

β -Lactamase-Degradable Biomaterials for the Controlled Release of Therapeutics

By

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science in Biomedical Engineering at Brown University.

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3. Dykstra, H., Fisk, C., **LaRose, C.**, Walhart, A., Meng, X., Zhao, G., and Wu, N.* Mouse long-chain Acyl-CoA synthetase 1 is active as a monomer. *Arch Biochem Biophys* 2021. [doi.org/10.1016/j.abb.2021.108773]
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5. Travis, J., # **LaRose, C.**, # and LaDuca, R.* Control of dimensionality and topology in nickel and cobalt coordination polymers containing adamantane-based dicarboxylate and long-spanning dipyridyl ligands. *Inorg Chim Acta*. 2017. [doi.org/10.1016/j.ica.2016.10.043]
6. **LaRose, C.** and LaDuca, R.* Effect of pyridyl donor disposition and ligand flexibility on dimensionality in luminescent and nitrobenzene-detecting cadmium adamantanedicarboxylate coordination polymers. *Inorg Chim Acta*. 2016. [doi.org/10.1016/j.ica.2016.07.027]

CONFERENCE ABSTRACTS

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Table of Contents

Curriculum Vitae	4
Acknowledgements	7
Abstract	11
Chapter 1: Introduction	12
1.1 The Antibiotic Crisis and its Global Impact	12
1.2 Passive Drug Delivery Approaches for Bacterial Infection Prevention and Treatment	15
1.3 Smart Antibacterial Drug Delivery Systems	19
1.4 Our Approach: Bacteria-Degradable Drug Delivery Systems for On-Demand Infection Control	20
Chapter 2: Methods	22
2.0 Materials and Instrumentation	22
2.1 Synthesis of Compound 1	24
2.2 Synthesis of Compound 2	25
2.3 Synthesis of the Responsive Crosslinker (Compound 3)	25
2.4 Equilibrium Swelling and Rehydration of Hydrogels	26
2.5 Hydrogel Compression Testing	27
2.6 Examining Hydrogel Crosslinking Efficiency	27
2.7 Hydrogel Fabrication	28
2.8 Examining Hydrogel Response in β -Lactamase Enzyme Solutions	28
2.9 Modeling Responsive Hydrogel Degradation Kinetics	29
2.10 Examining In Vitro and Ex Vivo Hydrogel Response to Bacteria	30
2.11 Examining Responsive Hydrogel Cytocompatibility and Hemolytic Activity	31
2.12 Statistical Analyses	33
Chapter 3: Results and Discussion	34
3.1 Fabrication and Characterization of Hydrogels	34
3.2 Hydrogel Degradation and Nanoparticle Release in β -Lactamases	39
3.3 Effect of Hydrogel Polymer Concentration on Degradation	42
3.4 Hydrogel Degradation and Nanoparticle Release in β -Lactamase-Producing Bacteria	44
3.5 Cytocompatibility and Hemolytic Activity of β -Lactamase-Degraded Responsive Hydrogels	48
Chapter 4: Conclusions and Scientific Impact	51
Chapter 5: Future Directions	53
Appendices	64
Bibliography	82

Table of Tables

Table 1. Examples of naturally derived and synthetic antibiotics.....	13
Table 2. Physical properties of bacteria-responsive and non-responsive hydrogels.....	38
Table 3. Bacteria strains tested with bacteria-responsive and non-responsive hydrogels.....	44

Table of Figures

Figure 1. The mechanism of hydrolysis of β -lactam antibiotics by bacterial β -lactamase enzymes.....	14
Figure 2. Hydrolysis of a cephalosporin prodrug.....	21
Figure 3. Physical Properties of bacteria-responsive hydrogels.....	37
Figure 4. Effect of encapsulated nanoparticle diameter on β -lactamase-triggered hydrogel degradation and nanoparticle release from <i>P. aeruginosa</i>	40
Figure 5. Effect of encapsulated nanoparticle diameter on hydrogel degradation and nanoparticle release in β -lactamases from <i>E. cloacae</i>	41
Figure 6. Modeling hydrogel mass loss over time assuming surface erosion of hydrogels in β -lactamases from <i>P. aeruginosa</i>	43
Figure 7. Responsive hydrogel degradation by β -lactamase-producing bacteria on agar.....	46
Figure 8. Responsive hydrogel degradation by β -lactamase-producing bacteria on <i>ex vivo</i> porcine skin.....	48
Figure 9. Cytocompatibility of hydrogel degradation products.....	50
Figure 10. Pilot studies on the <i>in situ</i> formation of bacteria-responsive nanogels.....	56
Figure 11. Synthesis of a bacteria-responsive vancomycin conjugate.....	58
 Scheme 1. Schematic representation of β -lactamase-responsive and non-responsive hydrogels.....	 35

Abstract

Antibiotic resistance and the emergence of multidrug resistant bacteria poses a great public health threat and complicates treatment of infections. One of the most impactful mechanisms of bacterial resistance is the production of β -lactamase enzymes; these enzymes are produced by a broad range of bacteria and hydrolyze the β -lactam ring in commonly prescribed antibiotics, thus thwarting their efficacy. In this work, we developed hydrogels that macroscopically degrade in the presence of β -lactamases and β -lactamase-producing bacteria to serve as a platform for bacteria-triggered drug delivery. Within the hydrogel backbone is a β -lactamase-cleavable crosslinker which was functionalized on both ends with maleimides that undergo Michael-type addition with thiol-terminated, multiarm poly(ethylene glycol) macromers. The responsive behavior of these hydrogels was demonstrated *in vitro* in solutions of β -lactamases and β -lactamase-producing bacteria and in an *ex vivo* infected porcine skin model. To model controlled release of encapsulated cargo, fluorescent polystyrene nanoparticles were incorporated into the hydrogel. We observed highly linearly correlated degradation and nanoparticle release kinetics, thus demonstrating a bacteria-responsive, controlled release mechanism of our hydrogel platform technology. Non-responsive hydrogels lacking the β -lactam crosslinker remained stable in β -lactamases and β -lactamase-producing bacteria and exhibited no nanoparticle release. Altogether, these bacteria-responsive hydrogels have the potential to be used for site-specific, on-demand delivery of encapsulated antibacterial therapeutics to provide effective treatment, reduce harmful off-site toxicity, and potentially mitigate further antibiotic resistance development.

Chapter 1: Introduction

1.1 The Antibiotic Crisis and its Global Impact

Over the past half century, countless lives have been saved and many deaths have been prevented through the use of antibiotics, particularly naturally derived antibiotics.¹ The origin of naturally occurring antibiotics was attributed to an accidental discovery of penicillin in 1928; a groundbreaking event that changed the field of medicine. Penicillin was discovered by observing the inhibition of *Staphylococcus* bacterial growth on a laboratory culture plate due to the presence of an unknown mold.^{2,3} A little over a decade later, scientists were able to successfully extract and isolate penicillin from the mold and use it clinically for treating individuals presenting microbial infection. Penicillin quickly gained attention for its ability to effectively kill and protect humans and animals against pathogenic *Staphylococcus* and *Streptococcus* species.³ This groundbreaking discovery was one of the key factors that led to the “golden age” of antibiotic drug discovery research.

The golden era of antibiotic drug discovery research in the mid-1900s led to the rapid identification of over 20 classes of naturally produced antibiotics originating from bacterial and fungal sources (Table 1). Synthetic antibiotics were also developed, owing inspiration to synthesis processes of naturally derived products (i.e., azoles).⁴ The excitement surrounding antibiotic drug discovery research soon came to a head as

scientists were unable to continue identifying novel classes of antibiotics, which later proved to be a major hurdle in drug discovery research and one that is still a threat today.

Table 1. Examples of bacterial, fungal, and synthetically produced antibiotics discovered during the golden age of antibiotic discovery. Adapted from a prior report.⁴

Bacteria	Fungal	Synthetic
Carbapenems	Penicillins	Azoles
Mupirocin	Cephalosporins	Quinolones
Glycopeptides	Fusidic acid	Nitrofurans
Tetracyclines	Pleuromutilins	Sulfonamides

Alongside this, there was a drastic overuse of antibiotics which quickly gave rise to widespread antibiotic resistance. Antibiotic resistance is the generalized term for a microbe's ability to evade killing by antibiotics.⁵ In light of these issues, research then began focusing on developing antibiotic derivatives, mostly through chemical modification of existing antibiotics as a means to expand the antibiotic pipeline and potentially improve efficacy against resistant bacteria.⁶ Though great strides have been made with the development of these derivatives, many have shown short-term potency and eventually succumb to the mechanisms of resistance bacteria possess.⁵

Both gram-negative and gram-positive bacteria possess mechanisms to negate antibiotics' effects. In example, many bacteria have the capability to enzymatically modify antibiotics by altering functional groups or even enzymatically break down antibiotics.⁷ β -lactamases are one of the most notable enzymatic drivers of resistance; these enzymes are produced by a range of bacteria and specifically target the β -lactam ring in commonly used

antibiotics (i.e., Penicillin, carbapenems, etc).⁸ Their mechanism of action relies on initial complexation of the antibiotic in the active site of the β -lactamase enzyme leading to hydrolysis of the amide bond in the β -lactam ring (Figure 1).⁹ The hydrolysis of the β -lactam ring renders the antibiotic ineffective as the active component of β -lactam antibiotics is the ring itself. β -lactam antibiotics have been the largest group of antibiotics prescribed and the resistance against them remains a critical challenge to overcome.⁷

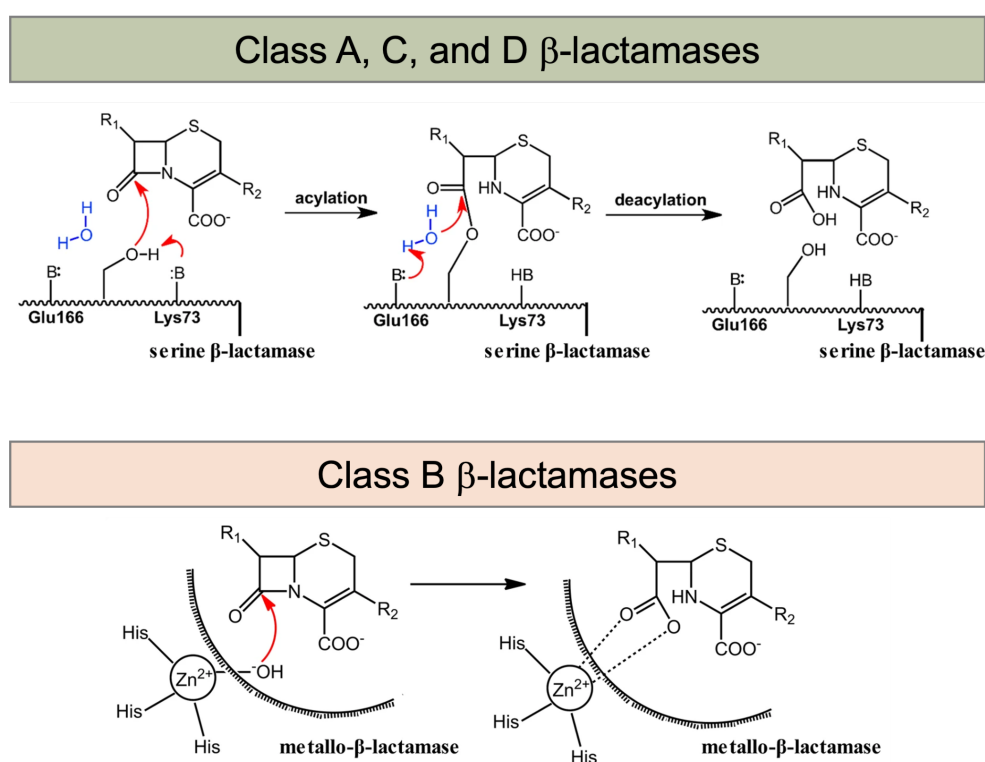


Figure 1. The mechanism of hydrolysis of β -lactam antibiotics by bacterial β -lactamase enzymes. Adapted from a previous report.⁹

