Ciprofloxacin-Loaded Keratin Hydrogels Prevent *Pseudomonas aeruginosa* Infection and Support Healing in a Porcine Full-Thickness Excisional Wound

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Objective: Cutaneous wound infection can lead to impaired healing, multiple surgical procedures, and increased hospitalization time. We tested the effectiveness of keratin-based hydrogels (termed “keratose”) loaded with ciprofloxacin to inhibit infection and support healing when topically administered to porcine excision wounds infected with *Pseudomonas aeruginosa*.

Approach: Using a porcine excisional wound model, 10 mm full-thickness wounds were inoculated with 10⁶ colony-forming units of *P. aeruginosa* and treated on days 1 and 3 postinoculation with ciprofloxacin-loaded keratose hydrogels. Bacteria enumeration and wound healing were assessed on days 3, 7, and 11 postinjury.

Results: Ciprofloxacin-loaded keratose hydrogels reduced the amount of *P. aeruginosa* in the wound bed by 99.9% compared with untreated wounds on days 3, 7, and 11 postinjury. Ciprofloxacin-loaded keratose hydrogels displayed decreased wound contraction and reepithelialization at day 7 postinjury. By day 11, wounds treated with ciprofloxacin-keratose hydrogels contained collagen-rich granulation tissue and myofibroblasts. Wounds treated with ciprofloxacin-loaded keratose hydrogels exhibited a transient increase in macrophages in the wound bed at day 7 postinjury that subsided by day 11.

Innovation: Current therapies for wound infection include systemic antibiotics, which could lead to antibiotic resistance, and topical antimicrobial treatments, which require multiple applications and can delay healing. Here, we show that ciprofloxacin-loaded keratose hydrogels inhibit cutaneous wound infection without interfering with key aspects of the healing process including granulation tissue deposition and remodeling.

Conclusions: Ciprofloxacin-loaded keratose hydrogels have the potential to serve as a point-of-injury antibiotic therapy that prevents infection and supports healing following cutaneous injury.

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INTRODUCTION

CUTANEOUS WOUND HEALING is a complex process involving cell signaling, matrix deposition, and remodeling.¹ Under ideal circumstances, healing is initiated by a short-lived inflammatory phase, followed by a tissue formatting stage marked by

granulation tissue deposition, neovascularization, and reepithelialization.² Healing culminates in a remodeling stage in which the newly deposited tissue is reorganized to resemble that of uninjured skin.² Several factors, both systemic and local, can affect the rate and

condition in which a wound heals.¹ For example, diabetic patients experience delayed healing due to neuropathy and lack of blood flow to the injured area.³ Similarly, bacterial infection stemming from environmental factors can impair healing by altering the inflammatory phase of wound healing, which in turn can delay the deposition and remodeling of new tissue.¹

Bacterial infections cause healing abnormalities in a variety of wound types including surgical wounds, acute soft tissue wounds, bite wounds, diabetic and pressure ulcers, and burn wounds.⁴ Burn wounds are particularly susceptible to complications stemming from infection, with an estimated 75% of deaths following burns having comorbid infectious complications.^{5,6} Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* are two opportunistic pathogens commonly associated with both surgical wounds and burn wounds.^{4,5,7,8} MRSA and *P. aeruginosa* have been shown to impair healing,^{9–11} in part due to their production of damaging virulence factors including Panton-Valentine leukocidin, staphylococcal protein A, α -hemolysin, exoenzyme S, and exotoxin A.^{12–17} Further, wounds infected with either MRSA or *P. aeruginosa* display increases in both neutrophil accumulation and in the expression levels of proinflammatory cytokines including tumor necrosis factor (TNF)- α , interleukin (IL)-1 α and IL-1 β .^{14,17} Therefore, infection prevention is an important design criterion when developing novel wound care products so as to abrogate the wound healing complications associated with infected wounds.

One potential solution to prevent wound infection is to use tailored biomaterials to slowly release antibiotics. Biomaterials have advantages with respect to drug release that many of the current wound healing products do not have. Keratin proteins derived from human hair make promising biomaterials for wound healing strategies due to their versatility and biocompatibility. Keratin proteins extracted from hair include α -keratins, which are α -helical structural proteins, and smaller, globular proteins, termed γ -keratins.¹⁸ Keratins can be formulated into a variety of dressing materials including foams, sponges, and hydrogels.^{19–22} Specifically, keratin hydrogels are advantageous over alternative dressings because they elicit minimal foreign body responses, possess tunable mechanical properties, and are capable of serving as sustained-release delivery vehicles.^{18,22,23} Additionally, keratins are unique as antibiotic carriers for infection control in that they are susceptible to enzymatic degradation by cutaneous bacteria that produce keratinases.²⁴ Given that mammals possess no natural mechanisms for proteolytic degradation

of keratin, this feature provides a mechanism of tailored antibiotic release kinetics where more infected wounds degrade the carrier faster and release more drug to combat the infection.

