

Novel Antimicrobial Peptides Formulated in Chitosan Matrices are Effective Against Biofilms of Multidrug-Resistant Wound Pathogens

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ABSTRACT Introduction: Infection frequently complicates the treatment of combat-related wounds, impairs healing, and leads to worse outcomes. To better manage wound infections, antimicrobial therapies that are effective against biofilm and designed for direct wound application are needed. The primary objective of this work was to evaluate a chitosan matrix for delivery of two engineered antimicrobial peptides, (ASP)-1 and ASP-2, to treat biofilm-associated bacteria. A secondary objective was to determine whether replacing the levorotatory (L) form amino acids in ASP-2 with dextrorotatory (D) form amino acids would impact peptide activity. Materials and Methods: Chitosan gels loaded with antimicrobial peptides were evaluated for peptide release over 7 days and tested for efficacy against biofilms grown both in vitro on polymer mesh and ex vivo on porcine skin. Results: When delivered via chitosan, 70% to 80% of peptides were released over 7 days. Gels eradicated biofilms of gram-positive and gram-negative, drug-resistant bacteria in vitro and ex vivo. Under the conditions tested, no meaningful differences in peptide activity between the L and D forms of ASP-2 were detected. Conclusions: Chitosan serves as an effective delivery platform for ASP-1 and ASP-2 to treat biofilm-embedded bacteria and warrants further development as a topical treatment.

INTRODUCTION

Infection remains a major cause of morbidity and mortality in injured military personnel.¹ The ability to effectively treat combat-related injuries is hampered by the increasing prevalence of multidrug-resistant organisms and the propensity for bacteria to form biofilms.² In many wounds, perfusion of systemically administered antibiotics is limited because of tissue and vessel damage. The impact of standard antibiotic use is further limited by bacterial resistance to certain antibiotics as well as the tolerance imposed by the structure of biofilms and behavior of bacteria within them. There is a need for a product that can deliver antimicrobial therapy directly to wounds that is effective against multidrug-resistant bacteria and their associated biofilms.

Antimicrobial peptides (AMPs) are promising candidates to address this need as many are broad spectrum, effective against drug-resistant pathogens, and capable of eradicating even dormant bacteria within biofilms.³ However, natural AMPs have some shortcomings that have limited their use as therapeutics, including lack of stability and reduced activity under conditions of high salt, divalent cations, and variable pH. These shortcomings have spurred the development of engineered peptides that mimic the function of natural peptides using sequences that have been optimized to improve

stability and potency within a broader range of physiologic environments. Stability of AMP in tissue might be strongly affected by proteases, which are abundant in the skin⁴ and in wounds.⁵ It is well recognized that uncontrolled activity of both endogenous proteases and exogenous bacterial proteases are major contributing factors in nonhealing and infected wounds.^{6–8} Peptides comprising amino acids in dextrorotatory (D)-isoform are known to be resistant to enzymatic degradation⁹; therefore, there is interest in understanding the potential impact on peptide activity of replacing levorotatory (L) with D amino acids to achieve greater stability of therapeutics for potential use in the challenging wound environment.

The purpose of this work was to examine chitosan as a matrix for delivery of broad-spectrum, engineered cationic AMP directly to wounds and to evaluate the performance of peptide-loaded chitosan matrices using both in vitro and ex vivo porcine skin biofilm models. The impact of replacing L amino acids with D amino acids on antimicrobial peptide activity was also evaluated.

METHODS

Antimicrobial Peptides, Gels, and Dressings

Antimicrobial peptides, (ASP)-1 (RRWVRRVRRWVR-RVVRRVRRWVRR) and ASP-2 (RWWRWRRWRR), were synthesized by American Peptide (now Bachem, Vista, CA) at > 96% purity using standard L-amino acids. The ASP-2D comprising all D isoform amino acids was synthesized by CASLO ApS (Kongens Lyngby, Denmark) at > 98% purity. Chitosan having an average molecular weight of 320 kDa (Siglufjordur Iceland, Primex) was used to prepare gels by first dissolving the material in acetic acid, then adjusting the pH to 5.9 ± 0.1 with sodium hydroxide. Gels were loaded with

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Presented as a poster at the 2018 Military Health System Research Symposium (Abstract number: MHSRS-18-1522).