Bacteria have also adapted mechanisms to actively remove antibiotics utilizing efflux pumps once it detects the presence of antibiotics.⁷ Efflux pumps are primarily porins that span the cell membrane to actively remove a drug or antibiotic that has entered into the bacterial cytoplasm.¹⁰ The last main mechanism of resistance bacteria possess is the

ability to modify an antibiotic target. In example, certain bacteria have developed mutations in genes (i.e., *mprF* gene) which alter the surface charge and prevent an antibiotic from targeting the cell membrane.¹⁰

The threat of bacterial resistance is an ongoing battle and continues to affect the lives of millions of individuals. In the US alone, it is estimated that nearly 2.8 million drug-resistant infections occur annually with a mortality rate of roughly 1.25%, or 35,000 deaths.¹¹ These numbers are projected to increase more than 100-fold if no action is taken to mitigate overuse and misuse of antibiotics and other antimicrobial agents.¹² In addition to properly prescribing antibiotics, it is also critical to tightly control the administration to the site of infection as a means to reduce toxicity and resistance development potential.¹³ Altogether, the stagnant development of new antibiotics and focus on developing antibiotic derivatives underscores the need for alternative methods and therapies to effectively control drug-resistant infections.¹⁴

1.2 Passive Drug Delivery Approaches for Bacterial Infection Prevention and Treatment

Bacterial infections are highly prevalent worldwide and with the continued development of antibiotic resistance, are becoming more deadly. Current treatment approaches for individuals with an infection commonly include oral or intravenous antibiotics depending on the location and severity of infection.^{15,16} Oftentimes, antibiotics will be prescribed without confirmatory testing, thus exacerbating antibiotic resistance development since pathogenic bacteria are continuously gaining exposure and can evolve to become more resistant or multidrug resistant.¹⁷ Additionally, this misuse and overuse of antibiotics can

have other harmful effects including off-site toxicity to healthy mammalian cells, cause allergic reactions, or even eliminate healthy bacteria making way for pathogenic bacteria to colonize and flourish.¹⁸

The Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have deemed many resistant and multidrug resistant bacteria as urgent public health threats and warn that the existing antibiotic pipeline may not be able to withstand the continuous rise in antibiotic resistance.¹⁹ Antibiotic stewardship programs and more stringent guidelines on proper antibiotic prescribing have been implemented by these organizations as means to slow resistance development, but this is only one part of the battle. To preserve the efficacy and longevity of existing antibiotics, controlled drug delivery approaches are necessary. Passive drug delivery systems that deliver antibiotic(s) locally, to the site of infection, can improve efficacy and mitigate off-site toxicity to non-pathogenic bacteria and mammalian cells.²⁰ Many novel antibacterial drug delivery systems have been developed to treat a range of infections at various sites in the body. Common antibacterial drug delivery platforms include nanoparticles, surface coatings, and wound dressings.

Nanoparticles

Nanoparticles such as polymeric nanoparticles, lipid vesicles or liposomes, and inorganic nanoparticles (i.e., metal or ceramic) serve as antibiotic delivery systems that possess many advantages over free antibiotic.²¹ These nanoparticles can encapsulate multiple types of antibiotics to aid in localizing delivery to reduce off-site toxicity, provide

protection from the host immune response, and increase stability of the encapsulated therapeutic(s).²² Polymeric nanoparticles that encapsulate free antibiotic(s) are one of the most notable advances in drug delivery research. Biologically inert polymers, such as poly(ethylene glycol), are commonly used to synthesize nanoparticles that trap free antibiotic inside the nanoparticle.²³ These nanoparticles could then be administered for systemic infection (i.e., sepsis)²⁴ or delivered to the lung via an inhaler to treat pulmonary infections, for example.²⁵ Similar nanoparticles have been developed for treating a range of infections using other materials including inorganic silica^{26,27} or lipids.²⁸ The development of nanoparticles as antibiotic drug carriers has proved to be a significant achievement in the fight against antibiotic resistance.

Surface Coatings

Surface coatings that act as drug delivery vehicles have been important developments for preventing and eradicating infection caused by surgical intervention or the use of indwelling or implantable medical devices. With invasive techniques comes risk for infection whether it is during the invasive procedure or following completion of the procedure. In the United States, surgical site infections occur in roughly 200,000-400,000 patients each year and nearly 3% of individuals who get a surgical site infection will die as a consequence.²⁹ Coinciding with this is the risk of surgical site infections progressing to implanted or indwelling medical device-related infection, which can lead to severe consequences such as revision surgery, removal of hardware, and surgical debridement of the infection site.³⁰ Common indwelling and implantable medical devices include catheters, vascular assist devices, and prosthetic joints. These devices are highly prone to

bacterial colonization because they can often become contaminated during the procedure or surgery and have desirable material properties for bacterial adhesion and growth.³¹ As many of the devices listed above are meant for long-term use, it is critical to prevent infection and preserve the longevity of the device to improve patient outcomes. Surface coatings that passively elute antibiotic(s) and prevent bacterial adhesion have shown great promise for mitigating indwelling and implantable medical device-related infection.³²

Wound Dressings

Wound dressings that can deliver drugs are also important developments in medicine especially for treating and preventing chronic wounds.¹⁵ It is estimated that nearly 2% of the United States population is affected by chronic wounds which are highly prone to infection.³³ Infected wound sites are commonly treated by administering antibiotics to a patient and preventing the wound site from contamination using traditional gauzes or cotton wound dressings/bandages.¹⁵ Antibiotic-loaded ointments and wound dressings are also commonly used for preventing or treating infection in chronic wounds as they provide localized antibiotic delivery, hydration (in the case of ointments), gaseous exchange, and enhancement of autolytic debridement (for ointments).³⁴ Advancements in wound dressing technology have centered around the development of hydrogels which are highly hydrated polymers that are physically or chemically crosslinked into three-dimensional structures designed to provide longer-lasting wound treatment.³⁵ These hydrogels can be loaded with antibiotics and other therapeutic cargo such as

antibiotic-loaded nanoparticles that can passively diffuse out to treat infection and aid in wound healing.

Passive drug delivery systems like the free antibiotic-loaded nanoparticles, surface coatings, and wound dressings described previously have shown great promise for treatment of a range of infections. However, the passive nature of these systems poses a threat for continued antibiotic resistance development.¹⁴ These systems uncontrollably release antibiotic(s) and do not specifically target bacteria which has been shown to exacerbate resistance. Passive release of antibiotic(s) has also been shown to have harmful effects on healthy bacteria and normal cells. By controlling the release of antibiotic(s) to only when bacteria are present, resistance development can be slowed and off-site toxicity can be significantly reduced.¹⁴

1.3 Smart Antibacterial Drug Delivery Systems

The need for more controlled antibacterial drug delivery has led to the development of “smart” drug delivery systems. Smart antibacterial drug delivery systems commonly employ exogenous or endogenous stimuli for triggered release of therapeutics. Triggers such as light, temperature, ultrasound, and magnetic or electric fields have been explored for site-specific drug delivery.³⁶ However, many exogenous stimuli have drawbacks as the target infection site becomes more internal. For example, if light or ultrasound are used to induce drug delivery, transmittance of these triggers can become skewed or ineffective.³⁷ Smart drug delivery systems that use endogenous stimuli have aided in overcoming these drawbacks^{38,39} and have been employed in a wider range of

applications. Endogenous triggers such as pH,^{40,41} bacterial toxins,⁴² and enzymes have been extensively used to design self-defensive drug delivery systems.⁴³ Bacteria can produce lactic and acetic acid, causing a decrease in local pH at infection sites,^{43,44} which makes pH-responsive biomaterials and drug delivery systems an attractive research area. However, decreases in local pH can occur naturally from injury or non-infectious diseases (i.e., wounds or tumors)^{45,46} and contribute to non-specificity, especially in the case of systemic drug delivery. To combat non-specific delivery, research has shifted focus to developing biomaterials or delivery systems that respond to bacterial enzymes such as hyaluronidases,^{47–49} proteases,⁵⁰ phosphatases,⁵¹ and lipases.^{52,53} However, many of these bacterial enzymes have mammalian analogs that operate similarly,⁵⁴ thus negating the specificity towards bacteria. β -lactamases are enzymes produced only by bacteria⁵⁵ and provide promise for developing truly bacteria-specific, responsive antibacterial treatments.

1.4 Our Approach: Bacteria-Degradable Drug Delivery Systems for On-Demand Infection Control

β -lactamase enzymes are produced by both gram-negative and positive bacteria and are one of the leading causes of antibiotic resistance;⁵⁶ they hydrolyze the β -lactam ring present in many highly prescribed β -lactam antibiotics (i.e., penicillins, carbapenems, and cephalosporins), rendering them ineffective.⁵⁷ The mechanism by which cephalosporins get hydrolyzed by β -lactamases is particularly interesting because this hydrolysis leads to release of the 3'-C position leaving group (Figure 2).⁵⁸ This mechanism has been explored for development of β -lactamase-responsive prodrugs for the release of

antibiotics^{59–61} or chromogenic molecules⁶² for treatment and detection of bacterial infection, respectively.

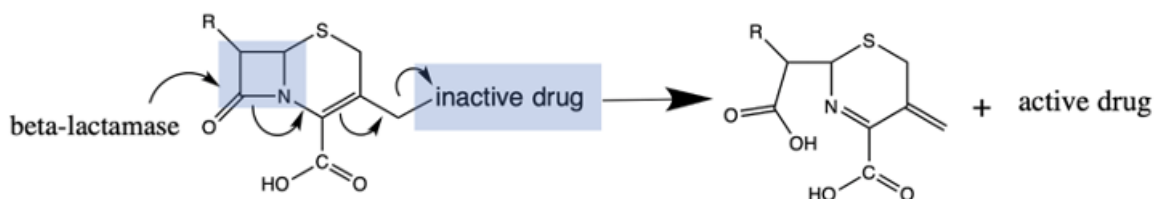


Figure 2. Hydrolysis of a cephalosporin prodrug leading to expulsion of the drug at the 3'-C position.

We have exploited this prodrug approach to develop hydrogels that macroscopically degrade in the presence of β -lactamases or β -lactamase-producing bacteria specifically. Our hydrogels undergo cleavage of a β -lactamase-labile crosslinker, which is incorporated into the backbone of the hydrogel. These hydrogels are designed to elicit a bacteria-specific drug delivery response with either encapsulated nanoparticles or tethered therapeutics upon hydrogel degradation. Encapsulating free antibiotic would negate the bacteria-specific response, as they are small molecules that can passively diffuse out of the hydrogel.⁶³ Altogether, our bacteria-responsive hydrogels that incorporate either antibacterial nanoparticles or tethered responsive antibiotic moieties offer promise for on-demand treatment of infections, reduction in harmful off-site effects, and potentially reduced susceptibility to antibiotic resistance.

Chapter 2: Methods

Chapter 2 is based on a previously published original article:

Alkekhia, D., **LaRose, C.**, and Shukla, A. “ β -Lactamase-Responsive Hydrogel Drug Delivery Platform for Bacteria-Triggered Cargo Release”, *ACS Applied Materials & Interfaces*, 2022, 14 (24): 27538-27550.