Keratin proteins can be extracted via oxidative techniques to yield proteins (termed “keratose”) that form hydrogels composed of physical entanglements but lack covalent disulfide cross-links.^{18,22,25} Compared to materials that release drugs simply via diffusion and are known to display a short burst of antibiotic release, keratose hydrogels have been shown to support the sustained release of the antibiotic ciprofloxacin for >1 week *in vitro*.²³ Further, keratose hydrogels loaded with 2 mg/mL ciprofloxacin inhibit *S. aureus* growth for 23 days *in vitro*.²³ The purpose of this study was to test the ability of ciprofloxacin-loaded keratose hydrogels to inhibit *P. aeruginosa* growth both *in vitro* and *in vivo*. We chose to use a porcine excisional wound model for this study due to the anatomical similarities between pig and human skin and because both heal through granulation tissue deposition and reepithelialization.^{26,27}

CLINICAL PROBLEM ADDRESSED

Several products and approaches are commercially available and clinically used to treat cutaneous wound infections, with the severity of the injury and infection dictating the method of treatment used. A spectrum of options are available for burn wound infections that range from sharp wound debridement and topical antimicrobial agents such as silver dressings; to the administration of systemic antibiotics.⁴ Topical silver dressings, while effective in reducing infection, are often associated with delayed healing and exhibit cytotoxicity toward fibroblasts and keratinocytes.^{28–30} Concerns exist over the routine use of systemic antibiotics due to their marginal effectiveness, poor tissue penetration, and propensity for antibiotic resistance.^{31–34} A great deal of current research and numerous treatments focus on localized antimicrobial therapies, however, these topical antibiotics and antiseptics typically require multiple applications and may delay wound healing.^{28,29} Therefore, the need exists for a topical antibiotic therapy that supports sustained antimicrobial activity, prevents infection, and facilitates timely healing.

MATERIALS AND METHODS

Keratose hydrogel preparation

Keratose was obtained by oxidative extraction of human hair and purified by KeraNetics, LLC using a patented process in a 21CFR820 validated facility

as described previously.^{21,23} For antibiotic solution preparation, ciprofloxacin (Sigma) was dissolved at 20 mg/mL in 0.1 N hydrochloric acid and the pH was adjusted to between 5.0 and 5.1. The 20 mg/mL ciprofloxacin stock was then diluted in sterile water to give final concentrations of 2, 5, 10, or 20 mg/mL. To make the hydrogel dressing, lyophilized keratose (95:5 α : γ keratose ratio) was weighed and hydrated with an aqueous solution containing ciprofloxacin or sterile water (to serve as a keratose only control) to achieve a 15% weight-to-volume ratio. Hydrogels were allowed to equilibrate overnight at 37°C prior to use.

Broth inhibition assay

All bacteria work was performed using *P. aeruginosa* strain PA01, a well characterized wound isolate and widely used laboratory strain.^{35,36} Mueller-Hinton broth (BD) was inoculated with 10 colonies of *P. aeruginosa* and grown at 37°C with agitation until a concentration of $\sim 10^8$ colony-forming units per mL (cfu/mL) was achieved as determined by the optical density. The bacterial suspension was then diluted to a concentration of 10^5 cfu/mL in Mueller-Hinton broth. Two milliliters of the 10^5 cfu/mL broth was added to 5 mL polystyrene round bottom tubes (BD) containing 200 μ L of keratose hydrogels with or without 2 mg/mL ciprofloxacin. Control tubes did not contain any gel. The bacterial suspension was allowed to grow at 37°C with agitation and was sampled and replaced daily. Samples were serially diluted 1–10 and plated onto Mueller-Hinton agar (Remel), incubated at 37°C for 18–24 h, and colonies were counted to determine the number of cfu/mL of broth.

Ciprofloxacin release assays

One hundred microliter aliquots of the keratose hydrogels containing either 0, 2, 5, 10, or 20 mg/mL ciprofloxacin were placed in microcentrifuge tubes, overlaid with 100 μ L phosphate-buffered saline (PBS), and incubated at 37°C. PBS was collected and replaced with fresh PBS at 1.5, 3, 6, and 12 h, and 1, 2, 3, 4, 5, 6, 7, 8, and 9 days. The concentration of ciprofloxacin in the collected samples was quantified using the inherent fluorescence of ciprofloxacin. Samples were excited at 340 nm and emission was detected at 450 nm using a microplate reader. Fluorescence values were converted to ciprofloxacin concentration values using a standard curve generated with each set of samples.