The views, opinions, and/or findings contained in this article are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

doi:10.1093/milmed/usz222

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1% peptide as well as proprietary moisturizing and viscosity modifying excipients. Chitosan sponges were prepared in the same way as gels but without excipients. These gels were dispensed into 2" × 3" silicone molds, frozen, and then lyophilized to form dry sponges using a Freezone 4.5 (Labconco, Kansas City, MO). The lyophilized cakes were released from molds and pressed to form 3 mm thick sheets with a density of about 30 mg/cm².

In Vitro Release of Peptides From Gels

Release of peptides from gels was carried out in saline at 37°C for up to 7 days. Lyophilized sponges were die cut into discs, 9 mm in diameter and about 19 mg by weight. The discs were placed in 5-mL polypropylene cylindrical containers and weighed. At time zero, the dry sponges were reconstituted into gels by adding 2-mL saline, and the containers were closed and incubated at 37°C with mild shaking. At time points 1, 2, 3, 4 and 7 days, extracts above gels were collected, replaced with 1 mL of fresh saline, and placed back in the incubator at 37°C. Collected extracts were weighed and the samples were assayed for peptide content and purity by reverse phase high pressure liquid chromatography using a Waters Alliance e2695 Separations Module equipped with a Waters 2998 photodiode array detector. A Cortecs C18 + 2.7 μm column was used in conjunction with an acetonitrile/water gradient mobile phase supplemented with 0.2% trifluoroacetic acid. All tests were carried out in triplicate.

Bacterial Isolates and Growth Conditions

Methicillin-resistant *Staphylococcus aureus* (USA 300 ATCC BAA-1717), *Pseudomonas aeruginosa* (ATCC 15692), and *Acinetobacter baumannii* (ATCC BAA-1605) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Bacteria were stored at -80°C and subcultured on trypticase soy broth (TSB) agar plates at 37°C before use. A single bacterial colony was aseptically picked from an agar overlay and released in 25 to 30 mL of prewarmed cation-adjusted Mueller Hinton Broth (CAMHB) in a culture flask. Each bacteria culture was incubated overnight in a 37°C incubator with shaking.

Minimum Bactericidal Concentration

Peptide stocks were serially diluted in acetate saline buffer, pH 6.0 within 96 well plates. An overnight bacterial culture was diluted to ~10⁶ colony forming units (CFU)/mL using CAMHB and added to serially diluted peptide solutions for 24 hours at 37°C. The resulting solutions were serially diluted and plated on TSB agar to count the number of remaining viable bacteria. The minimum bactericidal concentrations (MBCs) were assigned to the lowest peptide concentrations at which ≥3 log₁₀ reductions in CFU relative to controls were achieved.

Efficacy of Antimicrobial Gels Against Biofilms Grown on Mesh Substrates In Vitro

Polyethylene terephthalate (PET) mesh, 70 microns thick and 28% open area, was die cut into 9 mm discs and autoclaved (Component Supply, Sparta, TN). These PET substrates were selected because they have a high percentage of open area to allow biofilm ingrowth. A freshly prepared overnight bacteria culture was diluted with CAMHB to a concentration of 10⁶ bacteria per mL and then 0.5 mL of the bacteria culture was added to each well within a 48 well plate. A PET disk was added to each well within the plate followed by incubation at 37°C for 24 hours to allow biofilms to form on the disk. Disks with adhered biofilms were then transferred to a fresh 48 well plate containing 150 μL of 10% porcine serum in saline per well. A 150 μL aliquot of antimicrobial gel was then applied to each disk and incubated at 37°C for 24 hours. After treatment, PET meshes were removed and sonicated in Dey/Engley neutralizing broth for 15 minutes, and sonicates were serially diluted and plated on TSB/agar to count the number of viable bacteria recovered from the mesh. Opticell Ag+, a commercially available gelling chitosan fiber dressing containing silver (Medline Industries, Inc.), and saline controls were used for comparison. All tests were performed in triplicate.

