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Hydrogel delivery of lysostaphin eliminates orthopedic implant infection by *Staphylococcus aureus* and supports fracture healing

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Orthopedic implant infections are a significant clinical problem, with current therapies limited to surgical debridement and systemic antibiotic regimens. Lysostaphin is a bacteriolytic enzyme with high antistaphylococcal activity. We engineered a lysostaphin-delivering injectable PEG hydrogel to treat *Staphylococcus aureus* infections in bone fractures. The injectable hydrogel formulation adheres to exposed tissue and fracture surfaces, ensuring effective, local delivery of lysostaphin. Lysostaphin encapsulation within this synthetic hydrogel maintained enzyme stability and activity. Lysostaphin-delivering hydrogels exhibited enhanced antibiofilm activity compared with soluble lysostaphin. Lysostaphin-delivering hydrogels eradicated *S. aureus* infection and outperformed prophylactic antibiotic and soluble lysostaphin therapy in a murine model of femur fracture. Analysis of the local inflammatory response to infections treated with lysostaphin-delivering hydrogels revealed indistinguishable differences in cytokine secretion profiles compared with untreated fractures, demonstrating clearance of bacteria and associated inflammation. Importantly, infected fractures treated with lysostaphin-delivering hydrogels fully healed by 5 wk with bone formation and mechanical properties equivalent to those of untreated fractures, whereas fractures treated without the hydrogel carrier were equivalent to untreated infections. Finally, lysostaphin-delivering hydrogels eliminate methicillin-resistant *S. aureus* infections, supporting this therapy as an alternative to antibiotics. These results indicate that lysostaphin-delivering hydrogels effectively eliminate orthopedic *S. aureus* infections while simultaneously supporting fracture repair.

Orthopedic disease and injuries often require biomaterial implant and devices for successful clinical treatment. In 2011, 1.2 million prosthetic joint arthroplasty procedures were performed in the United States, and this number is projected to increase to 3.8 million procedures by 2030 (1). Infection of these devices is a major limitation with ineffective treatment options (2). For example, over 1 million joint prostheses (3) and 6 million fracture-fixation devices (4) are deployed each year, with 2% and 5% of these procedures, respectively, developing infection at an economic cost of over $2 billion (5). In the United States, 112,000 orthopedic device-related infections occur annually, with ~66% of these bacterial infections involving *Staphylococcus* species (6). Fracture fixation devices have infection rates ranging from 1 to 2% for closed fractures and rates as high as 30% for open fractures (7). Orthopedic implant infection occurs through three routes: direct contamination of the implant, infection spreading to the implant from a nearby source, and implant infection due to transient bacteremia, leading to implant colonization. Clinically, implant infections are primarily prevented by administration of antibiotics, the placement of antibiotic-laden bone cements, and the use of minimally invasive surgical techniques (6). Current treatment of orthopedic implant infections is limited to a combination of aggressive surgical debridement, device removal, and long-term systemic antibiotic regimens. Antibiotic treatment can lead to the development of opportunistic infections through perturbations to the gut microbiota (8) and the development of antibiotic resistance (9). Further complicating the scenario is the formation of bacterial biofilms, populations of sessile and slowly dividing bacteria encapsulated within extracellular polymeric substances (10, 11). The biofilm matrix provides significant protection from the host immune system and acts as a diffusion barrier for antibiotics, allowing for bacteria to be resistant to antibiotic concentrations 1,000 times higher than that required to kill the same planktonic strain (12). Bacteria in biofilms can be exposed to subinhibitory antibiotic concentrations, further driving the development of antibiotic resistance (9). As such, current treatment strategies for device-related infections are significantly limited, often requiring one to two revision surgeries.

**Significance**

Orthopedic implant infections require long-term antibiotic therapy and surgical debridement to successfully retain the implant; however, therapeutic failure can lead to implant removal. Here an injectable PEG-based hydrogel that adheres to exposed tissue and fracture surfaces is engineered to deliver the antimicrobial enzyme lysostaphin to infected, implant-fixed, mouse femoral fractures. Lysostaphin encapsulation within the hydrogel enhances enzyme stability while providing enhanced antibiofilm activity and serving as a controlled delivery platform. In a preclinical animal model of orthopedic-implant infection, we show that lysostaphin-delivering hydrogels outperform prophylactic antibiotic therapy and soluble lysostaphin, by eradicating infection while promoting bone repair. Importantly, lysostaphin-delivering hydrogels are effective against antibiotic-resistant infections. This lysostaphin delivery platform could be highly effective at treating and preventing implant infections.


Conflict of interest statement: C.T.J. and A.J.G. are inventors on a patent application filed by the Georgia Tech Research Corp. based on the results in this study.

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Editor's Summary

Orthopedic device-related infections require complex treatments including aggressive surgical debridement, antibiotic therapy, and local delivery of antimicrobial agents such as lysostaphin. This study describes the development of injectable hydrogels that contain lysostaphin and demonstrate effective antibacterial activity against *Staphylococcus aureus* infections, including infections with antibiotic-resistant bacteria. These results indicate that lysostaphin-delivering hydrogels effectively eliminate orthopedic *S. aureus* infections while facilitating bone fracture repair.
and causing significant patient morbidity, at a high economic cost of over $50,000 per case (6).

The widespread emergence of antibiotic-resistant bacteria is a growing public health threat, leading to a postantibiotic era, where current therapies are no longer effective (13). This threat has prompted the investigation of alternative strategies to traditional systemic antibiotic therapy. Lysostaphin is a 27-kDa antimicrobial enzyme with activity specific to Staphylococcus species (14). The enzyme has two domains, a cell wall-targeting domain, responsible for its specificity, and a lytic domain that cleaves the pentaglycine cross-bridges present in the bacterial cell wall (i.e., peptidoglycan) (15). Lysostaphin exhibits activity against antibiotic-resistant Staphylococcus aureus strains, including methicillin-resistant S. aureus (MRSA), vancomycin-intermediate S. aureus, vancomycin-resistant S. aureus (16–18), and Staphylococcus epidermidis (19). Importantly, lysostaphin kills planktonic and quiescent bacteria as well as cells growing in a biofilm (20), in contrast to most antibiotics that require active cellular metabolism to be effective (21).