Bacteria preparation for *in vivo* studies

P. aeruginosa was plated onto Mueller-Hinton agar and grown overnight at 37°C. The challenge inoculum was prepared the following day by scraping 10 colonies from the overnight culture into a conical

tube containing 10 mL of Mueller-Hinton broth. Bacteria were grown in suspension at 37°C with agitation until a concentration $>10^8$ cfu/mL was achieved. Dilutions of *P. aeruginosa* were made in sterile saline to achieve a final concentration of 4×10^7 cfu/mL.

Animals

All animal work was approved by the Institutional Animal Care and Use Committee, United States Army Institute of Surgical Research (USAISR) and the United States Army Medical Research and Materiel Command (USAMRMC) Animal Care and Use Review Office. This study has been conducted in compliance with the Animal Welfare Act, implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals. Two female Yorkshire pigs (Midwest Research Swine) weighing between 35 and 42 kg were used in this study. Animals had access to food and water *ad libitum*, and were allowed to acclimate to the facilities for at least 7 days prior to the experiment.

Anesthesia

On the day before anesthesia, animals were fasted and a transdermal fentanyl patch (100 μ g/h) was placed on the ear of the animal. Prior to anesthesia on day 0, the pig was premedicated with glycopyrrolate [0.01 mg/kg, intramuscular (IM) injection] to minimize salivation and bradycardia. Anesthesia was induced with an IM injection of tiletamine-zolazepam (Telazol, 6 mg/kg). Animals were then intubated with an endotracheal tube and placed on an automatic ventilator. Anesthesia was maintained with 1–3% isoflurane in 100% oxygen. On postinjury days 1, 3, 7, and 11, the animal was also briefly anesthetized to treat and/or obtain biopsies. For these follow-ups, animals were sedated with 10–20 mg/kg ketamine IM, and maintained under mask anesthesia (3–5% isoflurane).

Wounding, infection, and treatment

On day 0, anesthetized pigs were clipped and shaved on the back in the wounding area using a straight blade razor. Fifty-four full-thickness excision wounds were made on the dorsum of each pig using a 10 mm biopsy punch (Acuderm). All wounds were located at least 5 cm from the spine and wounds were separated from each other by 3 cm. Sterile gauze was applied to the wounds until bleeding ceased. Wounds were then inoculated with 25 μ L of the 4×10^7 cfu/mL *P. aeruginosa* stock for a final inoculum of 1×10^6 cfu per wound.

On days 1 and 3 postinjury, infected wounds were administered either 200 μ L of keratose hy-

drogels containing 5 or 10 mg/mL ciprofloxacin, keratose hydrogels without antibiotic, or saline (to serve as an untreated control). Following both infection and treatment, each wound was covered with an individual $1.5 \times 1.5 \text{ cm}^2$ piece of Tegaderm (3M) to prevent cross-contamination between wounds. Wounds were further sealed from the environment by bandaging with nonadherent Telfa gauze (Kendall), Elastikon elastic tape (Johnson & Johnson) and an adhesive Ioban dressing (3M).

***In vivo* bacteria quantification**

On days 3, 7, and 11 postinjury, the animal was anesthetized and bandages were removed from a subset of wounds for biopsy collection. Biopsy strips through the wound area were collected for bacteria quantification using a scalpel and cutting down to the fascia. Any intact keratose gel was removed from the excised tissue prior to bacteria quantification. Wound tissue was weighed, homogenized in a known volume of saline, serially diluted at 1–10 to account for wound bacterial loads exceeding 10^8 cfu per gram of tissue, and plated onto *Pseudomonas*-selective agar (Neogen). Plates were incubated for 18–24 h at 37°C prior to counting viable cfu. Data from bacteria quantification studies are presented as the log of cfu per gram of tissue.

Histological processing and analysis

Biopsy strips were collected through the wound area on days 3, 7, and 11 postinjury and were immediately placed in 10% formalin for at least 48 h. Tissue samples were then processed, embedded in paraffin, cut into $6 \mu\text{m}$ thick sections, and mounted onto glass slides. Sections were stained using Masson's Trichrome (Sigma) or immunohistochemistry (IHC). For the MAC387 antibody recognizing Calprotectin, slides underwent enzymatic antigen retrieval with 0.6 U/mL Proteinase K (Sigma; P2308) for 20 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 20 min and nonspecific Immunoglobulin G (IgG) was blocked with 10% horse serum in Hanks' balanced salt solution (HBSS). Tissue sections were then incubated with primary antibodies directed against either α -smooth muscle actin (Abcam; ab7817, mouse monoclonal, 1:100 dilution) or macrophages (MAC387; Abcam; ab22506, mouse monoclonal, 1:100 dilution) overnight at 4°C . Slides were washed with HBSS and treated with a biotinylated anti-mouse (Vector Labs; BA-2000) secondary antibody for 60 min. Immunostaining was completed with a 30 min incubation with Vectastain-RTU Kit solution (Vector Labs; PK-7100) followed by a 5–10 min incubation with ImmPACT Diaminobenzidine (Vector Labs; SK-4105). Slides were then counter-

stained with hematoxylin and dehydrated for cover slipping. Images were obtained using an Axio Scan digital slide scanner (ZEISS).