Efficacy of Antimicrobial Gels Against Biofilms Grown on Ex Vivo Porcine Skin

An ex vivo porcine skin biofilm model was previously described by Yang et al.^{10,11} and adapted here to allow for repeatable and higher throughput testing of antimicrobial gels. The dermal/epidermal layer of porcine skin, cut to 1.5 mm thickness, was obtained from Stellen Medical, St. Paul, Minnesota and stored frozen at -15°C. Porcine skin was die cut into 9-mm discs and sanitized in 70% ethanol overnight followed by thorough rinsing in sterile saline. A freshly prepared overnight bacteria culture was diluted with CAMHB to a concentration of 10⁶ bacteria per mL and then 0.5 mL of the bacteria culture was added to each well within a 48 well plate. A porcine skin disk was added to each well within the plate followed by incubation at 37°C for 72 hours to allow biofilms to form on the skin. Porcine skin discs with adhered biofilms were then transferred to a fresh 48 well plate containing 150 μL of 10% porcine serum in saline per well. A 150-μL aliquot of antimicrobial gel was applied to each porcine skin disk and incubated at 37°C for 24 to 72 hours. After 1, 2, or 3 days, the porcine skin was removed, sonicated in Dey/Engley neutralizing broth for 15 minutes twice, with the first sonicate being discarded, and the second sonicate being serially diluted and plated on TSB/agar to count bacteria recovered from the skin. Opticell Ag+, a commercially available gelling chitosan fiber dressing containing silver (Medline Industries, Inc.), and saline controls were used for comparison. All tests were performed in triplicate.

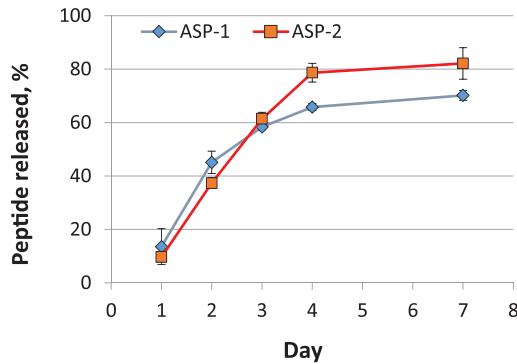


FIGURE 1. Recovery of ASP-1 and ASP-2 peptides from chitosan gels in saline at 37°C.

Statistical Analysis

All data are presented as the mean value \pm standard deviation. Datasets were evaluated for statistical significance by performing a one-way analysis of variance using SAS Statistical Software. Pairwise comparisons between groups were performed using the Tukey method. A p value of <0.05 ($P < 0.05$) was considered significant. For simplicity, significant differences between groups are only indicated on figures for comparisons of a group to ASP-1, ASP-2, or ASP-2D.

RESULTS

In Vitro Release of Peptides

Release of ASP-1 and ASP-2 from chitosan gels was measured over a 7-day period at 37°C in saline. The results shown in Figure 1 indicate that the gels provide consistent and strong release out to day 4 followed by slower release out to day 7. Both ASP-1 and ASP-2 demonstrated high recovery from chitosan gels in the range of 70% to 80% of the total peptide loaded and over the course of the experiment, only about 1% of the total peptide released had degraded. It should be noted that complete collection of liquid extracts from gels without disruption of gels was not possible, and therefore, the total amounts of peptides released were likely slightly higher than measured because of accumulation of sampling errors.

Minimum Bactericidal Concentration and In Vitro and Ex Vivo Efficacy

Minimum bactericidal concentration values for ASP-1, ASP-2, and ASP-2D are displayed in Table I and ranged from 4.8 to 19.3 $\mu\text{g}/\text{mL}$ depending on the peptide and bacteria strain. The potencies of ASP-1 and ASP-2 against planktonic bacteria were found to be similar, and potencies for ASP-2 and ASP-2D were nearly identical.

