 weeks at room temperature, we detected no colonies on any of the plates (data not shown). Further experiments will be conducted to examine the potential emergence of resistant mutants after prolonged exposure to NxtSc formulations.

Another critical factor in wound care management is the frequency of wound dressing changes. More-stable antimicrobial activity of the agent used in dressings for chronic wounds leads to longer wear times and reduces the frequency of dressing changes. One of the desirable features of Acticoat, as proved by animal experiments and several clinical trials, is its extended wear time (51, 56). Our results suggest that NxtSc-G5 has a similar possibility for extended wear time, as neither *P. aeruginosa*- nor *S. aureus*-infected wounds showed regrowth of these microorganisms 5 days after the application of NxtSc-G5 (Fig. 6; data not shown).

In conclusion, these results establish that NxtSc-G5 is an effective inhibitor of biofilm development by diverse wound pathogens when applied at the time of wounding, as well as an efficient disruptor of biofilms already established by these pathogens. This novel antimicrobial agent, which is nontoxic and has a usefully long shelf life, shows promise as an effective agent for the prevention of biofilm-related infections.

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