All wound measurements were made using Masson's Trichrome-stained slides and ImagePro software (Media Cybernetics). Briefly, wound contraction was determined by measuring the distance from one wound edge (indicated by intense, blue collagen staining resembling that of uninjured skin) to the other edge. Percent reepithelialization was determined by measuring the distance migrated by the leading edge of the epithelium from the wound edge toward the wound center. The distance migrated from both wound edges were summed to give a distance of epithelial cell migration per wound. The distance of epithelial migration per wound was then divided by the total distance between wound edges to give a percent reepithelialization for each biopsy. Granulation tissue deposition at the center of the wound was calculated by measuring the distance from the top of the granulation tissue to the top of the subcutaneous fat layer.

Macrophages were quantified using images collected using the $10\times$ objective on an Olympus BX60 microscope and DP71 camera. Three images were obtained within each wound bed exclusive of any epithelium or intact gel at the surface of the wound. The total number of macrophages present in each image was quantified by using ImageJ software. Briefly, the black color threshold in the hue, saturation, and brightness color space was adjusted to 140. The "Analyze Particles" feature was then used to count objects between 50 and 500 pixels², corresponding to $\sim 35\text{--}350 \mu\text{m}^2$. The macrophage counts from the three different images of the same biopsy were then averaged to give a mean value of macrophages per field of view for that wound.

Statistical analysis

All data are presented as a mean value $\pm$ standard error of the mean. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc). All statistical comparisons were made using either a one-way or two-way analysis of variance with a Bonferroni *post-hoc* test to determine significance between treatments. A value less than $p = 0.05$ was considered statistically significant.

RESULTS

Ciprofloxacin-loaded keratose hydrogels inhibit *P. aeruginosa* growth *in vitro*

Here we asked whether ciprofloxacin-loaded keratose hydrogels inhibit *P. aeruginosa* (Gram-negative)

Figure 1. Ciprofloxacin-loaded keratose hydrogels inhibit *P. aeruginosa* growth *in vitro*. Mueller-Hinton broth (10 mL) was inoculated with *P. aeruginosa*, diluted to a concentration of 10^5 colony-forming units (cfu)/mL, and added to 200 μ L of keratose hydrogels with or without 2 mg/mL ciprofloxacin. Broth was sampled and replaced daily, and samples were serially diluted at 1–10 to determine the number of cfu/mL present in the broth. Data are presented as mean log of cfu per mL of media $\pm$ standard error of the mean of three samples per condition and represent one of three individual experiments. *, significant from “No Gel,” $p < 0.05$ (Two-way analysis of variance).

growth *in vitro*. Keratose hydrogels loaded with 2 mg/mL ciprofloxacin completely inhibited bacteria growth for >9 days *in vitro* (Fig. 1). Conversely, keratose hydrogels alone did not possess any antimicrobial activity, and they were completely digested/degraded within 5 days (Fig. 1). These data indicate that ciprofloxacin-loaded keratose hydrogels are capable of inhibiting *P. aeruginosa* growth for a minimum of 9 days *in vitro*.

Ciprofloxacin release profiles are dependent on initial ciprofloxacin concentration

In vivo microenvironments present challenges that may require increased antibiotic release to prevent infection. Thus, we sought to determine whether we could control the amount of antibiotic released from the keratose hydrogel by varying the amount of antibiotic initially loaded within the gel. Keratose hydrogels were loaded with ciprofloxacin at concentrations ranging from 0 to 20 mg/mL. Keratose hydrogels loaded with 20 mg/mL ciprofloxacin released 1.01 ± 0.03 mg of ciprofloxacin within the first 5 days (Fig. 2). Keratose hydrogels loaded with 10 mg/mL ciprofloxacin released 0.63 ± 0.02 mg of ciprofloxacin within 5 days (Fig. 2). Hydrogels loaded with 5 and 2 mg/mL ciprofloxacin released 0.23 ± 0.00 and 0.11 ± 0.00 mg of ciprofloxacin within the first

Figure 2. Keratose hydrogels loaded with increasing ciprofloxacin concentrations display increased ciprofloxacin release. One hundred microliter aliquots of keratose hydrogels containing either 20, 10, 5, 2, or 0 mg/mL ciprofloxacin were placed in microcentrifuge tubes and overlaid with 100 μ L phosphate-buffered saline (PBS). PBS was exchanged and analyzed at regular intervals. Ciprofloxacin was quantified by fluorescence. Data are presented as mean values $\pm$ standard error of the mean of three separate gels and represent one of three individual experiments. *, significant from all other groups, $p < 0.05$ (Two-way analysis of variance).