The efficacies of gels loaded with the AMP, ASP-1 and ASP-2, were evaluated in vitro by application to biofilms grown for 24 hours on PET mesh substrates. The results in Figure 2 show that ASP-1 gels eradicated *Pseudomonas*

aeruginosa and both ASP-1 and ASP-2 gels eradicated methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms within 1 day. In the case of *Pseudomonas aeruginosa* biofilms, ASP-1 gels outperformed the commercially available silver-based gel.

An ex vivo porcine skin biofilm model was employed to further evaluate antimicrobial gels under conditions thought to better simulate in vivo conditions. In this model, biofilms were allowed to develop and mature for 3 days followed by a single antimicrobial treatment that was left in place for 1, 2, or 3 days. Gels loaded with ASP-1, ASP-2, and ASP-2D reduced MRSA counts by 4 to over 7 logs within the first day of treatment with some samples showing complete or nearly complete biofilm eradication (Fig. 3). Similar results were obtained for treatments left in place for 2 or 3 days. The silver-based gels resulted in <1 log reductions over the same 3-day treatment period as shown in Figure 3. Similar results were obtained using *Pseudomonas aeruginosa* and *Acinetobacter baumannii* biofilms grown on ex vivo porcine skin as shown in Figures 4 and 5.

DISCUSSION

Chitosan has been studied and used extensively for wound care, tissue regeneration, and drug delivery applications largely because of its inherent biocompatibility, biodegradability, and mild antimicrobial properties.^{12–14} In the context of the present work, chitosan has been shown to be a suitable biodegradable platform for delivery of antibiotics to prevent wound and orthopedic device-related infections.^{15–19} Depending on the antibiotic and chitosan formulation, drug delivery on the order of hours to several days has been shown.^{17,18,20} The ASP-1 and ASP-2 chitosan gels developed herein provided sustained peptide release over a 4- to 7-day period, and the chitosan matrix met requirements for compatibility with both peptides and maintenance of peptide stability.

Although effective delivery of antibiotics from chitosan materials shows promise for inhibiting biofilm formation with prophylactic use,^{15,18} antibiotic-loaded materials do not appear to be effective for clearing preestablished biofilms.¹⁸ For many chronic wounds, including combat-related wounds, bacteria biofilms are thought to play a role in delayed healing. Debridement and irrigation are mainstays of wound management and are critical in the removal of bioburden from wounds. However, even with deep debridement, pockets of biofilm within the tissue have been shown to persist and result in rapid regrowth.^{21–23} Once established, wound biofilms are largely resistant to current antibiotic and antiseptic treatments and new technologies are needed that have potential to act on biofilm precursors and established biofilms.²⁴ The ASP-1 and ASP-2-loaded chitosan formulations were found to be effective on biofilms that had been allowed to grow and mature for 3 days on ex vivo porcine skin before peptide exposure. Similar porcine skin models employed by others

TABLE I. Activity of Peptides: Minimal Bactericidal Concentrations (MBCs). *N* = 3

Organism	Peptide	ASP-1	ASP-2	ASP-2D
	Strain	MBC, µg/mL	MBC, µg/mL	MBC, µg/mL
Methicillin-resistant <i>Staphylococcus aureus</i>	ATCC BAA-1717	9.6	9.7	8.9
<i>Pseudomonas aeruginosa</i>	ATCC 15692	19.2	19.3	17.8
<i>Acinetobacter baumannii</i>	ATCC BAA-1605	4.8	9.7	8.9