Widespread resistance to lysostaphin has not been reported from clinical samples (22), although several isolates have been generated in laboratory settings (23, 24). Additionally, the specificity of lysostaphin allows for only offending staphylococcal species to be eliminated, thus preventing adverse effects of gut microbiota perturbation, which is associated with systemic antibiotic therapy. These characteristics make lysostaphin an ideal candidate to treat infections primarily limited to Staphylococcus species and where biofilm formation is often implicated in the disease process (25). Lysostaphin has been delivered topically, systemically, or as microbicides in several small animal models to target S. aureus infections (18, 26–28). In humans, topical application of lysostaphin reduces S. aureus nasal carriage 5 d after treatment with no reported toxicity (29). Additionally, parental administration in a human patient has been reported without major side effects (30).

Despite these attributes, lysostaphin therapy has been severely limited by the lack of effective delivery vehicles. Conjugation of PEG to lysostaphin increases the in vivo half-life of systemically administered enzyme from less than 1 h to up to 24 h, but at the expense of reduced enzymatic activity (31). Biomaterial carriers have focused on surface conjugation of lysostaphin to a material to prevent bacterial colonization (28, 32–34), as opposed to developing enzyme delivery vehicles. Localized delivery of antimicrobial therapeutics allows for higher drug concentrations to be achieved at the infection site over a longer period of time, with a lower risk of toxicity compared with systemic delivery (35, 36). Hydrogels are water-swollen polymer networks that exhibit significant therapeutic versatility for localized protein delivery (37).

We previously engineered injectable PEG-based hydrogels for controlled delivery of protein- and cell-based therapeutics (38–44). In this platform, four-arm PEG macromers functionalized with terminal maleimide groups (PEG-4MAL) that react specifically with thiols are functionalized with cell adhesive peptides and cross-linked into a network using thiolated molecules such as protease-degradable peptides with terminal cysteines. These synthetic hydrogels exhibit significant advantages over other delivery vehicles including well-defined composition and structure, minimal toxicity, stoichiometric incorporation of biomolecules, controlled polymerization kinetics, and nontoxic degradation products that are excreted in the urine (41, 42).

Here, we engineered lysostaphin-delivering injectable hydrogels to treat S. aureus orthopedic infections and support fracture repair (Fig. 1A). We characterized the activity, stability, and release of hydrogel-encapsulated lysostaphin, as well as antimicrobial and antibiofilm performance. The efficacy of lysostaphin-delivering hydrogels was tested in vivo using a murine femur fracture infection model. Bacterial reduction, cytokine profiling, and functional healing were measured to assess the therapeutic potential of lysostaphin-delivering hydrogel therapy. Finally, the antimicrobial efficacy of lysostaphin-delivering hydrogels against antibiotic-resistant bacteria was tested.