2.0 Materials and Instrumentation

7-amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (ACLE) was purchased from AK Scientific (Union City, CA). 3-maleimidopropionic acid was purchased from TCI Chemicals (Tokyo, Japan). 4-aminothiophenol (ATP), triethylamine (TEA), N-methylmorpholine (NMM), anhydrous dichloromethane (DCM), anhydrous dimethylformamide (DMF), hexanes, ethyl acetate (EtAc), dimethyl sulfoxide (DMSO), trifluoroacetic acid (TFA), anisole, diethyl ether, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), N,N-diisopropylethylamine (DIPEA), thin layer chromatography (TLC) Silica Gel 60 on glass plates, β -lactamases from *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*, Fluorometric Thiol Quantitation Kit, phosphate buffered saline (PBS), calcium chloride dihydrate, Triton X-100, and 4-arm-PEG-thiol (20 kDa) were acquired from Millipore Sigma (St. Louis, MO). 1,11-bismaleimido-triethyleneglycol (mal-PEG-mal, 494.5 Da) was purchased from BroadPharm (San Diego, CA). FluoSpheresTM carboxylate-modified fluorescent polystyrene nanoparticles (25, 50, and 100 nm diameter), silica gel, tryptic soy broth (TSB), and SYLGARDTM 184 silicone

elastomer kit were purchased from Thermo Fisher Scientific (Waltham, MA). Bacto agar and BD BBL™ Cefinase™ paper discs were purchased from BD (Franklin Lakes, NJ). Deuterated acetone (acetone-d₆) and chloroform (CDCl₃) were obtained from Cambridge Isotope Laboratories (Andover, MA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (Grand Island, NY). Penicillin-streptomycin was purchased from Caisson Laboratories (Smithfield, UT). Endothelial Cell Growth Medium-2 (EGM-2) BulletKit was purchased from Lonza (Basel, Switzerland). Cell Counting Kit-8 (CCK-8) was obtained from ApexBio (Houston, TX). Single donor human red blood cells (HRBCs) were obtained from Innovative Research (Novi, MI). *Staphylococcus aureus* 25923, *Bacillus cereus* 13061, *Pseudomonas aeruginosa* 27853, *Enterobacter cloacae* 13047, mouse embryonic fibroblasts (NIH 3T3), and human umbilical vein endothelial cells (HUVEC) were purchased from American Type Culture Collection (ATCC, Manassas, VA). *Klebsiella pneumoniae* 1603 and 3834 were generously donated by Dr. Louis Rice at Rhode Island Hospital (Providence, RI). Cleaned, shaven, and frozen porcine skin tissue was obtained from a butcher shop (Providence, RI). Ultra-high purity nitrogen gas (99.999% N₂) was obtained from Corp Brothers, Inc. (Providence, RI). Ultrapure deionized water (18.2 MΩ·cm, Millipore Sigma, Billerica, MA) was utilized in all experiments. Room temperature (RT) referred to in this article was ~23°C.

Proton nuclear magnetic resonance (¹H-NMR) was recorded on a Bruker DRX Avance 400 MHz spectrometer and reported with chemical shifts stated as δ in units of parts per million (ppm) relative to CDCl₃ (7.26 ppm) or acetone-d₆ (2.05 ppm). High resolution

mass spectrometry (HRMS) electrospray ionization (ESI) was conducted on an Agilent 6530 liquid chromatograph tandem mass spectrometer (LC-MS). Fluorescence spectroscopy was performed using a BioTek Cytation 3 microplate reader (Winooski, VT) using 96-well black plates (Thermo Fisher Scientific, Waltham, MA). Confocal images of fluorescent polystyrene nanoparticle-loaded hydrogels were captured using a Nikon Eclipse Ti-A1R microscope (Tokyo, Japan) equipped with a water immersion objective (25 $\times$, NA 1.1, WD 2 mm), a 561 nm laser, 570 - 620 nm filter, and NIS Elements software. Uniaxial compression tests were performed using an Instron 5942 universal testing system (Instron, MA) at RT with a crosshead speed of 0.1 mm/s and a load cell of 500 N to obtain elastic moduli.

2.1 Synthesis of Compound 1

The core of the β -lactamase-cleavable crosslinker, compound 1, was synthesized based on previous reports.⁶⁴ ACLE (1.48 mmol) was dissolved in anhydrous DCM (20 mL) and stirred on ice under N₂. TEA (2.8 mmol) was slowly added to the ACLE mixture over 20 min. NMM (1.8 mmol) and ATP (2.4 mmol) were added sequentially thereafter. The reaction was stirred on ice and monitored by TLC (30% EtAc/70% DCM). After 3 h, the solvent was removed by rotary evaporation, and the product was purified using column chromatography (silica gel, 20% to 80% gradient of EtAc in DCM) to yield compound 1 (~65% yield). HRMS-ESI: m/z calculated for C₂₂H₂₃N₃O₄S₂⁺ [M+H]⁺: 458.1129; found: 458.1199. ¹H-NMR (Appendix A, Figure S2, 400 MHz, CDCl₃) δ (ppm): 7.31 (d, J= 8.5, 2H), 7.15 (d, J= 8.5, 2H), 6.88 (d, J= 8.5, 2H), 6.52 (d, J= 8.6, 2H), 5.10 - 4.94 (dd, J₁ = 11.9 Hz, J₂ = 43.1 Hz, 2H), 4.83 (d, J= 4.9, 1H), 4.65 (d, J= 4.9, 1H), 4.19 -

3.51 (dd, $J_1 = 13.3$ Hz, $J_2 = 245.2$ Hz, 2H), 3.80 (s, 3H), 3.72 - 3.27 (dd, $J_1 = 17.9$ Hz, $J_2 = 143.8$ Hz, 2H).

2.2 Synthesis of Compound 2

To synthesize the protected mal- β -lactam-mal (compound 2), compound 1 (0.22 mmol), 3-maleimidopropionic acid (0.76 mmol), and HATU (0.92 mmol) were dissolved in anhydrous DMF (3 mL). The mixture was stirred for 15 min under N_2 at RT before adding DIPEA (1.3 mmol); the reaction was allowed to proceed for an additional 75 min and was monitored by TLC (80% EtAc/20% DCM). The crude reaction was partitioned between DCM and water, washed twice with water and once with brine, before it was dried over sodium sulfate, filtered, and concentrated via rotary evaporation. The product was purified by flash chromatography (silica gel, 40 to 80% gradient of EtAc in DCM) then dried by rotary evaporation to yield compound 2 (~80% yield). HRMS-ESI: m/z calculated for $C_{36}H_{33}N_5O_{10}S_2^+$ $[M+H]^+$: 760.1678; found: 760.1746. 1H -NMR (Appendix A, Figure S3, 400 MHz, acetone- d_6) δ (ppm): 9.34 (s, 1H), 8.02 (d, $J = 8.5$, 1H), 7.59 (d, $J = 8.7$, 2H), 7.35 - 7.28 (dd, $J_1 = 8.6$ Hz, $J_2 = 12.7$ Hz, 4H), 6.91 (d, $J = 8.7$, 2H), 6.85 (d, $J = 5.5$, 4H), 5.71 - 5.66 (dd, $J_1 = 4.7$ Hz, $J_2 =$ Hz, 1H), 5.06 - 4.88 (dd, $J_1 = 12.0$ Hz, $J_2 = 49.2$ Hz, 2H), 5.03 (d, $J = 4.8$, 1H), 4.29 (d, $J = 13.3$ Hz, 1H), 3.86 - 3.72 (m, 9H), 3.51 (d, $J = 17.8$ Hz, 1H), 2.71 - 2.60 (m, 4H).

2.3 Synthesis of the Responsive Crosslinker (Compound 3)

Compound 2 (0.16 mmol) was deprotected in a solution of 1:1:5 (v/v/v) TFA:anisole:DCM (42 mL) under N_2 on ice for 1.5 h and monitored by TLC (silica gel, 100% EtAc). Solvents were evaporated via rotary evaporation. The deprotected

crosslinker, compound 3, was dissolved in acetone, precipitated in cold diethyl ether three times, and then centrifuged and dried via rotary evaporation (~54% yield). HRMS-ESI: m/z calculated for $C_{28}H_{25}N_5O_9S_2^+$ $[M+H]^+$: 640.1083; found: 640.1152. 1H -NMR (Appendix A, Figure S4, 400 MHz, acetone- d_6) δ (ppm): 9.34 (s, 1H), 8.02 (d, $J = 8.5$, 1H), 7.57 (d, $J = 8.6$, 2H), 7.37 (d, $J = 8.6$, 2H), 6.85 (d, $J = 5$, 4H), 5.71 - 5.66 (dd, $J_1 = 4.7$ Hz, $J_2 = 9$ Hz, 1H), 5.03 (d, $J = 4.8$, 1H), 4.28 - 3.89 (dd, $J_1 = 13.3$ Hz, $J_2 = 136.6$ Hz, 2H), 3.84 - 3.69 (m, 5H), 3.51 (d, $J = 17.7$ Hz, 1H), 2.70 - 2.61 (m, 4H).

2.4 Equilibrium Swelling and Rehydration of Hydrogels

For swelling experiments, 5, 10, and 15% (w/v) hydrogels fabricated with and without 100 nm polystyrene nanoparticles were swollen by incubation in 2 mL of 1× PBS (pH 7.4) at 37°C with shaking (100 rpm) for 48 h, followed by measuring their wet mass, M_w . The swollen gels were then lyophilized at RT for 72 h, and the dry mass was measured, M_d . The equilibrium swelling ratio, Q_s , was calculated using Equation 1, and then used in estimation of the average mesh size.^{65–67} The same methods were used in estimating overall hydrogel mesh size for hydrogels fabricated with and without nanoparticles, due to the extremely low volume fraction of nanoparticles within the hydrogels (~0.097 % (v/v)).

$$Q_s = \frac{M_w - M_d}{M_d} \quad (1)$$

The rehydration ability of lyophilized hydrogels stored at RT for 9 months was also evaluated, specifically for 5 and 15% (w/v) hydrogels fabricated with and without 100 nm polystyrene nanoparticles. These hydrogels were rehydrated in 2 mL of 1× PBS (pH

7.4) at 37°C with shaking (100 rpm) for a total of 120 h, following which hydrogel wet mass was determined, M_t . The rehydration ratio, RR, was assessed using Equation 2.

$$RR = \frac{M_t}{M_w} \quad (2)$$

2.5 Hydrogel Compression Testing

Prior to compression experiments, 5, 10, and 15% (w/v) hydrogels with and without 100 nm polystyrene nanoparticles were fabricated and swollen following the same protocol used in the swelling and rehydration studies. Before each compression test, the compression platen was lowered to initially load the hydrogel at 0.04-0.05 N and set the initial sample height. Compression of the hydrogel was concluded at half the initial height to prevent potential volume change through water loss. The elastic modulus was determined by examining the slope of the linear portion of the stress-strain curve obtained.

2.6 Examining Hydrogel Crosslinking Efficiency

To quantify unreacted thiols in responsive and non-responsive hydrogel formulations [5, 10, 15% (w/v) with and without 100 nm polystyrene nanoparticles], a fluorometric thiol quantitation kit was utilized. Hydrogels were fabricated and swollen following the same protocol used in swelling ratio studies. Hydrogels were then immersed in the thiol detection solution and incubated at RT for 1 h with shaking (100 rpm). 4-arm-PEG-thiol (20kDa) at various concentrations [% (w/v)], in the same volume of thiol detection solution, were used as unreacted thiol controls. Following incubation, fluorescence was measured on a plate reader with excitation at 490 nm and emission at 535 nm. A

crosslinking efficiency was calculated based on the fluorescence values obtained for the hydrogel formulations compared with the unreacted 4-arm-PEG-thiol controls.

2.7 Hydrogel Fabrication

Responsive and non-responsive hydrogels were formed using the β -lactamase-cleavable crosslinker, mal- β -lactam-mal (compound 3), and a non-cleavable crosslinker, mal-PEG-mal, respectively. The appropriate crosslinker was dissolved in dimethyl sulfoxide (DMSO). 4-arm-PEG-thiol was dissolved in 1 $\times$ PBS (pH 7) or in 0.1 $\times$ PBS (pH 3) for responsive or non-responsive hydrogel fabrication, respectively. These buffer concentrations and pH values were selected to achieve similar gelation times between the two hydrogel types. Hydrogels of varying polymer density [5, 10, and 15% (w/v)] were formed by mixing the crosslinker and PEG at a 1:1 maleimide/thiol stoichiometric ratio with the resulting hydrogel containing 25% (w/w) DMSO in PBS. The prepolymer mixture was rapidly vortexed, and 50 μ L was transferred into a 2 mm (height) $\times$ 5 mm (diameter) cylindrical polydimethylsiloxane (PDMS) mold. Gelation occurred within $\sim$ 10 min; however, samples were left for 1.5 h at 37°C to ensure complete gelation throughout the full hydrogel volume. For nanoparticle-loaded hydrogel studies, fluorescent polystyrene nanoparticles of the desired size were added to the prepolymer PEG solution at a final concentration of 0.08% (w/v).