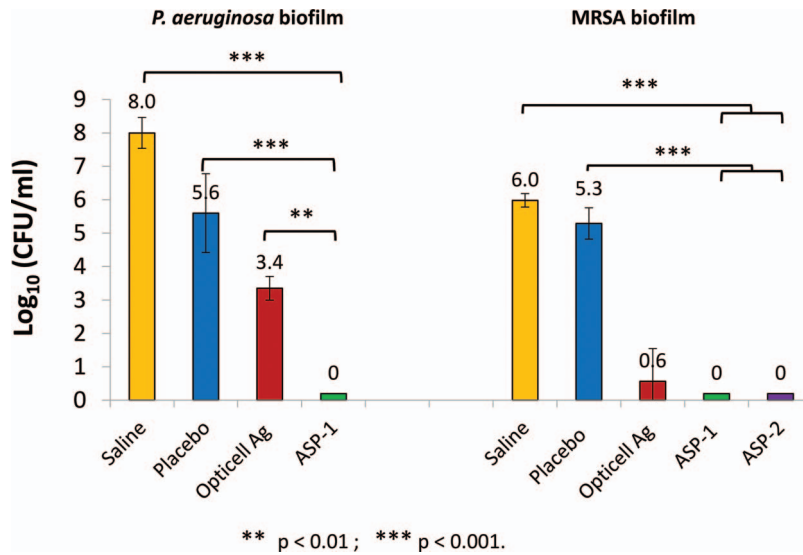


FIGURE 2. In vitro efficacy of ASP-1, ASP-2, and opticeil Ag + Gels against biofilms of *pseudomonas aeruginosa* and MRSA grown on PET mesh for 1 day and treated for 1 day. Saline and placebo gels having no peptide served as controls. *N* = 3.

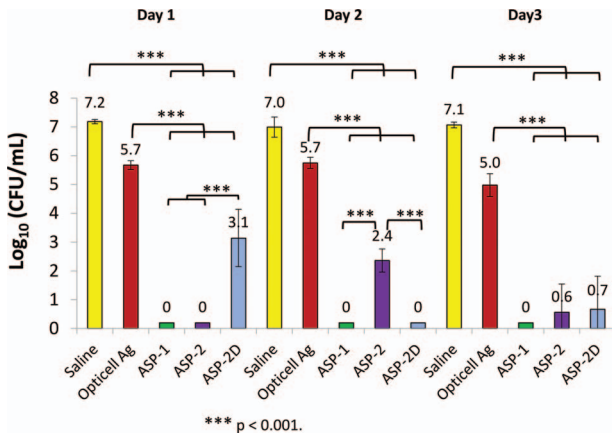


FIGURE 3. Ex vivo efficacy of ASP-1, ASP-2, ASP-2D, and opticeil Ag + Gels against biofilms of MRSA grown on pig skin for 3 days and treated for 3 days. Saline served as a control. *N* = 3.

typically allow biofilm maturation for 4 to 24 hours before use for antimicrobial evaluation.^{25–27} In our hands, the use of biofilms allowed to mature for 3 days on pig skin followed by 1, 2, and 3 days of treatment provides more challenging conditions for evaluation relative to biofilms grown for ≤ 24 hours. This more challenging model was useful in that it allowed for better differentiation between various

formulations under development as well as commercial products.

D form peptides are highly resistant to degradation by proteases, and there is potential for D form peptides to provide greater activity in vivo because of greater stability and a longer retention time.⁹ Several authors reported that replacement of L-peptides with D-isomers for some short AMPs led to higher in vitro antibacterial and antibiofilm activity.^{28,29} In the other cases, D-isomers of short AMPs had activity similar to their L-counterparts, at the same time demonstrating ~30 times lower hemolytic activity.³⁰ Although further work is needed to evaluate the potential impact of replacing L amino acids with D amino acids, the early in vitro studies reported here indicate that gels loaded with ASP-2D perform similarly to those loaded with ASP-2 and that substitution of L amino acids with D amino acids did not adversely impact peptide activity under the conditions tested. This is consistent with the results of MBC testing, which showed similar MBC values for ASP-2 and ASP-2 D. The porcine skin used in this work was sanitized with 70% alcohol before use to avoid contamination of samples with intrinsic skin flora. Sanitization leads to reduced protease activity, and this may be one reason differences between ASP-2 and ASP-2D were not detected in this model. Further testing is needed to understand if the D form peptide would have a greater impact in vivo, where the