5 days, respectively. All keratose hydrogels released between 50% and 63% of their total ciprofloxacin within the first 5 days, independent of the initial ciprofloxacin concentration. These data indicate that the amount of ciprofloxacin released from the keratose hydrogels can be controlled in a dose-dependent manner by increasing the initial amount of ciprofloxacin loaded within the hydrogel.

Ciprofloxacin-loaded keratose hydrogels inhibit *P. aeruginosa* growth *in vivo*

To determine *in vivo* efficacy of keratose-ciprofloxacin formulations, full-thickness excision wounds in a pig were infected with *P. aeruginosa* and treated on days 1 and 3 postinjury with keratose hydrogels with or without ciprofloxacin. Untreated wounds contained $\sim 10^6$ cfu of *P. aeruginosa* per gram of tissue on days 3, 7, and 11 (Fig. 3). Treatment with keratose hydrogels loaded with 5 mg/mL ciprofloxacin or 10 mg/mL ciprofloxacin significantly reduced the amount of *P. aeruginosa* in the wound by $>99.9\%$ compared to untreated wounds at days 3, 7, and 11 postinjury (Fig. 3). As expected, keratose hydrogel alone did not possess any antimicrobial activity against *P. aeruginosa* (Fig. 3).

Ciprofloxacin-loaded keratose hydrogels decrease wound contraction and reepithelialization

In addition to preventing infection, it is important that any wound treatment support wound

Figure 3. Ciprofloxacin-loaded keratose hydrogels inhibit *P. aeruginosa* growth in a porcine excisional wound. Full-thickness wounds were infected with *P. aeruginosa* and treated with 200 μ L of keratose hydrogels with or without ciprofloxacin on days 1 and 3 post-injury. Untreated wounds were administered an equal volume of saline. Wound tissue was biopsied on days 3, 7, and 11, homogenized, serially diluted at 1–10, and plated onto *Pseudomonas*-selective agar to determine cfu. Data are presented as mean log of cfu per gram of tissue $\pm$ standard error of the mean of six wounds per condition. *, significant from “Untreated,” $p < 0.05$ (Two-way analysis of variance).

healing in a timely fashion. On day 7 postinjury, untreated and keratose-treated wounds consisted of a fibrin clot that was being displaced by an epithelial layer that was migrating atop granulation tissue (Fig. 4A). Conversely, wounds that were

treated with ciprofloxacin-loaded keratose hydrogels contained areas of intact gel atop the wound bed (Fig. 4A; yellow dotted line).

Following injury, excessive contraction of normal skin could result in painful contractures that restrict movement. On day 7, untreated wound beds were 5.80 ± 0.24 mm wide (Fig. 4B). Similarly, keratose-treated wounds were 6.63 ± 0.47 mm wide (Fig. 4B). Wounds treated with keratose hydrogels loaded with either 5 or 10 mg/mL ciprofloxacin were 7.46 ± 0.67 or 7.51 ± 0.41 mm wide, respectively (Fig. 4B). These data suggest that ciprofloxacin-loaded keratose hydrogels decrease contraction of excisional wounds.

Reepithelialization at days 7 and 11 postinjury was quantified by measuring the distance migrated by the leading edge of the epithelium. There were no differences in reepithelialization between untreated and keratose-treated wounds at day 7 ($74.2 \pm 8.54\%$ and $66.4 \pm 7.60\%$ closed, respectively) or day 11 ($97.8 \pm 2.22\%$ closed and $100 \pm 0.00\%$ closed, respectively) (Fig. 5). However, wounds treated with 5 mg/mL ciprofloxacin-loaded keratose hydrogels were $33.2 \pm 7.60\%$ closed on day 7 and $80.7 \pm 10.6\%$ closed on day 11 (Fig. 5). Similarly, wounds treated with 10 mg/mL ciprofloxacin-loaded keratose hydrogels were $31.9 \pm 5.85\%$ and $90.0 \pm 5.84\%$ closed at days 7 and 11, respectively (Fig. 5). While there was a significant decrease in percent reepithelialization of ciprofloxacin-treated wounds compared with untreated wounds on day 7, these differences were not significant on day 11.