**Fig. 1.** Lysostaphin-delivering hydrogel synthesis and characterization. (A) Outline of overall study design. (B) Schematic diagram of lysostaphin encapsulation within protease-degradable PEG-MAL hydrogel and subsequent application to infected femurs, which leads to fracture callus formation and healing. (C) Passive lysostaphin release with one-phase association fit with extra sum of squares F test to compare K values are different. (D) Optical density curves of lysostaphin-laden hydrogels placed in S. aureus UAMS-1 suspensions as a function of incubation time. (E) Lysostaphin activity as measured by the average half-life of the kinetic bacteria reduction assay (SI Appendix, Fig. S3 A–D) at 1, 3, 7, and 14 d after hydrogel polymerization. (F) Protease-triggered release of lysostaphin with one-phase association fit using extra sum of squares F test to compare all K values are different.
Results
Encapsulation of Lysostaphin Within Injectable PEG Hydrogels Maintains Activity. Orthopedic fractures range from simple closed fracture patterns with minimal soft tissue injury to complex open compound fractures with significant muscle injury, making delivery by injection and in situ polymerization desirable features in an antimicrobial delivery system. This property allows for the material to adhere to exposed tissue and fracture surfaces, ensuring local delivery to the injury. We synthesized PEG hydrogels in a one-step reaction by mixing PEG-4MAL macromers with the protease-degradable peptide cross-linker GCRDVPSMGRGDR (VPM) (45) and cell adhesive peptides (e.g., RGDF and GFOGER), which were covalently incorporated into the network by terminal cysteine groups that react specifically with maleimides on the PEG-4MAL macromer (Fig. 1B). Lysostaphin enzyme was physically entrapped within the hydrogel without covalent incorporation onto the polymer network due to the lack of free thiol groups in the protein (46). This injectable format allows for lysostaphin to maintain its activity throughout the hydrogel synthesis process. Sustained release of lysostaphin, both via passive diffusion through the hydrogel network and protease-dependent degradation of the hydrogel, results in lysis of target bacteria and supports bone formation and subsequent fracture repair (Fig. 1B). To assess the effect of hydrogel mesh size on diffusion-dependent release of lysostaphin, we labeled lysostaphin with a fluorescent dye (SI Appendix, Fig. S1) and measured its diffusion out of the hydrogel. Exponential one-phase association curves were then fit to these data. We synthesized hydrogels using different-sized PEG-4MAL macromers (10 kDa and 20 kDa) at 8.0% and 4.0% wt/vol to generate hydrogels with different mesh sizes. These PEG-4MAL macromers are chemically equivalent, except for the arm length, allowing for modulation of the hydrogel mesh size, thereby allowing for control of lysostaphin diffusion from the hydrogel. The 20-kDa hydrogels with a relatively larger mesh size exhibit more rapid lysostaphin release compared with the 10-kDa hydrogels with a tighter mesh structure (Fig. 1C). This result shows that as the mesh size is reduced the rate of diffusion-mediated release of lysostaphin is decreased. Both hydrogel formulations fully released all of the encapsulated lysostaphin within the first 24 h of swelling. Lysostaphin release could be prolonged by further reducing the hydrogel mesh size, or by engineering a free cysteine into the lysostaphin protein, allowing for covalent tethering into the hydrogel. We also assessed the activity of the released lysostaphin after 24 h of swelling by assaying the swelling supernatant for lysostaphin activity, which showed that the released enzyme retained 50% activity after release from the hydrogel (SI Appendix, Fig. S2). To evaluate lysostaphin activity following encapsulation and release, hydrogels were synthesized and placed in a bacterial suspension of S. aureus UAMS-1, a clinical isolate from a pediatric case of osteomyelitis (47), and reduction of bacteria was monitored over time by optical density measurements. In this experiment, no protease was included so lysostaphin is released from the hydrogel only by diffusion. Lysostaphin-containing hydrogels rapidly and completely reduced bacteria levels in suspension (Fig. 1D). Importantly, the rate of bacterial clearance was dependent on the dose of encapsulated lysostaphin. Retention of enzyme activity after hydrogel polymerization is a critical design criterion. We assessed the long-term activity of lysostaphin encapsulated within the hydrogel carrier and compared it to enzyme maintained in solution and fresh, reference lysostaphin. Hydrogels were synthesized with lysostaphin and not swollen to prevent loss of enzyme to directly assess the hydrogel’s capacity to maintain enzyme stability. Enzyme activity was determined by calculating the rate of bacterial killing, as defined by the time required to kill 50% of a UAMS-1 bacterial suspension. The rate of bacterial killing was determined by fully degrading the hydrogels in protease and immediately incubating this product with bacteria, then monitoring the reduction in optical density over the course of 1 h (SI Appendix, Fig. S3). A one-phase decay curve was then fit to these data to obtain the half-life metric. Remarkably, hydrogel encapsulation preserved lysostaphin activity over 14 d when kept at 25 °C compared with soluble unencapsulated lysostaphin, which rapidly degraded (Fig. 1E). There was no difference in lysostaphin activity between hydrogel-encapsulated enzyme throughout 14 d and fresh enzyme.

Bacterial infection often triggers an inflammatory response, including locally elevated protease levels (48). The inclusion of protease-degradable peptide cross-links in lysostaphin-delivering hydrogels allows for lysostaphin to be released on-demand in response to infection and local protease activity. Protease-dependent release of lysostaphin was characterized by monitoring the release of fluorescently labeled lysostaphin from lysostaphin-laden hydrogels (20 kDa, 4.0% wt/vol) exposed to different levels of protease (Fig. 1F). The results show that higher levels of protease cause faster lysostaphin release, indicating protease-responsive release. Importantly, nearly all of the loaded enzyme was released in these assays. Measurement of the mechanical properties of lysostaphin-delivering hydrogels reveals that the addition of lysostaphin does not affect the elastic or viscous properties of the hydrogel, as determined by measuring the storage and loss moduli of the gels, respectively (SI Appendix, Fig. S4).

Encapsulated Lysostaphin Kills Bacteria, Including in Biofilms. Lysostaphin is highly active against both S. aureus and S. epidermidis (20). We examined the bactericidal activity of the enzyme encapsulated in the hydrogel delivery system. Hydrogels were synthesized with different strains of S. aureus (Xen29, UAMS-1, and 46106) or S. epidermidis (IDRL8885, a clinical strain isolated from a prosthetic joint infection) trapped within the hydrogel matrix with or without lysostaphin (SI Appendix, Table S1). The gels were then cultured overnight in bacterial growth media and after 24 h were assayed for viable bacteria. Encapsulated lysostaphin reduced viable bacteria to undetectable levels for all bacterial strains tested (Fig. 2A–D). After confirming that lysostaphin-laden hydrogels are effective against various strains of bacteria, we tested the in vitro cytocompatibility of lysostaphin using human mesenchymal stem cells. We induced human mesenchymal stem cells to differentiate toward an osteogenic lineage and added lysostaphin to the culture media. Lysostaphin had no effects on the osteogenic differentiation of human mesenchymal stem cells as assessed by alkaline phosphatase activity (SI Appendix, Fig. SS4A) and calcium deposition (SI Appendix, Fig. SS B and C), demonstrating that lysostaphin effectively kills staphylococcal species but does not interfere with the osteogenic differentiation of human cells.