2.8 Examining Hydrogel Response in β -Lactamase Enzyme Solutions

The hydrogel degradation response and nanoparticle release in enzyme solutions were investigated by fabricating 5, 10, and/or 15% (w/v) responsive and non-responsive hydrogels with 100 nm polystyrene nanoparticles and then swelling the hydrogels in 2

mL of 1× PBS (pH 7.4) at 37°C with shaking (100 rpm) for ~16–18 h to remove DMSO (and potential surface bound nanoparticles).

These swollen hydrogels were incubated with β -lactamases in 1 mL of 1× PBS (pH 7.4) at 37°C with shaking (100 rpm). For all β -lactamases tested, activity was reported by the vendor with one unit (U) defined as hydrolyzing 1.0 μ mol of benzylpenicillin per min at pH 7.0 at 25°C. At predetermined time points, hydrogels were removed from the β -lactamase solution and weighed. Simultaneously, 150 μ L of the incubation solution was removed and nanoparticle fluorescence (excitation 580 nm, emission 607 nm) was measured. This fluorescence signal was compared with standard curves of just nanoparticles in enzyme solution to determine released nanoparticle concentration from the hydrogels. These incubation solutions and hydrogels were placed back into the β -lactamase solution for continued monitoring of degradation and release.

2.9 Modeling Responsive Hydrogel Degradation Kinetics

We examined the degradation kinetics of these responsive hydrogels by fitting the hydrogel mass time course from Section 2.8 using Equations 3–9 below.

$$m(t) = \rho \pi r(t)^2 h(t) \quad (3)$$

$$r(t) = r_o - kt \quad (4)$$

$$h(t) = h_o - kt \quad (5)$$

$$X = \left(\frac{m_o}{\rho \pi r_{mold}^2 h_{mold}} \right)^{1/3} \quad (6)$$

$$r_o = X \cdot r_{mold} \quad (7)$$

$$h_o = X \cdot h_{mold} \quad (8)$$

$$m(t) = \rho\pi[r_o^2 h_o - kr_o(2h_o + r_o)t + k^2(2r_o + h_o)t^2 - k^3 t^3] \quad (9)$$

Here, the mass, $m(t)$, of the cylindrical hydrogels at any time, t , is a function of the hydrogel density, ρ ; radius, $r(t)$; and height, $h(t)$. Equations 4 and 5 describe the linear change in hydrogel dimensions over time due to surface erosion, assuming a rate constant, k . Here, r_o and h_o are the initial dimensions of the swollen hydrogel. These initial dimensions were estimated by calculating a swelling factor, X , on the basis of the initial hydrogel mass measured, m_o , and the known radius and height of the mold used to fabricate the hydrogels, r_{mold} and h_{mold} , respectively (Equations 6-8). By substituting equations 4 and 5 in Equation 3, Equation 9 was obtained to describe the hydrogel mass. Fitting hydrogel mass over time using this equation, we obtained k for 5, 10, and 15% (w/v) responsive hydrogels incubated in β -lactamases from *P. aeruginosa*.

2.10 Examining *In Vitro* and *Ex Vivo* Hydrogel Response to Bacteria

Hydrogel response was examined upon incubation of 10% (w/v) hydrogels with β -lactamase-producing *B. cereus* 13061, *P. aeruginosa* 27853, *E. cloacae* 13047, *K. pneumoniae* 1603 and 3834 and non- β -lactamase-producing *S. aureus* 25923. All bacteria were utilized in their logarithmic growth phase in tryptic soy broth (TSB), as determined by optical density measurements at 600 nm. For all assays, responsive and non-responsive hydrogels were swollen after fabrication, as done for the hydrogel response studies in enzyme solutions, and then sterilized via ultraviolet light in a Nuair Class II Type A2 biosafety cabinet for 15 min at RT.

To examine bacteria-mediated degradation of hydrogels *in vitro* on nutrient agar, bacteria at 1×10^8 CFU/mL were streaked on TSB agar plates. Hydrogels were then immediately placed on the agar and incubated at 37°C until complete degradation of responsive hydrogels was observed. Digital images of the hydrogels were taken over time. Controls of non-inoculated agar and no hydrogel on agar were included.

We also investigated the degradation of hydrogels *ex vivo* on bacteria-inoculated porcine skin. The tissue was thawed at RT for 1 h and sterilized by soaking in 70% ethanol for 30 min, followed by soaking in sterile $1 \times$ PBS three times. Bacteria at 1×10^7 CFU/mL were streaked three times on the tissue using a bacteria-soaked cotton swab. Hydrogels were immediately placed on the inoculated *ex vivo* tissue samples and incubated at 37°C until complete degradation of responsive hydrogels was observed. Digital images of the hydrogels were taken over time. Controls of non-inoculated porcine skin and no hydrogel on skin were included.

2.11 Examining Responsive Hydrogel Cytocompatibility and Hemolytic Activity

Cytocompatibility of 15% (w/v) hydrogel degradation products was evaluated for NIH 3T3 and HUVEC using a CCK-8 cell viability assay. Briefly, NIH 3T3 and HUVEC were seeded at a density of $\sim 3,200$ cells/cm² in DMEM and EGM-2 media supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin, respectively, at 37°C with 5% CO₂. After 24 h, cells were treated with solutions of the following: $1 \times$ PBS (pH 7.4), 4-arm-PEG-thiol in $1 \times$ PBS (pH 7.4), mal- β -lactam-mal in 1% (v/v) DMSO in $1 \times$ PBS (pH 7.4), degraded hydrogel products obtained by incubating hydrogels for 24 hours in β -lactamases from *P. aeruginosa* at 30 U/mL, *B. cereus* at 400 U/mL, and *E. cloacae* at 1

U/mL in 1× PBS (pH 7.4), and the same concentrations of β-lactamases in 1× PBS (pH 7.4). The 4-arm-PEG-thiol and mal-β-lactam-mal concentrations used were matched to the concentrations in the degraded hydrogel products. Cells incubated with media only served as positive controls (untreated) and cells incubated with 10% DMSO in media served as negative controls. After 24 h, the media was refreshed and a 10 μL CCK-8 solution was added to each well and incubated for 2 h at 37°C and 5% CO₂. Absorbance at 450 nm (Abs₄₅₀) was measured using a plate reader. Cell viability was calculated using Equation 10.

$$Cell\ viability\ (\%) = \frac{Abs_{450, sample} - Abs_{450, negative}}{Abs_{450, positive} - Abs_{450, negative}} \times 100 \quad (10)$$

HRBC hemolysis was investigated using 15% (w/v) hydrogel degradation products. Briefly, 25% (v/v) HRBCs were incubated for 1 h at 37°C in a 96-well plate with 20 μL of the following: 1× PBS (pH 7.4), 4-arm-PEG-thiol in 1× PBS (pH 7.4), mal-β-lactam-mal in 1% (v/v) DMSO in 1× PBS (pH 7.4), degraded hydrogel products obtained by incubating hydrogels for 24 hours in β-lactamases from *P. aeruginosa* at 30 U/mL, *B. cereus* at 400 U/mL, and *E. cloacae* at 1 U/mL in 1× PBS (pH 7.4), and the same concentrations of β-lactamases in 1× PBS (pH 7.4). The 4-arm-PEG-thiol and mal-β-lactam-mal concentrations used were matched to the concentration in the degraded hydrogel products. HRBCs incubated with 2% (v/v) Triton X-100 served as positive controls and untreated HRBCs served as negative controls. After incubation, HRBCs were centrifuged for 5 min at 500 g, following which 100 μL of the supernatant in each well was transferred to a new 96-well plate. Absorbance at 540 nm (Abs₅₄₀) was measured using a plate reader. Hemolysis was calculated using Equation 11.

$$Hemolysis (\%) = \frac{Abs_{540, sample} - Abs_{540, negative}}{Abs_{540, positive} - Abs_{540, negative}} \times 100 \quad (11)$$

2.12 Statistical Analyses

Results are reported as mean $\pm$ standard deviation whenever appropriate. All experiments were repeated three times with three samples at minimum. Statistical significance was calculated using unpaired, two-tailed Student's t-test or one- or two-way analysis of variance (ANOVA; $\alpha = 0.05$) with Tukey's post hoc analysis using GraphPad PRISM™ software. A value of $p < 0.05$ was considered statistically significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. MATLAB was used for data fitting and obtaining goodness of fit.

Chapter 3: Results and Discussion

Chapter 3 is based on a previously published original article:

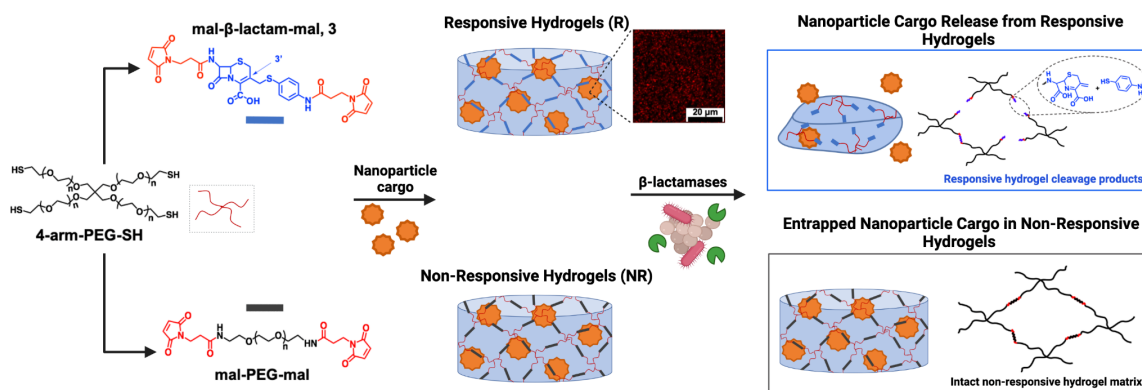
Alkekha, D., **LaRose, C.**, and Shukla, A. “ β -Lactamase-Responsive Hydrogel Drug Delivery Platform for Bacteria-Triggered Cargo Release”, *ACS Applied Materials & Interfaces*, 2022, 14 (24): 27538-27550.

3.1 Fabrication and Characterization of Hydrogels

A maleimide-functionalized β -lactamase-cleavable cephalosporin (Appendix A, Figure S1, compound 3) was synthesized in order to fabricate β -lactamase-degradable hydrogels, hereinafter termed “responsive hydrogels.” In the presence of β -lactamases, the 4-aminothiophenol (ATP) at the 3'-C site of the 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (ACLE) β -lactam core (Appendix A, Figure S1, compound 1) serves as a leaving group and is expelled. We functionalized the terminal amines on either end of compound 1 with maleimide moieties by conjugating 3-maleimidopropionic acid via amidation, forming compound 2 (Appendix A, Figure S1). After functionalization with maleimides, the carboxylic acid on the β -lactam was deprotected by the removal of the *p*-methoxybenzyl protecting group, yielding the β -lactamase-responsive crosslinker, mal- β -lactam-mal (Appendix A, Figure S1, compound 3). The carboxylic acid is an enzyme–substrate recognition site for some β -lactamases,⁶⁸ rendering the deprotection step critical for activity with a broader range of β -lactamases. Proton nuclear magnetic resonance (¹H NMR) (Appendix A, Figure S2–S4) and high resolution mass spectrometry (HRMS) electrospray ionization (ESI) confirmed successful synthesis of this crosslinker.