Figure 4. Ciprofloxacin-loaded keratose hydrogels decrease wound contraction. Sections through the wound center on day 7 postinjury were stained with Masson's Trichrome. (A) Representative images of untreated wounds ($n=6$) and wounds treated with keratose only ($n=6$), keratose with 5 mg/mL ciprofloxacin ($n=6$), and keratose with 10 mg/mL ciprofloxacin ($n=5$). The area above the yellow dashed line indicates areas of intact keratose gel. Scale bar = 1 mm. (B) Wound contraction was quantified by measuring the width of the wound bed. Data represent mean wound width $\pm$ standard error of the mean. *, significant from “Untreated,” $p < 0.05$ (One-way analysis of variance).

Figure 5. Ciprofloxacin-loaded keratose hydrogels delay wound reepithelialization. Sections through the wound center on days 7 and 11 postinjury were stained with Masson's Trichrome. Percent wound reepithelialization was quantified by measuring the distance of reepithelialized wound bed and normalizing it to the total width of the wound bed. Data represent mean percent reepithelialization $\pm$ standard error of the mean for untreated wounds ($n=6$) and wounds treated with keratose only ($n=6$), keratose with 5 mg/mL ciprofloxacin ($n=5$), and keratose with 10 mg/mL ciprofloxacin ($n=5$). *, significant from "Untreated," $p < 0.05$ (Two-way analysis of variance).

Ciprofloxacin-loaded keratose hydrogels do not interfere with granulation tissue deposition or remodeling

During the later stages of wound healing, collagenous granulation tissue is deposited and subsequently remodeled by myofibroblasts within the wound space.² Masson's Trichrome-stained tissue sections displayed collagen-rich granulation tissue in all treatment conditions at day 11 postinjury (Fig. 6A). Measurements of granulation tissue thickness from wounds treated with ciprofloxacin-loaded keratose hydrogels and keratose hydrogel alone showed no significant difference at the center of the wound from the untreated wounds (Fig. 6B).

To identify myofibroblasts within the wound space, IHC was performed by using an antibody

Figure 6. Ciprofloxacin-loaded keratose hydrogels support granulation tissue deposition and remodeling. Sections through the center of the wound on day 11 postinjury were stained with (A) Masson's Trichrome or (C) an α -smooth muscle actin antibody (brown). (A, C) Representative images of untreated wounds ($n=6$) and wounds treated with keratose only ($n=6$), keratose with 5 mg/mL ciprofloxacin ($n=5$), and keratose with 10 mg/mL ciprofloxacin ($n=5$). Scale bar = 1 mm. (B) Granulation tissue thickness was quantified by measuring the distance from the top of the granulation tissue to the subcutaneous fat. Data represent mean thickness $\pm$ standard error of the mean. There were no significant differences between any of the groups tested, $p > 0.05$ (One-way analysis of variance).

Figure 7. Macrophage infiltration of keratose-treated wounds. Sections through the center of the wound on days 7 and 11 postinjury were stained with a macrophage-specific antibody (MAC387). **(A)** Representative images of the wound bed on day 7 for untreated wounds ($n=6$) and wounds treated with keratose only ($n=6$), keratose with 5 mg/mL ciprofloxacin ($n=5$), and keratose with 10 mg/mL ciprofloxacin ($n=5$). Scale bar = 100 μ m. **(B)** Macrophages were quantified by using ImageJ. Data represent the mean number of macrophages $\pm$ standard error of the mean per field of view. *, significant from "Untreated," $p < 0.05$ (Two-way analysis of variance).

directed against α -smooth muscle actin. Myofibroblasts were distributed within all wounds at day 11 postinjury (Fig. 6C). There were no differences in the distribution of myofibroblasts between untreated wounds and wounds treated with ciprofloxacin-loaded keratose hydrogels or keratose hydrogel alone. Together, these data suggest that ciprofloxacin-loaded keratose hydrogels do not interfere with granulation tissue deposition or remodeling by day 11.

Wounds treated with ciprofloxacin-loaded keratose hydrogels exhibit transient macrophage infiltration

Macrophages within the wound bed were visualized on days 7 and 11 postinjury (Fig. 7A). On day 7, untreated wounds contained 116 ± 27.1 macrophages per field of view (Fig. 7B). Wounds treated with keratose alone contained 241 ± 48.9 macrophages per field of view (Fig. 7B). Treatment with 5 mg/mL ciprofloxacin- and 10 mg/mL ciprofloxacin-loaded keratose hydrogels resulted in a significant increase in macrophages detected at day 7 (Fig. 7B; 561 ± 91.2 and 422 ± 66.9 macrophages per field of view, respectively). By day 11 postinjury, macrophage levels in the wound space decreased under all treatment conditions, with no significant differences seen between treatments (Fig. 7B).