Orthopedic implant infections typically involve formation of a bacterial biofilm. The biofilm protects the bacteria from the host immune response and acts as a diffusion barrier for antibiotics, making them particularly difficult to eliminate. We evaluated the antibiofilm activity of lysostaphin-delivering hydrogels. We hypothesized that delivery via the hydrogel carrier would improve the antibiofilm activity of the enzyme compared with soluble enzyme alone based on our observation that encapsulation within the hydrogel prolonged enzyme stability (Fig. 1C). S. aureus strain UAMS-1 is a prolific biofilm former (49). We grew UAMS-1 biofilms for 24 h and then treated them with lysostaphin-laden hydrogels or soluble enzyme. After 18 h of treatment, bacterial reduction was assessed by staining for live bacteria and subsequently imaging the biofilm. Fig. 2E shows representative images of biofilms after treatment. There is a clear lysostaphin dose-dependent reduction in live bacteria for the hydrogel-treated group, which is confirmed by image quantification (Fig. 2F). Comparisons between equivalent concentrations of lysostaphin demonstrate that hydrogel-mediated delivery of lysostaphin significantly reduces bacteria compared with delivery without a carrier (Fig. 2F).
Lysostaphin-Laden Hydrogels Effectively Reduce *S. aureus* Infection of Bone Fractures. Treatment of long bone fractures, such as the femur, often require fixation devices to stabilize the injury, enable healing, and promote return to mobility. However, bacterial infection of devices used to stabilize orthopedic injuries leads to the inability of fractures to heal, characterized by bone resorption, reactive bone formation, implant loosening, and, ultimately, device failure (2). To model this situation in vivo, we developed a mouse model of orthopedic implant infection. In this model, the femur is fractured using a custom three-point bending device; the fracture is then stabilized with a 25-gauge needle placed in the femoral shaft (50), and then a hydrogel is polymerized in situ over the fracture (Fig. 3A). Importantly, the injectable hydrogel formulation adheres to exposed tissue and fracture surfaces, ensuring efficient, local delivery. For mice receiving an infection, bacteria is mixed with the hydrogel components and polymerized in situ over the fracture. We then measure bacterial counts 1 wk after fracture or assess fracture healing 5 wk postimplantation.

Lysostaphin-delivering hydrogels should support fracture repair in the absence of infection to be an acceptable therapy for preventing staphylococcal infections. We hypothesized that the application of a lysostaphin-laden hydrogel would not impair normal (sterile) fracture healing. To test this, femoral fractures were treated with a sterile, lysostaphin-delivering hydrogel or left untreated. No bacteria were detected in this experiment. After 5 wk, femora were explanted and analyzed by microcomputed tomography (μCT), mechanical testing, and histology to evaluate fracture repair. μCT reconstructions revealed no gross morphologic differences in the fracture callus (SI Appendix, Fig. S6A). Similarly, no differences in fracture callus volume (P = 0.26, SI Appendix, Fig. S6B) or mechanical strength (P = 0.94, SI Appendix, Fig. S6C) of the repaired femora were detected between untreated and lysostaphin-delivering hydrogel-treated mice. Histological staining with H&E for tissue morphology and safranin-O and fast green (Safr-O/FG) for cartilage also showed no gross differences in healing between sterile control fractures and fractures treated with lysostaphin-delivering hydrogels (SI Appendix, Fig. S6D).

We next evaluated the ability of lysostaphin-delivering hydrogels to prevent *S. aureus* infection in vivo using the murine infected femur fracture model. Mouse femora were fractured and hydrogels containing methicillin-sensitive *S. aureus* UAMS-1 were polymerized in situ over the fracture to induce infection with or without lysostaphin. We included a group treated with soluble lysostaphin (no hydrogel) as well as an antibiotic prophylaxis group that received a single injection of oxacillin (100 mg/kg) preoperatively to directly compare the lysostaphin-delivering hydrogel to antibiotic-based therapy. Seven days postoperation, animals were killed, tissue was separated, and viable bacteria were enumerated in the tissue surrounding the femur (Fig. 3B), the femur (Fig. 3C), and the stabilization needle (Fig. 3D). Untreated infected controls had high numbers of recovered bacteria, indicating a persistent infection. Mice receiving prophylactic oxacillin therapy before the operation also had elevated bacteria counts, demonstrating that prophylactic antibiotic treatment did not prevent bacterial infection in this model. This result is consistent with clinical experience with systemic antibiotic regimens that do not consistently eliminate *S. aureus* infections (51). Treatment with lysostaphin-laden hydrogels significantly reduced the amount of recovered bacteria compared with the infection-only control and infections receiving systemic oxacillin. Importantly, the lysostaphin-laden hydrogels reduced bacteria counts to the same level as sterile controls. For the soluble lysostaphin-treated group, variable levels of bacteria were recovered and there was no difference in bacteria counts between this group and the infection-only control or the oxacillin-treated group, demonstrating that the hydrogel delivery vehicle is necessary to effectively treat these infections. Histologic analysis demonstrated significant leukocyte infiltration for infection-only and oxacillin-treated fractures compared with sterile fractures and fractures treated with lysostaphin-laden hydrogels (Fig. 3E). Safr-O/FG staining indicated poor collagen staining at the fracture site, characteristic of inhibited fracture repair, for the infection-only and oxacillin-treated fractures. In contrast, the lysostaphin-laden hydrogel-treated samples showed collagen deposition at the fracture site, consistent with the sterile control. Gram-positive bacteria were detected in the infection-only control and oxacillin-treated groups, showing that the infection persisted over the course of the experiment. We note that the sample shown from the highly variable soluble lysostaphin-treated group corresponds to a sample with no bacterial counts and as expected has features comparable to the sterile control and lysostaphin-delivering hydrogel sample. No gram-positive bacteria were detected in the lysostaphin-treated and sterile groups. Taken together, these results demonstrate that lysostaphin-delivering hydrogels eliminate *S. aureus* infections of bone fractures and outperform systemic antibiotic and direct delivery of soluble lysostaphin.