Compound 3 was then utilized in the thiol-ene-mediated crosslinking of 4-arm-PEG-thiol macromers, thus incorporating the β -lactamase-responsive moiety into the backbone of the responsive hydrogel network. Control, non-responsive hydrogels were instead fabricated using a maleimide-functionalized PEG crosslinker, mal-PEG-mal (Scheme 1).

Scheme 1. Schematic representation of β -lactamase-responsive (R) and non-responsive (NR) hydrogel fabrication; degradation of responsive hydrogels and their subsequent release of entrapped nanoparticle cargo. Non-responsive hydrogels remain intact and nanoparticle cargo remains entrapped.



Hydrogels were fabricated at varying 4-arm-PEG-thiol concentrations, with or without fluorescent polystyrene nanoparticles to assess controlled release behavior. The polystyrene nanoparticles serve as model cargo and are representative of therapeutic antibacterial nanoparticles, which could be encapsulated in the hydrogel drug delivery platform. The reaction conditions for hydrogel fabrication for the two types of hydrogels were empirically determined in order to yield similar reaction times between formulations. Responsive hydrogels fabricated at room temperature in $1\times$ phosphate buffered saline (PBS) (pH 7) were polymerized within ~ 10 min, while the non-responsive hydrogels required $0.1\times$ PBS (pH 3) to achieve a similar gelation time. Thiol reactivity may have been the differentiating factor that influenced polymerization time between the

two formulations. The carboxylic acid of compound 3, which is negatively charged at pH 7, potentially increases the pK_a of the thiols on the 4-arm-PEG-thiol macromers, thus decreasing their reactivity. The concentration of reactive thiolates can be increased to enable more rapid polymerization by increasing the pH during responsive hydrogel fabrication, which is an approach that has been used in various reports.^{69,70}

Following hydrogel fabrication, we measured the hydrogel crosslinking efficiency, elastic modulus, and equilibrium swelling ratio, and we also estimated the hydrogel mesh size (Figure 3a–d and Table 2). The crosslinking efficiency was found to be ~99% for all formulations. A decrease in PEG concentration led to an increase in swelling and mesh size and a decrease in stiffness, which was consistent with previous work.^{71–73} Although differences in reaction times for responsive and non-responsive formulations were minimized by optimizing gelation buffer concentration and pH, the responsive hydrogels typically exhibited greater swelling ratios and mesh sizes and decreased stiffness as compared to non-responsive hydrogels. The hydrogel network may have expanded due to charge repulsion caused by the deprotonated carboxylate of the β -lactam in the responsive crosslinker at neutral pH. Additionally, the deprotonated carboxylate can also enable hydrogen bonding with water molecules which likely contributed to increased water uptake.^{74,75} For hydrogels fabricated with nanoparticles, the concentration of nanoparticles was selected to obtain a similar gelation time as for formulations lacking nanoparticles. In comparing hydrogels with nanoparticles to those without nanoparticles for each polymer concentration, the swelling ratio and average mesh sizes remained

largely unchanged, with a decrease in elastic modulus observed only for 15% (w/v) responsive hydrogels with nanoparticles.

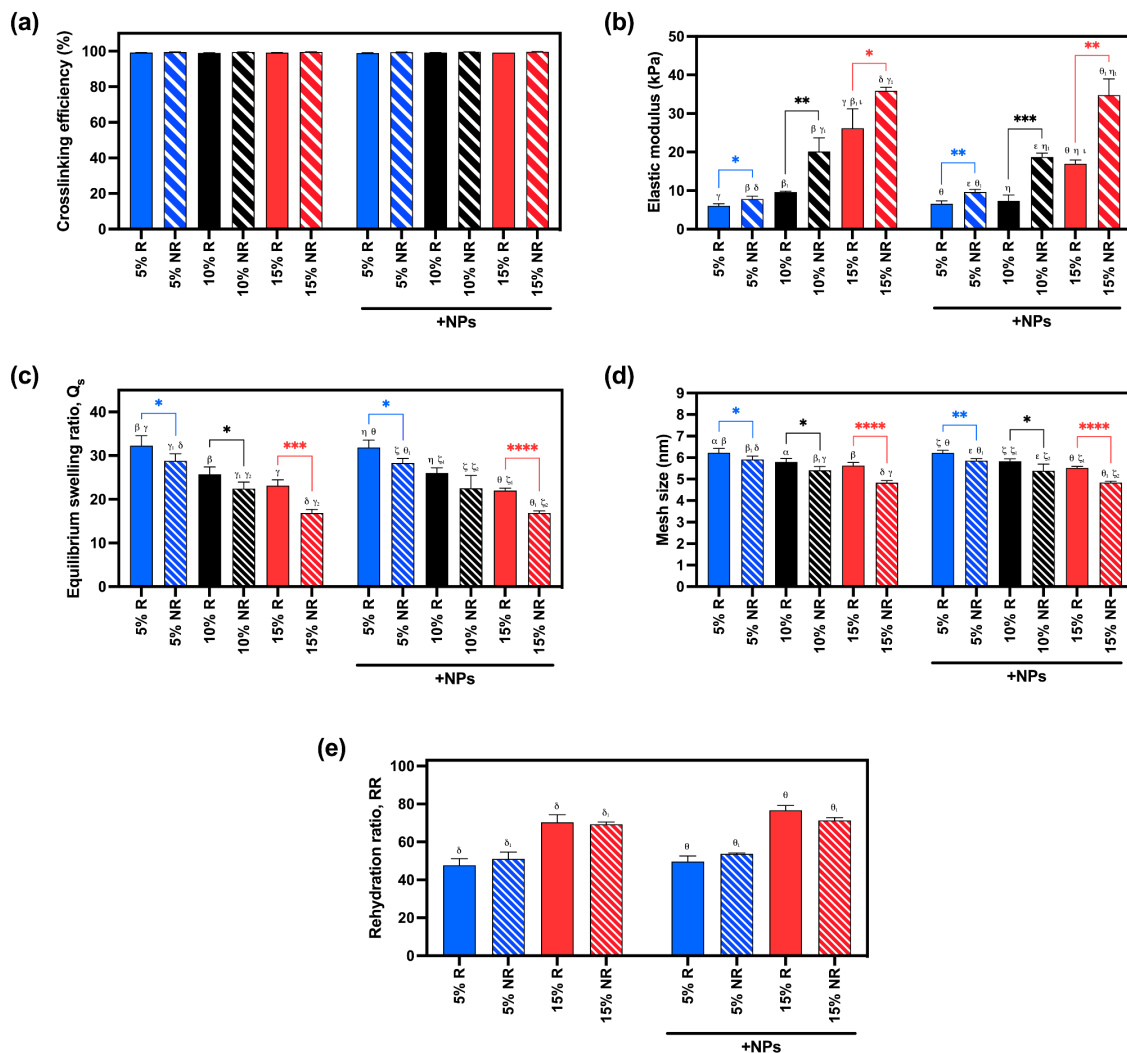


Figure 3. Crosslinking efficiency (a), elastic modulus (b), equilibrium swelling ratio (Q_s) (c), theoretical mesh size (d), and rehydration ratio (RR) (e) of responsive (R) and non-responsive (NR) hydrogels of varying polymer density [5, 10, 15% (w/v)] fabricated with (+) or without (-) 100 nm nanoparticles (NPs). Data is presented as mean $\pm$ standard deviation; $n \geq 3$. Statistical significance was examined using unpaired, two-tailed Student's t-test comparing hydrogels $\pm$ NPs for each formulation and for comparing R and NR hydrogels for each formulation. One-way ANOVA was used to compare hydrogels with different percent (w/v) PEG for each formulation. Note, matching Greek letters indicate significant differences ($p < 0.05$; *, α , and ϵ are $p < 0.05$; **, β , and ζ are $p < 0.01$; ***, γ , and η are $p < 0.001$; ****, δ , and θ are $p < 0.0001$).

Table 2. Elastic modulus, equilibrium swelling ratio, and theoretical mesh size of responsive (R) and non-responsive (NR) hydrogels of different polymer densities, with (+) and without (-) encapsulated 100 nm nanoparticles.

Formulation		Elastic Modulus (kPa)		Equilibrium Swelling Ratio, Q_s		Mesh Size (nm)	
PEG % (w/v)	Nanoparticles (+/-)	R	NR	R	NR	R	NR
5	+	6.6 ± 0.8	9.6 ± 0.7	31.8 ± 1.7	28.3 ± 1.1	6.2 ± 0.1	5.9 ± 0.1
	-	6.0 ± 0.5	7.8 ± 0.8	32.3 ± 2.3	28.7 ± 1.7	6.2 ± 0.2	5.9 ± 0.2
10	+	7.4 ± 1.5	18.7 ± 1.0	26.0 ± 1.2	22.5 ± 2.9	5.8 ± 0.1	5.4 ± 0.3
	-	9.5 ± 0.3	20.1 ± 3.5	25.6 ± 1.7	22.5 ± 1.5	5.8 ± 0.2	5.4 ± 0.2
15	+	17.0 ± 1.0	34.8 ± 4.2	22.0 ± 0.6	16.9 ± 0.5	5.5 ± 0.1	4.8 ± 0.1
	-	26.1 ± 5.0	35.8 ± 1.0	23.1 ± 1.4	16.8 ± 0.8	5.6 ± 0.1	4.8 ± 0.1

We also evaluated the rehydration ratio for 5 and 15% (w/v) hydrogels to investigate the ability of dried hydrogels to regain their initial swollen mass when incubated in $1 \times$ PBS. As shown in Figure 3e, none of the formulations were able to regain their original post-fabrication wet mass and there were no notable differences between hydrogels of a given formulation fabricated with or without nanoparticles. Both responsive and non-responsive 5% (w/v) hydrogel formulations, with and without encapsulated nanoparticles, reached ~50% of their initial post-fabrication wet mass after rehydration. All 15% (w/v) formulations were able to re-swell to ~70–75% of their initial post-fabrication wet mass. During hydrogel drying, the micro- and nanostructure of the hydrogel is likely affected by factors such as pore collapse,⁷⁶ which can affect rehydration.

3.2 Hydrogel Degradation and Nanoparticle Release in β -Lactamases

To investigate the behavior of hydrogels in β -lactamases, 10% (w/v) responsive and non-responsive hydrogels formulated with 25, 50, and 100 nm polystyrene nanoparticles were incubated in β -lactamases isolated from *Pseudomonas aeruginosa*. *P. aeruginosa* is a common pathogenic, gram-negative bacteria that is known to cause a range of acute and chronic infections and to produce multiple classes of β -lactamases.⁷⁷ As shown in Figure 4a, nanoparticle release from the responsive hydrogels followed hydrogel degradation for all nanoparticle sizes, yet the release of 25 and 50 nm nanoparticles exhibited slightly accelerated release kinetics compared with 100 nm nanoparticle-loaded responsive hydrogels. All responsive hydrogel formulations exhibited a highly linear correlation between release of the encapsulated nanoparticles and hydrogel mass loss in β -lactamases from *P. aeruginosa* (Fig. 4b). An overall decrease in the rate of mass loss was observed over time for these formulations, suggesting a surface degradation mechanism as opposed to bulk degradation.^{78,79} Surface erosion is common in enzymatically degradable PEG hydrogels where enzyme hydrodynamic diameter is on the same order of magnitude as hydrogel mesh size, preventing or slowing enzyme penetration and diffusion into the hydrogel.⁸⁰ Many β -lactamases have been characterized as having dimensions that are comparable to the estimated mesh sizes of the hydrogels developed in this work of ~5-6 nm.⁸¹