DISCUSSION

Keratin hydrogels show great promise as natural biomaterial-based wound care products due to their ability to serve as sustained-release delivery vehicles, their minimal foreign body responses, and the fact that they are susceptible to bacterial enzymatic degradation.^{22–24} Here, we show that keratose

hydrogels loaded with ciprofloxacin inhibited *P. aeruginosa* growth both *in vitro* and in a clinically relevant porcine excisional wound. Ciprofloxacin-loaded keratose hydrogels were still present in the wound space by day 7 postinjury and delayed initial wound reepithelialization. However, wounds treated with ciprofloxacin-loaded keratose hydrogels exhibited less wound contraction than untreated wounds and granulation tissue was deposited and being remodeled by day 11 postinjury.

Infected, untreated wounds did not experience any noticeable abnormalities in healing. By day 11, wounds were completely reepithelialized and consisted of collagen-rich granulation tissue. It is generally accepted that infections $>10^5$ cfu per gram of tissue (as seen in these wounds) cause impaired healing.³⁷ Conversely, others have shown that inoculating rodent wounds with *P. aeruginosa* strain PA01 at concentrations $>10^7$ cfu per wound accelerates reepithelialization by triggering an immediate neutrophil response and subsequent TNF- α production.³⁸ The primary objective of this study was to assess the ability of keratose hydrogels with and without ciprofloxacin to inhibit infection in a clinically relevant model. Further, we wanted to ensure that locally delivered keratin-ciprofloxacin formulations did not cause any healing irregularities, as seen with therapies such as silver creams.^{28,29} By day 11 postinjury, ciprofloxacin-loaded keratose hydrogel-treated wounds were void of *P. aeruginosa* infection, were 80% closed, and contained collagen-rich granulation tissue with myofibroblast infiltration.

In vitro, *P. aeruginosa* rapidly degrades keratose hydrogels, whereas hydrogels that contain ciprofloxacin persist for greater than a week. Mammals

are not known to possess keratin-specific degradative enzymes (keratinases). However, bacteria such as *P. aeruginosa* express keratinases, which may lead to enzymatic degradation of the keratose hydrogels.³⁹ We have previously shown a direct correlation between hydrogel degradation and antibiotic release.²³ Together, this suggests a unique mechanism in which the amount of *P. aeruginosa* in the area of the keratose material could increase the rate of hydrogel degradation, thus increasing antibiotic release.²³

Related to this, we observed that keratin hydrogels without ciprofloxacin were readily degraded *in vivo*. However, ciprofloxacin-loaded keratin hydrogels were detected intact, and atop the granulation tissue on day 7. This may be due to the mechanism above where the released antibiotic would reduce bacterial load, thereby reducing the local concentration of keratinase enzymes and subsequent rate of gel degradation. It is also possible that the presence of the ciprofloxacin-loaded keratose hydrogel served as a physical barrier during the early stages of the healing process, decreasing both reepithelialization and wound contraction. The reduced contraction seen with ciprofloxacin-loaded keratose treatment may be in part responsible for the decrease in percent reepithelialization reported in Fig. 5, as percent reepithelialization was calculated as a function of total wound width. Importantly, keratose hydrogels were not detected by day 11. Therefore, while initial wound contraction and reepithelialization are reduced, reepithelialization and granulation tissue deposition are allowed to proceed as usual during the second week of healing.

Macrophages are critical to the inflammatory phase of wound healing, both by removing damaged tissue and cells from the wound bed and by releasing cytokines and growth factors to initiate reparative processes including angiogenesis and granulation tissue formation.⁴⁰ However, sustained macrophage infiltration could be an indicator of prolonged inflammation or could serve as a precursor for scar formation.⁴⁰ Treatment with ciprofloxacin-loaded keratose hydrogels significantly increased the number of macrophages seen in the wound at day 7 postinjury. However, the subsequent decrease seen by day 11 under these same conditions suggests that the macrophage presence is transient, and not an indicator of prolonged inflammation or scar formation. Despite the presence of *P. aeruginosa* in untreated wounds, there were few macrophages detected at days 7 and 11 postinjury. However, it is possible that macrophages were upregulated at earlier time points in untreated wounds. Other groups have shown that

macrophages exposed to keratin proteins produced by crude extraction processes, preferentially differentiate to the M2 anti-inflammatory and reparative phenotype.⁴¹ Though it is tempting to make the argument that the increases of macrophages in ciprofloxacin-loaded keratose hydrogels are due to an increase in M2 macrophages based on this study, there were no increases in macrophages seen in the keratose only-treated wounds. This suggests that the macrophage increase in the ciprofloxacin-loaded keratose hydrogels is unrelated to the keratin used in this study, and it is likely due to the presence of ciprofloxacin in these wounds.