Lysostaphin Delivery to Infections Restores a Sterile Inflammatory Environment. Bone healing is characterized by three primary phases. The inflammatory phase is the initial step in the healing process, lasting approximately 1 wk, and is followed by the remodeling phase over the next 4–6 wk where the fracture callus is formed and mineralized. The final remodeling phase occurs...
over the next 3–6 mo, leading to complete fracture healing (52). This initial inflammatory phase is critical to successful fracture repair. A major concern of bactericidal therapy is an elevated and sustained inflammatory response to bacterial degradation products that negatively affects healing responses (10). Lysostaphin catalytically degrades the bacterial cell wall, leading to cell lysis and subsequent release of bacterial debris; the release of these bacterial products could cause a significant inflammatory response. To analyze this inflammatory response and assess the safety profile of lysostaphin therapy, we treated fractures with UAMS-1–infected hydrogels containing lysostaphin or empty hydrogels. Sterile hydrogels devoid of S. aureus UAMS-1 containing lysostaphin were included as the healing control. One week postinfection, we performed a multiplexed cytokine array assay on explanted tissue. Hierarchal cluster analysis using the Ward method revealed clear separation between the infected scaffolds and the sterile and lysostaphin-hydrogel–treated gels (Fig. 4A). Multivariate ANOVA with a sum combination across all cytokines demonstrated significant differences ($P < 0.001$) between the infection-only group and the sterile and lysostaphin-gel treated fractures (Fig. 4B). Importantly, significant overlap was observed between the sterile and lysostaphin-laden hydrogel groups, suggesting that the lysostaphin-delivering hydrogels restored the local inflammatory environment to a sterile state. Elevated levels of G-CSF, IL-1a, IL-1b, IL-6, KC, IP-10, MIP-1a, MIP-1b, and MIP-2 (Fig. 4 C–K), important cytokines in the inflammatory response to infection, were present in the UAMS-1–only group compared with the sterile control and lysostaphin-hydrogel–treated infections. No differences were detected between the sterile and lysostaphin hydrogel-treated groups for any of the cytokines. These results provide further evidence that lysostaphin-delivering hydrogels clear infecting bacteria and restore an inflammatory environment that could support fracture repair.

**Fig. 3.** Lysostaphin-delivering hydrogels eliminate bacteria in infected fractures. (A) Schematic diagram of mouse femur infection model. Quantification of S. aureus UAMS-1 recovered from the (B) tissue surrounding the femur, (C) femur bone, and (D) stabilization needle 7 d postfracture. Dashed line indicates detection limit. $*P < 0.05$, $**P < 0.01$. (E) Histological sections of femurs 7 d postfracture stained for H&E, Saf-O/FG, and Gram. Black arrows indicate gram-positive bacteria. Kruskal–Wallis test with Dunn’s multiple comparisons test. Ox., oxacillin; Sol., soluble. Mean ± SD n = 4–8, compilation of four independent experiments.
the infection-only control ($P < 0.001$), and these high torque values were equivalent to those for the sterile control group (Fig. 5D). Bacterial counts performed at 5 wk postfracture (SI Appendix, Fig. S8) indicate that the infection persists and remains stable in the untreated infected controls over the 5-wk experimental time course and that lysostaphin-delivering hydrogel-treated mice remain sterile, confirming the results at 7 d postsurgery. Together, these data demonstrate that lysostaphin-delivering hydrogels clear the bacterial infection and support effective and complete fracture repair. No differences were observed between infected fractures treated with soluble lysostaphin and infection-only controls, again showing poor outcomes for lysostaphin therapy without the hydrogel carrier. Histologic sections of infected fractures treated with lysostaphin-delivering hydrogels and sterile fractures show no notable morphological differences (Fig. 5E), providing further support of successful fracture repair. The infection-only group shows significant leukocytic infiltrate on H&E staining and the presence of gram-positive bacteria (black arrows). The sample selected from the soluble lysostaphin treatment group shows the presence of gram-positive bacteria, indicating the sample was infected. This result clearly demonstrates persistent infection and inflammation for infected fractures that were not treated with lysostaphin-laden hydrogel. Finally, as an initial assessment of the potential systemic toxicity of the lysostaphin-delivering hydrogels, serum liver enzyme tests and liver and kidney histology were performed at 5 wk postinfection. Liver enzyme testing revealed all values within the normal range (SI Appendix, Fig. S9), and no gross histological changes (SI Appendix, Fig. S10) were observed in the liver or kidney, supporting the safety of lysostaphin-delivering hydrogel therapy.

**Lysostaphin-Delivering Hydrogels Clear MRSA Bone Infections.** To test the efficacy of lysostaphin-delivery hydrogels to combat antibiotic-resistant bacteria, we measured bacterial numbers of the persistent infection at 7 d postoperation with the MRSA strain USA300. Mouse femora were fractured and hydrogels containing MRSA were placed at the fracture sites to induce infection. These hydrogels contained lysostaphin or were left empty as controls. Sterile lysostaphin-delivering hydrogels were used as controls. Consistent with the results obtained with UAMS-1, lysostaphin-delivering hydrogels significantly reduced MRSA bacteria counts compared with the infection-only control for the tissue surrounding the femur (Fig. 6A), the femur (Fig. 6B), and the stabilization needle (Fig. 6C). Notably, all of the...
lysostaphin hydrogel-treated mice had undetectable levels of bacteria. This shows that lysostaphin-delivering hydrogels eradicate antibiotic-resistant bacteria.

Discussion

Orthopedic implant infections are a significant clinical problem and lack effective therapies. Current interventions are limited to long-term systemic antibiotics, surgical debridement, and implant removal. Alternative therapy with antimicrobial enzymes such as lysostaphin provides for effective killing of specific bacterial species; however, these approaches are limited by the lack of suitable delivery vehicles. Here, we engineered synthetic hydrogels to deliver active lysostaphin to infected bone fractures to clear the infection and promote fracture healing. The synthetic hydrogel delivery vehicle maintained lysostaphin activity over 14 d and controlled the release of active enzyme via passive and protease-triggered mechanisms. These hydrogels displayed high activity against various strains of \textit{S. aureus}, as well as a methicillin-resistant \textit{S. epidermidis} clinical isolate from a prosthetic joint infection in vitro. Importantly, lysostaphin delivery via the hydrogel carrier outperformed soluble enzyme when treating biofilms, most likely due to the sustained release of active lysostaphin.