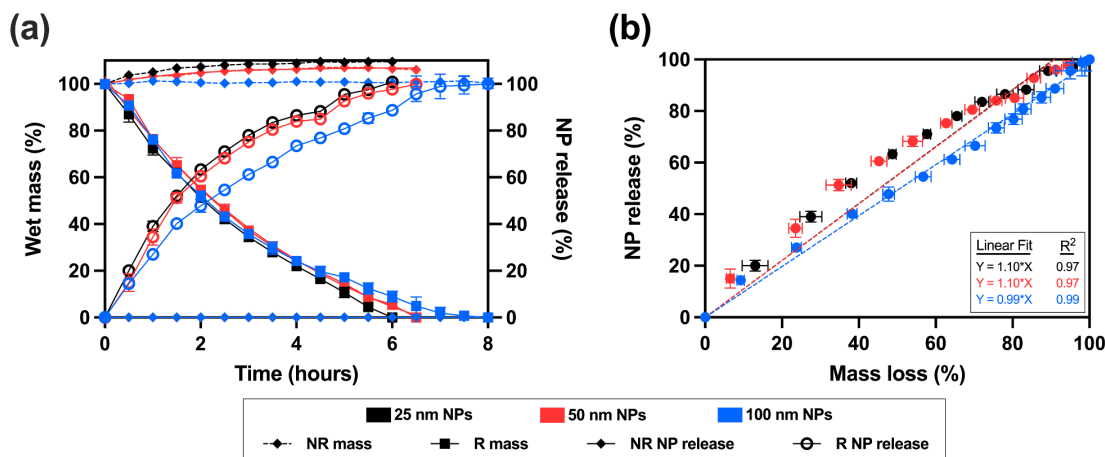


Figure 4. Effect of encapsulated nanoparticle (NP) diameter on β -lactamase-triggered hydrogel degradation and NP release. (a) Normalized wet mass and NP release over time from 10% (w/v) responsive (R) and non-responsive (NR) NP-loaded hydrogels incubated in solutions of 30 U/mL of β -lactamases from *P. aeruginosa*. Wet mass is normalized to the initial mass of the swollen hydrogels before incubation with β -lactamases. NP release is normalized to the NP fluorescence signal measured in the incubation solution after complete hydrogel degradation. (b) Normalized NP release as a function of normalized wet mass loss of 25, 50, and 100 nm NP-loaded 10% (w/v) R hydrogels with each data point representing one time point. The linear fit and coefficient of determination, R^2 , are shown for each formulation in black (25 nm NPs), red (50 nm NPs), and blue (100 nm NPs). Data are presented as mean $\pm$ standard deviation; $n = 4$.

We observed that the bacterial source of the β -lactamases had a significant impact on hydrogel degradation rate. There are four major classes of β -lactamases, and at the time of writing, there have already been ~7400 different β -lactamases reported.⁸² Bacteria produce varying levels of different β -lactamases in a dynamic manner, which can affect the hydrolysis kinetics of the β -lactamase-cleavable crosslinker in the responsive hydrogel backbone. We investigated hydrogel response in β -lactamases from gram-negative *Enterobacter cloacae* to compare hydrogel degradation and nanoparticle release kinetic differences. While non-responsive hydrogels remained stable in β -lactamases, responsive hydrogels (with 25, 50, and 100 nm nanoparticles) incubated in

β -lactamases isolated from *P. aeruginosa* and *E. cloacae* resulted in vastly different timescales to achieve complete degradation. We observed that hydrogels incubated in 1 U/mL β -lactamases from *E. cloacae* exhibited a rapid, linear degradation over ~ 3.5 hours (Figure 5a), while hydrogels incubated in 30 U/mL β -lactamases from *P. aeruginosa* degraded in 6-8 hours (Figure 4a). The differences between the hydrogel responses observed may be due to differences in the types of β -lactamases produced by the two bacterial species, as well as the specific strains that were utilized. β -lactamases exhibit varying specificity and activity towards different β -lactam containing compounds.⁸³ For example, some β -lactamases have a larger or more flexible substrate binding pocket as compared to other classes of β -lactamases and can better accommodate polymers or bulky moieties near the β -lactam ring.^{84–86}

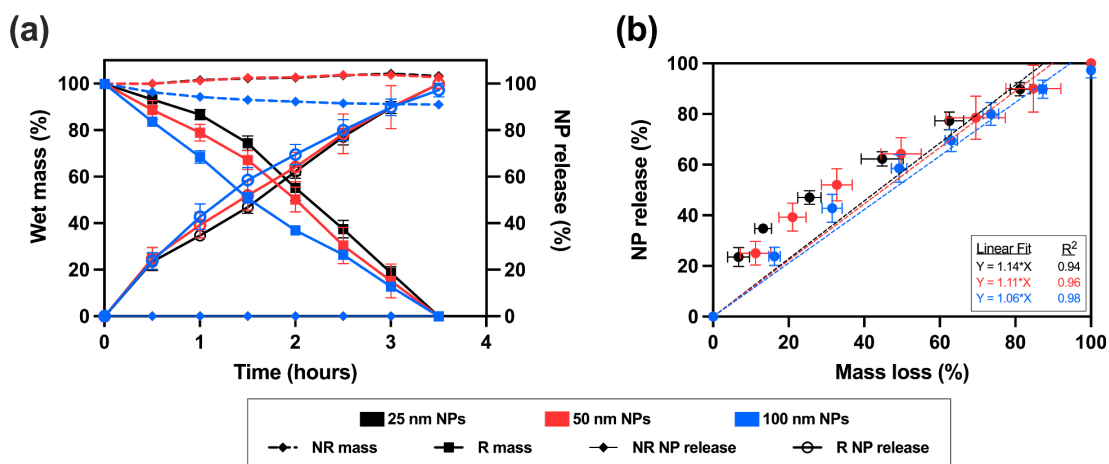


Figure 5. Effect of encapsulated nanoparticle (NP) diameter on hydrogel degradation and NP release in β -lactamases from *E. cloacae*. (a) Normalized wet mass and NP release from 25, 50, and 100 nm NP-loaded 10% (w/v) responsive (R) and non-responsive (NR) hydrogels incubated in solutions of 1 U/mL β -lactamases from *E. cloacae* over time. Wet mass is normalized to initial mass after swelling and before addition of enzymes. NP release is normalized to the fluorescence measured in the solutions after complete hydrogel degradation. (b) Normalized NP release as a function of normalized wet mass loss of 25, 50, and 100 nm NP-loaded 10% (w/v) R hydrogels with each data point representing one timepoint. The linear fit and coefficient of determination, R^2 , are shown

for each formulation in black (25 nm NPs), red (50 nm NPs), and blue (100 nm NPs). Data is presented as mean $\pm$ standard deviation; n = 4.

3.3 Effect of Hydrogel Polymer Concentration on Degradation

Degradation rates of enzyme-responsive hydrogels depend on the concentration of the enzyme-labile moiety in the hydrogel matrix and polymer crosslinking density. We began probing these effects by examining the response of hydrogels fabricated with varying polymer concentrations. Along with examining the hydrogel mass over time as an indicator of degradation, nanoparticle release was also quantified. First, responsive and non-responsive 5, 10, and 15% (w/v) hydrogels with 100 nm nanoparticles were fabricated and incubated in 30 U/mL β -lactamases from *P. aeruginosa*. Non-responsive formulations remained stable in these conditions and exhibited no significant change in mass or release of nanoparticles (with a nanoparticle detection limit of 0.3 μ g/mL). These results were expected due to the lack of a β -lactamase-cleavable backbone and the much smaller average mesh size of these hydrogels compared with the nanoparticle diameter of 100 nm leading to physical entrapment. In contrast, all responsive hydrogels exhibited mass loss over time, with complete degradation in $\sim$ 3.5, 8, and 13 h for 5, 10, and 15% (w/v) hydrogels, respectively (Figure 6a-c).

To confirm our hypothesis of responsive hydrogels undergoing surface degradation in β -lactamases, we fit the mass time course of the different polymer concentration hydrogels using Equations 3-9 from Chapter 2, which are representative of surface erosion. After fitting Equation 9 to the hydrogel mass loss time course for 5, 10, and 15% (w/v) responsive hydrogels, we determined each of their goodness of fit (R^2) which all resulted in values of 0.99 (Figure 6d). These results, together with data from Section 3.2

confirm the ability to achieve an on-demand, β -lactamase-triggered release of encapsulated cargo (over a broad range of sizes) from these hydrogel drug delivery systems.

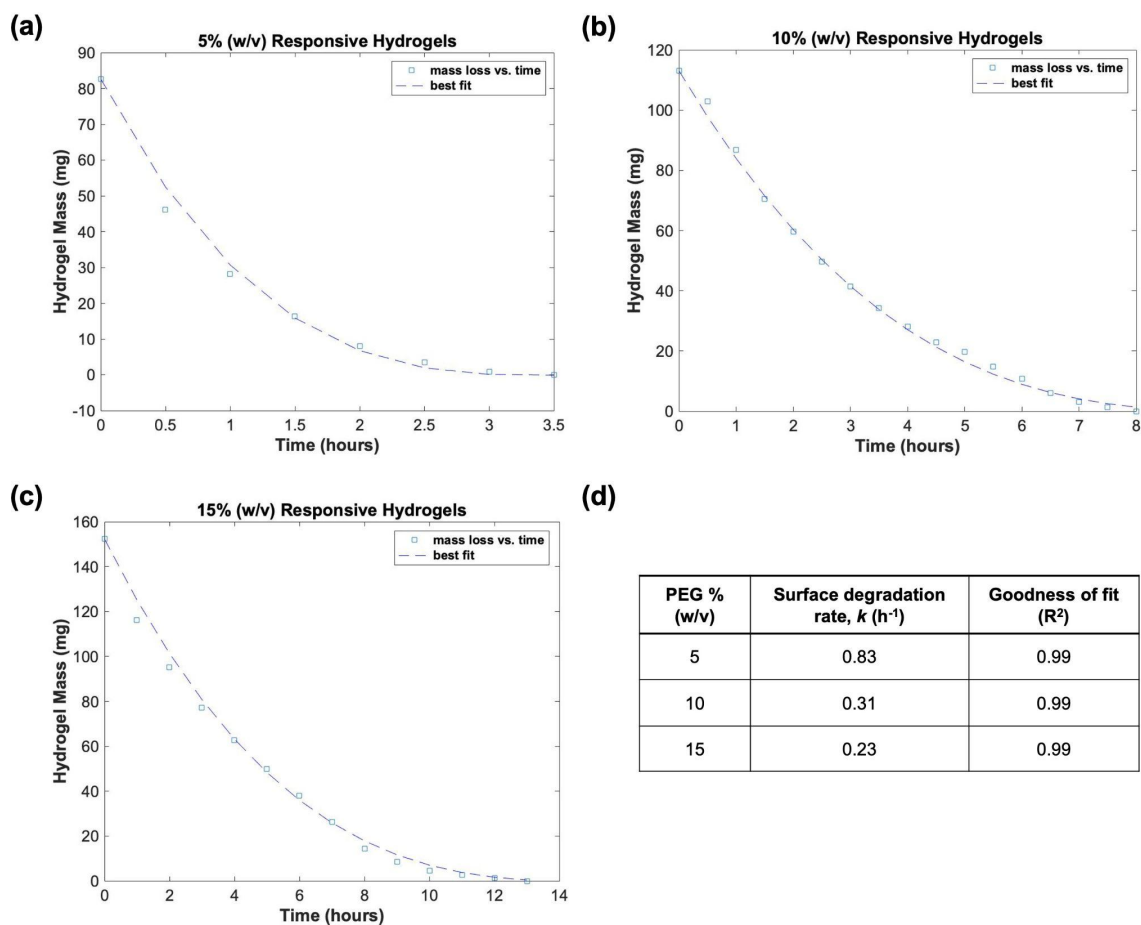


Figure 6. Modeling hydrogel mass loss over time assuming surface erosion of 100 nm-nanoparticle loaded hydrogels in 30 U/mL β -lactamases from *P. aeruginosa*. Hydrogel wet mass over time of 5% (w/v) (a), 10% (w/v) (b), and 15% (w/v) (c) R hydrogels incubated in β -lactamases. The dashed trace represents the best fit curve obtained by fitting Equation 9 to the experimental values obtained. (d) Summary table of the surface erosion rate constant (k) and goodness of fits (R^2) for these formulations.