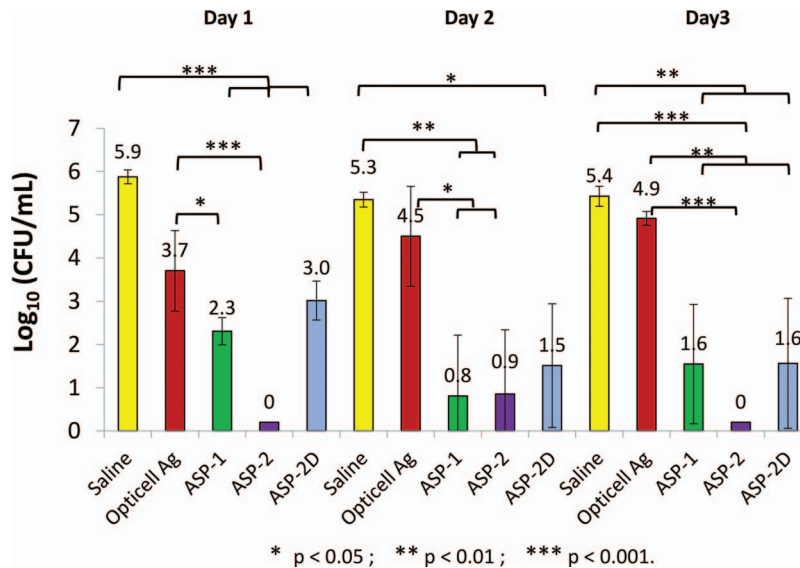


FIGURE 4. Ex vivo efficacy of ASP-1, ASP-2, ASP-2D, and opticell Ag + Gels against biofilms of *pseudomonas aeruginosa* grown on pig skin for 3 days and treated for 3 days. Saline served as a control. N = 3.

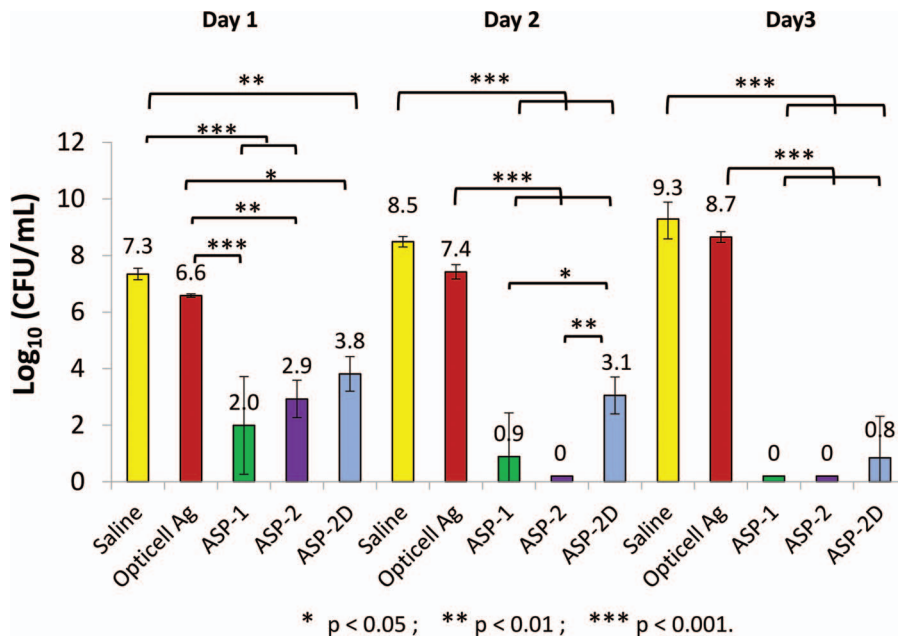


FIGURE 5. Ex vivo efficacy of ASP-1, ASP-2, ASP-2D, and opticell Ag + Gels against biofilms of *acinetobacter baumannii* grown on pig skin for 3 days and treated for 3 days. Saline served as a control. N = 3.

concentration of proteases is expected to be higher, especially in the case of nonhealing and chronic wounds.