In a murine model of fracture infection, lysostaphin-delivering hydrogels cleared the infections and supported fracture repair, with bone formation and mechanical properties equivalent to those of uninfected fractures. Lysostaphin-delivering hydrogels restored the local inflammatory environment to that of sterile fractures at 7 d. In contrast, infected fractures treated with either prophylactic antibiotics or soluble lysostaphin showed no differences in bacterial levels and impaired healing compared with the infection controls. Notably, delivery of lysostaphin with this hydrogel carrier significantly reduced MRSA infections in this fracture model. Finally, no signs of liver toxicity or histologic changes to the liver or kidneys were observed for mice treated with lysostaphin-delivering hydrogels at 5 wk. Taken together, these results show that hydrogel-mediated delivery of lysostaphin eliminates fracture infections, including antibiotic-resistant strains, allowing for the endogenous fracture repair mechanisms to progress and healing to occur.

Biomaterial strategies to deliver active lysostaphin have primarily focused on surface functionalization, either by passive adsorption (27, 53), covalent tethering (33, 34), or impregnation within a coating (28, 54). This is an effective way to reduce bacteria at the material surface but may not be practical for settings where infection is already established, or not localized to a material surface (e.g., surrounding tissue). Our injectable formulation allows for in situ polymerization of the hydrogel at the fracture site, adhering to the exposed tissue and fracture surfaces, which is an important feature for treating complex fractures. We demonstrate that lysostaphin-delivering hydrogels have greater antibiofilm activity compared with soluble lysostaphin. This effect may be attributed to the enhanced enzyme stability and higher concentrations of lysostaphin localized to the biofilm achieved using the hydrogel carrier. Together, these material properties could allow for a broader application of lysostaphin-delivering hydrogels to treat other types of staphylococcal infections.

We demonstrate that lysostaphin-delivering hydrogels are effective at reducing infection for both clinical osteomyelitis and MRSA isolates in vivo. Importantly, we did not observe any lysostaphin resistance in our in vivo studies as \textit{S. aureus} was effectively eradicated.

![Fig. 5. Lysostaphin-delivering hydrogels enable fracture healing. (A) \(\mu\)CT reconstructions of the fracture callus 5 wk postoperation. (Scale bar, 1 mm.) Quantification of \(\mu\)CT reconstructions showing the (B) fracture callus volume and (C) bone volume within the fracture callus at 5 wk. (D) Mechanical strength of femurs as assessed by ex vivo torsion to failure testing. \(*P < 0.05, **P < 0.01, ***P < 0.001. (E) H&E, Saf-O/FG, and Gram staining of femurs. Black arrows indicate gram-positive bacteria. Kruskal–Wallis test with Dunn’s multiple comparisons test. Mean ± SD, \(n = 6–8\), compilation of two independent experiments.](image)

![Fig. 6. Lysostaphin-laden hydrogels clear MRSA infections. Quantification of MRSA USA300 recovered from the (A) tissue surrounding the femur, (B) femur, and (C) stabilization needle at 7 d postfracture. Dashed line indicates detection limit. ANOVA with Tukey’s post hoc test for A and B. Kruskal–Wallis test with Dunn’s multiple comparisons test for C. Mean ± SD, \(n = 3–4\). \(*P < 0.05, ****P < 0.0001.](image)
However, the development of resistance to the treatment is still a potential concern. Interestingly, lysostaphin exhibits synergy with β-lactam antibiotics, and lysostaphin exposure can sensitize strains to the antibiotic they have resistance against (19, 24). Therefore, lysostaphin delivery together with antibiotics could broaden the activity of the enzyme, while also reducing the chance of resistance developing. A lysostaphin-based approach offers several advantages over traditional small-molecule antibiotics. The bacteriocin-specific nature of lysostaphin provides specific targeting of the infecting organism, which may reduce complications associated with disrupting commensal bacteria (8). Small-molecule antibiotics primarily function through disruption of bacterial metabolic processes, leading to growth inhibition and death. This reliance limits drug activity against biofilm bacteria. The enzymatic nature of lysostaphin sidesteps this requirement, as the enzyme directly disrupts and kills bacteria. This feature contributes to the low concentrations (nanograms per milliliter range) required to kill bacteria compared with antibiotics (micrograms per milliliter range) (20), thereby reducing the amount of enzyme needed to provide bactericidal activity to the infection site.

Lysostaphin-delivering hydrogel treatment assists in restoring a prohealing inflammatory environment, supported by the absence of differences in cytokine secretion profiles compared with the sterile control. We attribute this effect to the kinetics of bacterial debris clearance by inflammatory cells, which is complete by 7 d after treatment. This result also supports the translation of lysostaphin therapy, as rapid bacterial killing and clearance are critical features for materials designed to treat infections. Importantly, lysostaphin administration with the hydrogel carrier eradicates the infection while supporting fracture healing as assessed by both μCT imaging and mechanical testing. The protease-degradable nature of the hydrogel carrier, in addition to protease-triggered release of lysostaphin, allows for host cells to degrade the hydrogel during repair, resulting in replacement of the gel with repair tissue. This is in contrast to nondegradable scaffolds that are either never removed or only removed at revision surgery once the infection is cleared. A concern with lysostaphin use is the development of neutralizing antibodies. Indeed, several studies have reported antibody development (29, 31, 55), but bacteriolytic activity was preserved in rabbits immunized to lysostaphin before therapy (55). Additionally, deimmunization of lysostaphin by removing protein recognized by T-cell epitopes reduces the likelihood of antibody formation (56, 57), which could eliminate concerns of systemic immune response to therapeutic delivery. We found that one of five mice treated with lysostaphin-delivering hydrogels tested positive for antilysozyme IgG antibodies 5 wk after treatment, while none of the sterile or infected lysostaphin-free mice tested positive. However, preexposure serum was not tested for existing IgG titers, making it difficult to definitively conclude that the lysostaphin-delivering hydrogel generated an immune response. Furthermore, concerns over the development of neutralizing antibodies against lysostaphin are minimal for the bone repair application presented here as it would be exceedingly rare for a patient to have multiple independent infected or open fractures requiring lysostaphin therapy in a lifetime.