3.4 Hydrogel Degradation and Nanoparticle Release in β -Lactamase-Producing Bacteria

Having established the responsive degradation of these hydrogels in β -lactamase solutions, we next examined whether this triggered hydrogel degradation is also observed in the presence of β -lactamase-producing bacteria. Both responsive and non-responsive 10% (w/v) hydrogels were incubated with a range of gram-positive and negative bacteria, including several ESKAPE pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter spp.*). The highly virulent ESKAPE pathogens are particularly concerning due to the prevalence of antibiotic resistance in these species. Specifically, we examined a non- β -lactamase-producing strain of *S. aureus* and β -lactamase-producing strains of *K. pneumoniae*, *P. aeruginosa*, *E. cloacae*, and *B. cereus* (a non-ESKAPE pathogen). Table 3 below provides details on the strains of bacteria utilized in these studies and whether they produce β -lactamases, which were all confirmed based on previous reports.

Table 3. Bacteria strains tested with responsive and non-responsive hydrogels.

Bacteria	Strain	Gram	β -lactamase	Nitrocefin Disk Assay*
<i>S. aureus</i>	ATCC® 25923 TM	Positive	No	
<i>B. cereus</i>	ATCC® 13061 TM	Positive	Yes	
<i>P. aeruginosa</i>	ATCC® 27853 TM	Negative	Yes	
<i>E. cloacae</i>	ATCC® 13047 TM	Negative	Yes	
<i>K. pneumoniae</i>	Clinical isolate 3834	Negative	Yes	
<i>K. pneumoniae</i>	Clinical isolate 1603	Negative	Yes	
Water	—	—	—	

*Red color indicates β -lactamase production.

We investigated β -lactamase-triggered degradation of hydrogels incubated with bacteria cultured on agar to better represent a topical application of these hydrogels (Figure 7). Responsive hydrogels underwent degradation over time with all β -lactamase-producing bacteria examined, including the *K. pneumoniae* clinical isolates, while non-responsive hydrogels remained stable and visibly unchanged on the agar during this time. Responsive and non-responsive hydrogels incubated on the non- β -lactamase-producing *S. aureus* 25923 behaved identically and remained stable and unchanged on the bacteria inoculated agar over 7 days. These results matched controls of hydrogels incubated on sterile, non-bacteria-inoculated agar over 7 days. The timescales of degradation observed for responsive hydrogels on agar inoculated with the β -lactamase-producing strains varied widely, ranging from a day for *B. cereus* to 2–3 days for both *K. pneumoniae* clinical isolates to ~8 days for *P. aeruginosa* and *E. cloacae* (Figure 7). Note, the green-blue color seen in the hydrogels incubated on *P. aeruginosa* is likely the pyocyanin and pyoverdine metabolites secreted by *P. aeruginosa*,^{87,88} which can change the color of media and agar and here also seem to diffuse into the hydrogels. There are many factors that may contribute to the differences in degradation time observed, including differences in β -lactamase composition and concentration produced by these strains, which may also be influenced by differences in bacterial growth rates in the specific media and growth conditions used. Overall, these results indicate that the β -lactamase-responsive hydrogels retain their enzyme-responsive degradation capability in the presence of a broad range of β -lactamase-producing bacteria and that non- β -lactamase-cleavable hydrogels remain stable in these conditions.

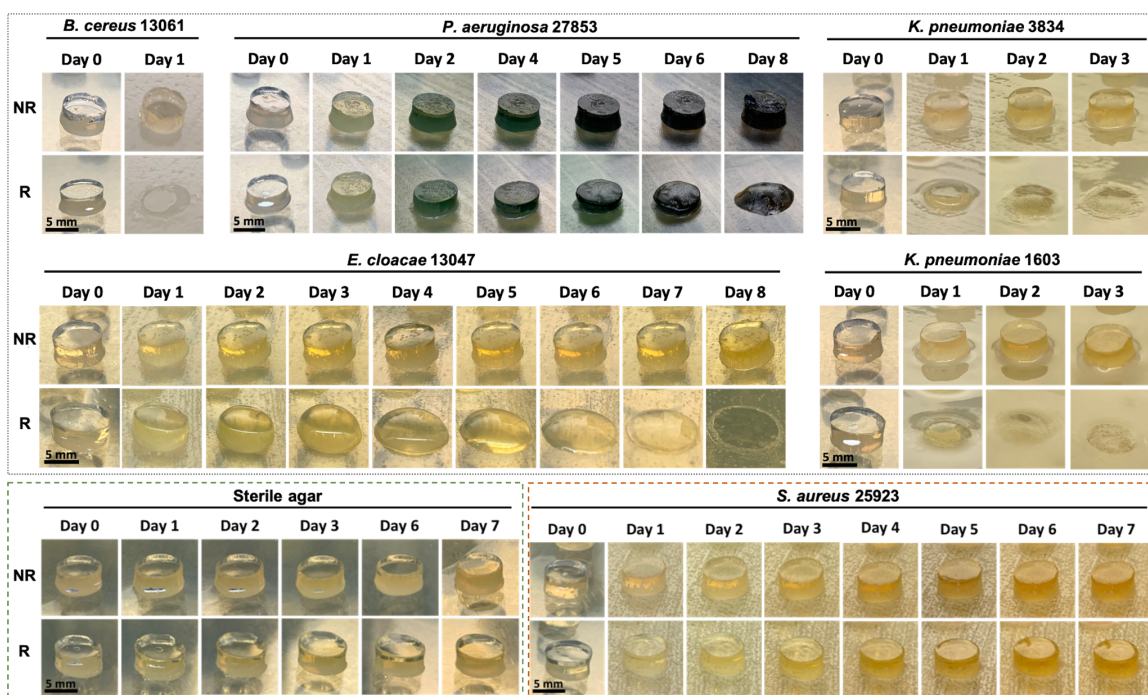


Figure 7. Responsive hydrogel degradation by β -lactamase-producing bacteria on agar. Digital images over time of 10% (w/v) non-responsive (NR) and responsive (R) hydrogels incubated on agar inoculated with β -lactamase-producing *B. cereus* 13061, *P. aeruginosa* 27853, *E. cloacae* 13047, *K. pneumoniae* 3834, and *K. pneumoniae* 1603; non- β -lactamase-producing *S. aureus* 25923; and a sterile agar control at 37°C. Images are representative of three repeats.

After demonstrating the robust β -lactamase response of the hydrogels developed here *in vitro*, we investigated the translational potential of the hydrogels using model infections of *ex vivo* porcine skin tissue. *Ex vivo* porcine skin inoculated with bacteria is commonly used as a model to study wound infections that closely mimic superficial tissue infections.⁸⁹ As shown in Figure 8, again, only the responsive hydrogels degraded when incubated on porcine skin inoculated with the β -lactamase-producing *B. cereus*, *P. aeruginosa*, *K. pneumoniae*, and *E. cloacae*, while the non-responsive hydrogels remained stable. As with the agar studies, responsive and non-responsive hydrogels remained stable and visibly unchanged on *S. aureus* 25923 inoculated and sterile porcine

tissue. These results further confirmed the selective degradability of the hydrogels by β -lactamase-producing bacteria. Interestingly, degradation induced by *P. aeruginosa* was faster on the skin (~4 days) than on agar (~8 days). A potential reason for this more rapid degradation by *P. aeruginosa* may be a higher bacteria growth rate on skin compared with agar, which has been observed in an *ex vivo* human skin model infected with *P. aeruginosa* PAO1,⁹⁰ potentially resulting in a higher concentration of β -lactamases. *K. pneumoniae* inoculated samples also demonstrated a faster hydrogel degradation on pig skin (1–2 days) compared with agar (~3 days), while *E. cloacae* demonstrated slower degradation on pig skin (~14 days) than on agar (~8 days). Future *in vivo* investigation of these materials will enable further understanding of the differences in hydrogel degradation rates on the basis of the type of bacteria and β -lactamases present and also factors such as host immune response and wound healing.

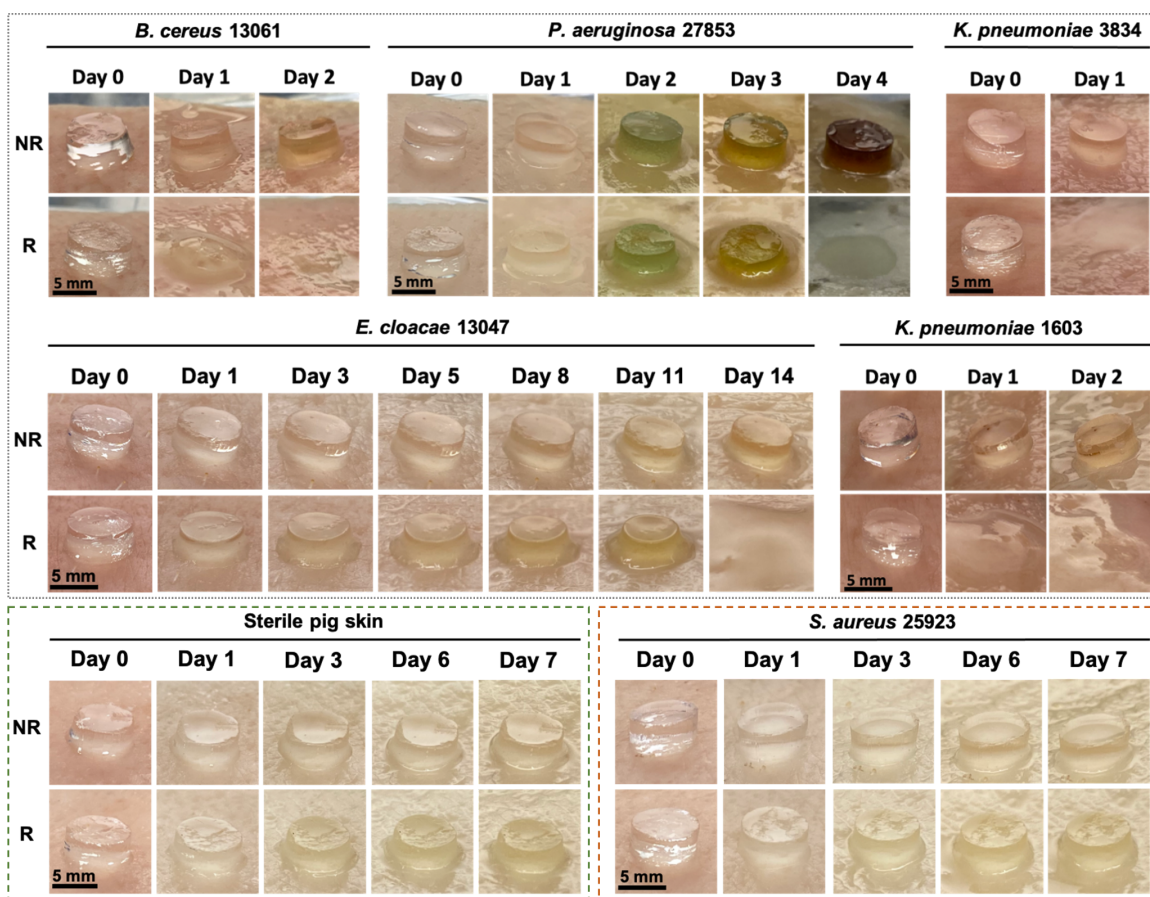


Figure 8. Responsive hydrogel degradation by β -lactamase-producing bacteria on *ex vivo* porcine skin. Digital images over time of 10% (w/v) non-responsive (NR) and responsive (R) hydrogels incubated on porcine *ex vivo* skin tissue inoculated with β -lactamase-producing *B. cereus* 13061, *P. aeruginosa* 27853, *E. cloacae* 13047, *K. pneumoniae* 3834, *K. pneumoniae* 1603; non- β -lactamase-producing *S. aureus* 25923; and a sterile agar control at 37°C. Images are representative of three repeats.