When designing an antimicrobial treatment regimen, careful thought must be given to the type of antibiotic chosen, route of administration, and potential for antibiotic resistance. Common antibiotics are often more potent toward either Gram-negative or Gram-positive bacteria. However, it is unlikely that wounds would be infected with only one microorganism.⁹ Polymicrobial wound infections are prevalent and require antibiotic approaches to clear the wound of all infection. Ciprofloxacin is a broad spectrum antibiotic that has been approved by the U.S. Food and Drug Administration for oral administration and is both safe and effective as an ophthalmic solution.⁴² When administered systemically, ciprofloxacin has been shown to treat cutaneous infection.^{43,44} Ciprofloxacin has also been shown to inhibit both Gram-negative (*P. aeruginosa*) and Gram-positive (*S. aureus*) bacteria growth when loaded into keratose hydrogels.²³ Additionally, *P. aeruginosa* exhibits less resistance toward ciprofloxacin than the commonly administered broad spectrum antibiotic imipenem.^{45,46} Together, these data suggest that ciprofloxacin is a suitable antibiotic to be loaded into keratose hydrogels to treat cutaneous infection.

This study and others are showing the potential advantages of biomaterials as antibiotic-delivery vehicles to prevent infection and support wound healing. Previous work has shown that antibiotic-loaded collagen scaffolds,⁴⁷ composite fibers comprised of polyglyconate and porous poly(DL-lactic-co-glycolic acid) (PDLGA),^{48,49} and electrospun nanofibers consisting of biodegradable poly vinyl alcohol and sodium alginate,⁵⁰ can reduce infection either *in vitro* or *in vivo*. Recently, Albaugh *et al.* combined topical antibiotics with cellulose dressing to fight infection of lower extremity chronic ulcers infected with MRSA.³² However, these wounds required dressing changes every 3 days in addition to sharp debridement.³² Similarly, Jacobsen *et al.* demonstrated the effectiveness of

the broad spectrum antibiotic moxifloxacin to decrease MRSA and *P. aeruginosa* infection in a porcine burn model. However, the antibiotic-gel combination used in this study was applied to the wounds 6 times over a 11-day span.⁵¹ Elsner *et al.* have shown that a porous PDLGA matrix can be loaded with gentamicin to create a sustained release scaffold that inhibits *P. aeruginosa* growth and improves healing outcomes by increasing closure and decreasing contraction in a guinea pig burn model.⁵² A major advantage to keratin hydrogels over these and other sustained-release matrices is the ease with which they are formulated and delivered. Once hydrated with an antibiotic solution, lyophilized keratin powder spontaneously forms an injectable hydrogel that can easily be applied to fill or cover any infected wound. Further, others have shown that keratin biomaterials promote wound closure following thermal burn injury.⁵³ Therefore, ciprofloxacin-loaded keratose hydrogels show great potential for their ability to serve as a point-of-injury antibiotic therapy that prevents infection and supports healing following cutaneous injury.

INNOVATION

Current therapies for wound infection include systemic antibiotics, which could lead to antibiotic resistance, and local antimicrobial treatments, which typically require multiple applications and can delay healing. Here, we show that ciprofloxacin-loaded keratose hydrogels inhibit *P. aeruginosa* growth by >99.9% compared with untreated controls in infected porcine, full-thickness excisional wounds. Wounds treated with ciprofloxacin-loaded keratose hydrogels are 80% reepithelialized by day 11 postinjury, and consist of collagen-rich granulation tissue and myofibroblasts. Taken together, these findings suggest that ciprofloxacin-loaded keratose hydrogels have the potential to both prevent wound infection and support cutaneous wound healing.

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KEY FINDINGS

- Ciprofloxacin-loaded keratose hydrogels inhibit *P. aeruginosa* infection *in vitro* and in a porcine excisional wound.
- Ciprofloxacin-keratose hydrogel treatment does not interfere with granulation tissue deposition or remodeling at day 11 postinjury.
- Wounds treated with ciprofloxacin-keratose hydrogels display a transient increase in macrophage infiltration at day 7 postinjury.

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Drs. Daniel C. Roy, Seth Tomblyn, and Luke R. Burnett are employees of KeraNetics, LLC. No ghostwriters were involved in the writing of this work.

The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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Abbreviations and Acronyms

Cfu = colony-forming units
 HBSS = Hanks' balanced salt solution
 IHC = immunohistochemistry
 IL = interleukin
 IM = intramuscular
 MRSA = Methicillin-resistant
 Staphylococcus aureus
 PBS = phosphate-buffered saline
 PDLGA = poly(DL-lactic-co-glycolic acid)
 TNF = tumor necrosis factor
 USAISR = United States Army Institute
 of Surgical Research
 USAMRMC = United States Army Medical
 Research and Materiel
 Command