The present application focused on a biomaterial to specifically reduce S. aureus infections using lysostaphin. This technology could be further enhanced by broadening the antimicrobial spectrum to target other relevant pathogens in osteomyelitis cases, such as other coagulase-negative Staphylococcus species, *Pseudomonas*, and *Enterococcus* (6). Broadening the bacterial targets of the material will increase its utility as an effective prophylactic (58, 59). It will also be important to evaluate the ability to treat established biofilms in vivo (60). Species-specific antimicrobial therapies with activity toward bacteria growing in biofilm will help to successfully treat these complicated infections with reduced side effects to patients, such as disruption of the gut microbiota. Finally, this strategy will need to be evaluated in larger animal models for safety and efficacy to further assess its clinical potential.

**Materials and Methods**

**Bacteria Strains and Culture.** The bacteria strains used in these studies were UAMS-1 [ATCC 49230 (47)], USA-300 [ATCC BAA-1556 (61)], Xen29 [PerkinElmer (62)], 46,106 (CDC Clinical and Environmental Microbiology Branch Culture Collection), and IDRL-8883 [clinical isolate (63)] and are compiled in **SI Appendix**, Table S1. All strains were cultured on tryptic soy agar (TSA) plates (BD Diagnostics) at 37 °C unless otherwise specified.

**Preparation of Lysostaphin-Delivering Hydrogels.** Twenty-kilodalton PEG-4MAL macromer (Lysan Bio) was mixed with recombinant lysostaphin protein (AMBI Products LLC) in 100 mM MES buffer, pH 5.5-6.0. Hydrogels were then cross-linked in a one-step reaction by combining PEG-lysostaphin with either the GFOGER peptide, GGYGGP(GPP)/GFOGER(GPP)/GPP (New England Peptide), or the RGD linker, GCRDVPMSMRGGDRCG (GenScript), and the bacterial suspension. Bacterial suspensions were prepared by picking individual colonies of bacteria grown on a TSA plate overnight and suspending them in Dulbecco's PBS supplemented with calcium and magnesium (PBS) to an optical density of 0.20 at 600 nm (MicroScan Turbidity Meter; Siemens) and then diluting this suspension 100-fold. The viable count for all bacterial inocula was determined by plate count on TSA medium. Unless otherwise noted the hydrogels were 4.0% wt/vol 20-kDa PEG-4MAL, 1 mM GFOGER, and 424 U/mL lysostaphin. The amount of PVM cross-linker added was determined stoichiometrically by matching the remaining malimide groups after accounting for GFOGER or RGD incorporation. After mixing, the hydrogels were allowed to gel for 15 min in a humidified incubator at 37 °C and 5.0% CO2 for in vitro studies or polymerized over the fracture for in vivo studies.

**Lysostaphin Activity and Stability Assays.** Lysostaphin was encapsulated within 25-μL sterile hydrogels (4.0% wt/vol 20-kDa PEG-MAL, 1 mM RGD, VPM, and 424 U/mL lysostaphin). The soluble lysostaphin group was 424 U/mL lysostaphin in an equivalent buffer to the hydrogel formulation in 25 μL aliquots. The reference lysostaphin group was prepared fresh from frozen at each time point. At 1, 3, 7, and 14 d, samples were incubated in 50 μL of 730 U/mL collagenase for 1 h at 37 °C and then 50 μL of each sample was assessed for activity by incubating with 150 μL UAMS-1 inoculum. The inoculum was prepared by culturing UAMS-1 overnight in brain heart infusion (BHI) broth with shaking at 37 °C, washing three times in 200 mM Tris-HCl, pH 8.0, by centrifugation, and adjusting the optical density to 0.25 to 0.60 nm (MicroScan Turbidity Meter; Siemens). Changes in optical density at 590 nm were measured using a HTS 7000 Plus plate reader (PerkinElmer) every minute for 1 h at 35 °C.

**Lysostaphin Release from PEG Hydrogels.** Amine groups on lysostaphin were fluorescently tagged using an AlexaFluor 488 dye conjugated to a 2-kDa PEG linker functionalized with an NHS ester (Nanoic). The reaction was performed in 100 mM NaHCO3 buffer at pH of 8.3 at room temperature for 1 h with continuous mixing in the dark. Excess dye was removed from labeled protein using an AKTA Pure 25 (GE Healthcare) in combination with a Superdex 75 increase size-exclusion column (GE Healthcare) using PBS as the running buffer, at 4 °C. Labeled lysostaphin was incorporated in the hydrogel conditions tested: 4.0% wt/vol 20-kDa PEG-4MAL, 1 mM RGD, VPM and 8.0% wt/vol 10-kDa PEG-4MAL, 1 mM RGD, VPM. For the diffusion release study, hydrogels were polymerized, swollen in PBS, and incubated statically at 37 °C and 5.0% CO2. For the protease-triggered release studies, 4.0% wt/vol 20-kDa PEG-4MAL, 1 mM RGD, VPM hydrogels were swollen in PBS supplemented with 2 U/mL 10 U/mL collagenase type 1 (Worthington) and incubated shaking at 200 rpm, 37 °C, and 5.0% CO2. At each time point, the supernatant was sampled and read (488/530 excitation/emission) on a Synergy H4 (BioTek) plate reader. The measured fluorescence values were normalized to the fluorescence of PEG-4MAL/lyostaphin mixtures of the respective hydrogel condition.