Today, combat-related infections are largely managed using systemic antibiotics, including early prophylactic administration, along with debridement, irrigation, and surgical management.³¹ In the case of burn injuries, clinical practice guidelines for US Military personnel recommend the use of topical antimicrobials for infection prevention, including mafenide acetate (MFA), silver sulfadiazine (SSD) creams, and silver-impregnated dressings.^{31,32} All of these treatments

provide broad-spectrum antibacterial activity; however, each one has limitations and there is a need for improved therapies. Although MFA has the ability to penetrate eschar, it is painful on application and pulmonary complications can occur with continuous use on large burns.³³ SSD is less painful on application but does not penetrate eschar.³² Both MFA and SSD require frequent application, typically twice a day, which is labor intensive and uncomfortable for the patient. With the use of SSD and silver dressings, there is some risk of silver staining but more importantly, there is lack

of evidence that these approaches reduce wound infection and some evidence indicates that they may slow healing.³⁴ The antimicrobial peptide-based treatments described herein have potential to overcome some of these limitations. We recently demonstrated that the biocompatibility indices for both ASP-1 and ASP-2 are substantially more favorable than those for SSD and silver, which may allow for better control of bioburden without impairment of healing.³⁵ Pathogens that commonly infect burns are known biofilm formers, and it has been shown that biofilms are present on burns.³⁶ The strong activity of ASP-1 and ASP-2 against biofilms relevant to burn wounds combined with sustained release from a chitosan formulation has potential to allow for better infection control with less frequent treatment application relative to MFA and SSD.

Another area where the antimicrobial peptide-based gels have potential to improve care is in the treatment of musculoskeletal injuries that require fixation. The use of antibiotic-loaded beads and gels has gained acceptance for use in treating these injuries, as an adjunct to systemic antibiotics.³⁷ In complex injuries, the local vascular anatomy is often damaged resulting in less effective delivery of systemic antibiotics to the site of injury. Under these conditions, local antibiotic delivery has been shown to reduce infection risks by enabling high local drug concentrations that would not be possible to achieve by systemic delivery without substantial side effects.^{37–39} The use of fixation devices increases risks for infection because they present artificial surfaces that can harbor biofilm containing bacteria that are recalcitrant to antibiotic treatment because of multiple tolerance mechanisms.^{40–42} Once established, biofilms formed on devices can be extremely difficult to treat, they often require surgical intervention, and in many cases, device removal.⁴³ Current products for local delivery rely on the use of front-line clinical antibiotics and pose a threat for increasing resistance. When delivered via a local depot, the concentration of antibiotic will decrease with distance from the source and depending on the duration of delivery and timing of removal, has potential to expose bacteria to sublethal concentrations.³ Most antibiotics also display limited efficacy against bacteria within biofilms. ASP-1 and ASP-2 kill bacteria rapidly and work by a different mechanism of action relative to most front-line clinical antibiotics. As such, their use would pose little threat for promoting resistance to the limited arsenal of antibiotics. Because they are highly effective against biofilm and biodegradable, the ASP-1 and ASP-2 gels presented in this work may be considered for local delivery to prevent infection of musculoskeletal injuries that require fixation.

The work presented here focused on monospecies biofilms. However, many in vivo biofilms are polymicrobial in nature where microbial synergy can lead to increased growth, antimicrobial tolerance, and infection persistence.^{44,45} It has previously been shown that ASP-1 containing dressings are effective against polymicrobial biofilms comprised of MRSA,

A. baumannii, *K. pneumoniae*, and *P. aeruginosa*, when studied using an in vitro poloxamer biofilm model.³⁵ Future work will include evaluation of both ASP-1 and ASP-2 chitosan gels against mixed species biofilms established on ex vivo pig skin.

CONCLUSIONS

Chitosan-based gels provide an effective delivery platform for the engineered cationic AMPs, ASP-1 and ASP-2. The peptide-loaded gels are highly active against biofilms formed by both gram-positive and gram-negative, drug-resistant bacteria that are relevant to wound infections. Replacement of the L-form peptide (ASP-2) with its protease resistant D-form (ASP-2D) did not result in a significant change in peptide activity. Future work will include studies to evaluate efficacy against mixed species biofilms as well as the safety and efficacy of ASP treatments in a porcine model of burn wound infection.

FUNDING

This work is supported in part by NIH NIAID Grant 1R43AI136195-01A1.

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