**Released Lysostaphin Activity Assay.** Hydrogels were swollen for 24 h, after which the swelling supernatant was assayed for enzymatic activity. A detailed protocol is available in **SI Appendix**, SI Materials and Methods.

**Measurement of Hydrogel Mechanical Properties.** The mechanical properties of the hydrogels were measured using a stress-controlled rheometer. A detailed protocol is available in **SI Appendix**, SI Materials and Methods.

**Human Mesenchymal Stem Cell Differentiation Potential and Calcium Deposition.** Bone marrow-derived human mesenchymal stem cells were cultured in osteogenic differentiation media supplemented with lysostaphin. Alkaline phosphatase and calcium deposition were measured. A detailed protocol is available in **SI Appendix**, SI Materials and Methods.
Antibiofilm Activity of Lysostaphin Hydrogels. Biofilms were grown by inoculating 500 μL of BHI supplemented with 1% glucose (64) in a 48-well tissue culture plate with 20 μL of UAMS-1 cells picked from a TSA plate and suspended in PBS to an optical density to 0.20 measured at 600 nm. The biofilm was cultured 24 h at 37 °C statically. Viable bacteria were enumerated by washing the hydrogels three times in PBS and detecting them in 365 U/mL collagenase Type 1 (Worthington) for 1 h. The degraded gels were serially diluted in PBS and 10 μL of each dilution was plated on TSA and grown overnight at 37 °C. Colony-forming units (cfu) were then enumerated.

Histology of Tissue Samples. At the designated time point, mice were killed by CO2 inhalation. Blood was collected via cardiac puncture and a comprehensive blood chemistry panel was performed by Anatech Diagnostics on the serum samples.

Recovery of Bacteria from Tissue Samples. The indicated bacterial strain was grown overnight on TSA at 37 °C. Bacterial cells were suspended in sterile PBS to an optical density to 0.20 measured at 600 nm (Microscan Turbidity Meter; Siemens). This suspension was then diluted 100x in 100 mM MES buffer and used as the inoculum. Hydrogels were synthesized (4.0% wt/vol 20-kDa PEG-MAL, 1 mM RGD, VPM, 424 μM lysostaphin), inoculated with the diluted bacterial culture, and incubated overnight in 25% trypsin soy broth at 37 °C statically. Viable bacteria were enumerated by washing the hydrogels three times in PBS and detecting them in 365 U/mL collagenase Type 1 (Worthington) for 1 h. The degraded gels were serially diluted in PBS and 10 μL of each dilution was plated on TSA and grown overnight at 37 °C. Colony-forming units (cfu) were then enumerated.

μCT and Mechanical Testing of Femurs. Five weeks postoperatively, mice were killed by CO2 inhalation. The femur was dissected and the needle was removed. The femur was placed in gauze soaked in 0.9% wt/vol saline and frozen at −20 °C until further analysis. Samples were thawed under running deionized water and imaged using the μCT50 (Scanco Medical) at 55 kVp and 145 μA with a 0.5-mm filter and 300-ms integration time to achieve a 10-μm voxel size. Three-dimensional reconstructions were generated by segmenting the fracture callus from cortical bone and applying a Gaussian filter (sigma = 0.8, support = 1) and threshold value equivalent to 50% of intact cortical bone (50). Immediately after imaging, samples were mounted in potting blocks filled with Wood’s metal and torsion to failure was assessed with an MRTG-0.2NM force transducer (Interface) interfaced with an ELF 3200 (Bose) mechanical testing system running WinTest7. A continuous ramp function of 3°/s was applied and the highest recorded torque value was reported. Femurs not able to be tested due to a lack of mechanical integrity were assigned a value of 0.

Dot Blot for Antilysoptaphin Antibody Generation. A dot blot assay on serum samples was performed to detect host-antibody generation against lysosta- phin. A detailed protocol is available in SI Appendix, SI Materials and Methods.

Liver Enzyme Testing. Five weeks postfemur surgery, animals were killed by CO2 inhalation. Blood was collected via cardiac puncture and a comprehensive blood chemistry panel was performed by Anatech Diagnostics on the serum samples.

In Vivo Cytokine Analysis. One week postoperatively, mice were killed via CO2 inhalation. The femur was dissected and the fracture site with surrounding tissue was removed and placed in radioimmunoprecipitation assay buffer samples. Samples were frozen and placed on ice. Samples were sonicated for 10 s and debris was removed by centrifugation. The supernatant was passed through a 0.45-μm filter and snap-frozen in liquid nitrogen, and stored at −80 °C until analysis. A Milliplex 25-plex mouse cytokine kit (Millipore Sigma) was used per the manufacturer's instructions to assay for tissue concentrations of G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IFN-α, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α. Samples with measurements below the detection limit of the assay were reported as the detection threshold. Similarly, samples with values greater than the standard curve were reported as the maximum. All cytokines were normalized to the total protein content of the individual sample, which was determined using a bicinchoninic acid assay kit (Pierce by Thermo Fisher) per the manufacturer’s instructions.

Statistics. Individual data points are plotted with a line representing the mean ± the standard error bars indicating the SD of the mean. Statistical significance (P < 0.05) was determined using the Student’s t test to evaluate differences between multivariate groups with a Tukey post hoc test or a Hold-Sidak comparison between preselected groups, or a Kruskal–Wallis test with Dunn's post hoc test for nonparametric data. One-phase association curves were fit to the release data and an extra sum of squares F test as used to compare that K values were different. All calculations were performed using Prism (GraphPad). The multivariate analysis of multiplex cytokine data were performed using JMP Pro-13. Multivariate ANOVA with a sum combination was used to compare across cytokines. A two-way ANOVA with a Bonferroni correction was used to make comparisons between groups for individual cytokines with Prism (GraphPad).

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