3.5 Cytocompatibility and Hemolytic Activity of β -Lactamase-Degraded Responsive Hydrogels

We investigated cytocompatibility of the responsive hydrogels by exploring the effect of the degraded hydrogel products on the metabolic activity of murine fibroblasts (NIH 3T3) and human umbilical vein endothelial cells (HUVEC), along with potential hemolysis of human red blood cells (HRBCs). These cells were selected as representative of

mammalian cells that would be encountered in wounds where these hydrogels may eventually be applied. Responsive hydrogels [15% (w/v)] were completely degraded in β -lactamases from *P. aeruginosa*, *B. cereus*, or *E. cloacae*. The mammalian cells were incubated with these degradation products, along with 4-arm-PEG-thiol macromer, responsive crosslinker (mal- β -lactam-mal, compound 3), β -lactamases, and the appropriate positive and negative controls. A CCK-8 assay indicated no significant difference in HUVEC or NIH 3T3 viability for any of the hydrogel degradation products compared with untreated controls (Figure 9a); the polymer backbone, crosslinker, and β -lactamases also had no impact on cell viability (Figure 9b). As expected, HRBC hemolysis was not observed for any of the hydrogel degradation products (Figure 9c). Overall, these results indicate that the responsive hydrogel degradation products are compatible with important mammalian cell types.

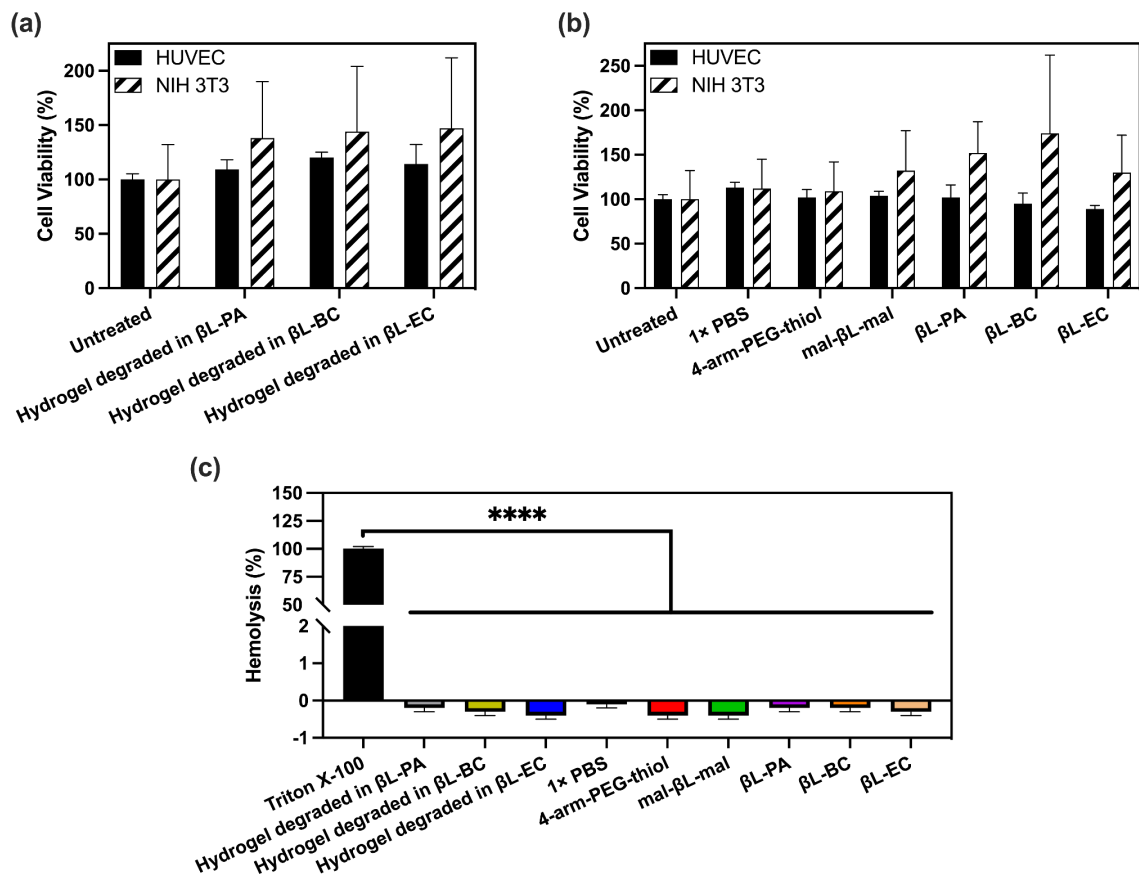


Figure 9. Cytocompatibility of hydrogel products. (a) Cell viability of HUVEC and NIH 3T3 fibroblasts incubated with 15% (w/v) hydrogels degraded in β -lactamases from *P. aeruginosa* (β L-PA, 30 U/mL), *B. cereus* (β L-BC, 400 U/mL), and *E. cloacae* (β L-EC, 1 U/mL) in 1x PBS (pH 7.4). (b) Cell viability of 1x PBS (pH 7.4), 4-arm-PEG-thiol in 1x PBS (pH 7.4), mal- β -lactam-mal (mal- β L-mal) in 1x PBS (pH 7.4), and 30 U/mL β L-PA, 400 U/mL β L-BC, and 1 U/mL β L-EC in 1x PBS (pH 7.4). 4-arm-PEG-thiol and mal- β L-mal concentrations used were matched to the concentrations in the degraded hydrogel products. Data is presented as average $\pm$ standard deviation; n = 9. One-way ANOVA showed no significant difference between any of the incubation conditions for HUVEC and NIH 3T3 with untreated controls ($p > 0.05$). (c) HRBC hemolysis of 15% (w/v) degraded hydrogel products in β -lactamases and their respective controls. Data is presented as average $\pm$ standard deviation; n = 6. One-way ANOVA was used to compare all incubation conditions to the positive control (Triton X-100). Statistically significant differences were observed for all incubation conditions (**** $p < 0.0001$).

Chapter 4: Conclusions and Scientific Impact

In this work, a β -lactamase-cleavable crosslinker was synthesized and used to fabricate β -lactamase-degradable hydrogels. These responsive hydrogels are the first hydrogel drug delivery platform demonstrated to achieve a β -lactamase-controlled release of encapsulated cargo. Responsive degradation of the hydrogels was observed in the presence of β -lactamases isolated from several different bacteria as well as β -lactamase-producing bacteria (including several ESKAPE species) both *in vitro* and in an *ex vivo* porcine skin infection model. In the conditions investigated, it was observed that nanoparticle cargo release from responsive hydrogels was enabled only by β -lactamase-triggered hydrogel degradation. Our bacterial enzyme-responsive hydrogel has the potential to be used for the on-demand delivery of encapsulated antibacterial nanoparticles. A broad range of nanoparticle cargo that is larger than the average hydrogel mesh size could be included, such as liposomes, micelles, nanogels, silver nanoparticles, and other polymeric drug carriers (e.g., drug-loaded or drug-coated nanoparticles and cationic nanoparticles). The hydrogel would thus prevent uncontrolled release of the encapsulated therapeutics and ensure triggered release only upon matrix cleavage by β -lactamases. Our results have demonstrated that a wide range of cargo sizes (25 to 100 nm) can achieve a triggered release using this hydrogel system. These hydrogels hold great promise for use as prophylactic biomaterial, such as a wound dressing that can deliver encapsulated therapeutics on-demand when triggered by the presence of pathogenic, β -lactamase-producing bacteria. The mechanism of action of this drug delivery system will allow for reduced unnecessary exposure to encapsulated

therapeutics and effective local delivery, which can reduce off-site toxicity and lower susceptibility for antibiotic resistance development.

Chapter 5: Future Directions

Smart biomaterials and drug delivery systems have the potential to substantially improve the field of medicine, especially with regard to mitigating the threat of antibiotic resistance. Our novel β -lactamase-cleavable moiety was designed to be versatile and used to develop a wide range of bacteria-responsive biomaterials and drug delivery systems. In this Chapter, the initial work and future theoretical plans for translating our β -lactamase-responsive biomaterials for other infection applications are described.

Nanogel Pilot Studies

Utilizing a similar chemical approach to the fabrication of our bacteria-responsive hydrogels, we have initiated pilot studies on the development of bacteria-responsive nanogels for use as topical antibacterial treatments or for systemic infections. Nanogels are nanometer scale hydrogel-like polymeric materials that have overlapping properties of traditional polymeric nanoparticles and hydrogels.⁹¹ The hydrogel-like properties associated with nanogels enables interaction with cells and can enhance uptake by cells as compared with their solid counterparts.⁹¹ In the case of our bacteria-responsive nanogels, these hydrogel-like properties can potentially facilitate better interaction with cells but be able to differentiate antibiotic release by cell type (i.e., bacterial vs mammalian cell). The nanogels would only release encapsulated or tethered antibiotic cargo when bacteria are present and remain intact in the presence of mammalian cells.

This controlled release mechanism could potentially reduce the toxicity to mammalian cells observed when high concentrations of antibiotics are released uncontrollably.

The fabrication of our β -lactamase-degradable nanogels utilizes *in situ* formation to synthesize nanogels within solution. *In situ* formation of our bacteria-responsive nanogels begins by empirically determining a 1:1 stoichiometric ratio of thiol:maleimide on 4-arm-PEG-thiol macromers and our β -lactamase-cleavable crosslinker. 4-arm-PEG-thiol (5 kDa, purchased from Jenkem Technology, USA) was dissolved in 1 $\times$ PBS and the β -lactamase-cleavable crosslinker, mal- β L-mal, was separately dissolved in equal parts DMSO and 1 $\times$ PBS. The total volumes of the 4-arm-PEG-thiol and mal- β L-mal mixtures should be equal prior to adding them to a heated solution of 1 $\times$ PBS (60°C). The 4-arm-PEG-thiol mixture was added to the PBS solution first and stirred vigorously to ensure homogeneity. When the mixture returned to 60°C, the mal- β L-mal mixture was quickly added to the heated solution of 4-arm-PEG-thiol. The reaction was allowed to continue for 1 h in a closed reaction vessel. After 1 h, the reaction was removed from heat and cooled to RT. The resultant nanogels were dialyzed overnight in ultrapure water to ensure any unreacted components were removed.

In our pilot studies, various parameters were altered to evaluate the effect on nanogel size. Size is an important parameter to investigate as it can influence the colloidal stability of the nanogels in solution. Larger nanogels will tend to aggregate more rapidly than smaller nanogels which introduces heterogeneity in the nanogel colloidal suspension.⁹² The parameters investigated include pH, stirring speed, and total reaction

volume. Figure 10 below depicts the results from our pilot studies on nanogel fabrication through hydrodynamic diameter (size) measurements using a Zetasizer dynamic light scattering instrument. We observed significant differences in nanogel size with various pH values tested, likely caused by differences in local reaction times. A reduction in pH led to smaller nanogels which can potentially be attributed to less reactive thiolates to undergo Michael-type addition with the maleimides on the responsive crosslinker along with the rapid mixing of components (Figure 10a). Stirring speed did not significantly affect bacteria-responsive nanogel size (Figure 10b). The statistically significant difference observed for the nanogels fabricated at 1000 rpm was likely attributed to more rapid evaporation of the solution as more condensation was observed at the top of the reaction vessel than the other two conditions. We would expect that more rapid mixing of the solution would lead to smaller nanogel size, as the components would have shorter local reaction times. We observed a statistically significant decrease in nanogel size with increasing total reaction volume (Figure 10c), which was expected as the components are dispersed more within larger reaction volumes, thus slowing local reaction time. Lastly, when comparing total reaction volumes of 3 and 4 mL at different stirring speeds (900 and 1100 rpm), the results were consistent with our hypothesis that increased volume and stirring speed led to smaller nanogels (Figure 10d). With the conditions and combinations of parameters tested, we were able to successfully fabricate bacteria-responsive nanogels ranging from roughly 100 - 400 nm. Many nanoparticles that are used clinically have sizes comparable to the nanogels fabricated in these studies,⁹³ which is promising for advancing these nanogels clinically.

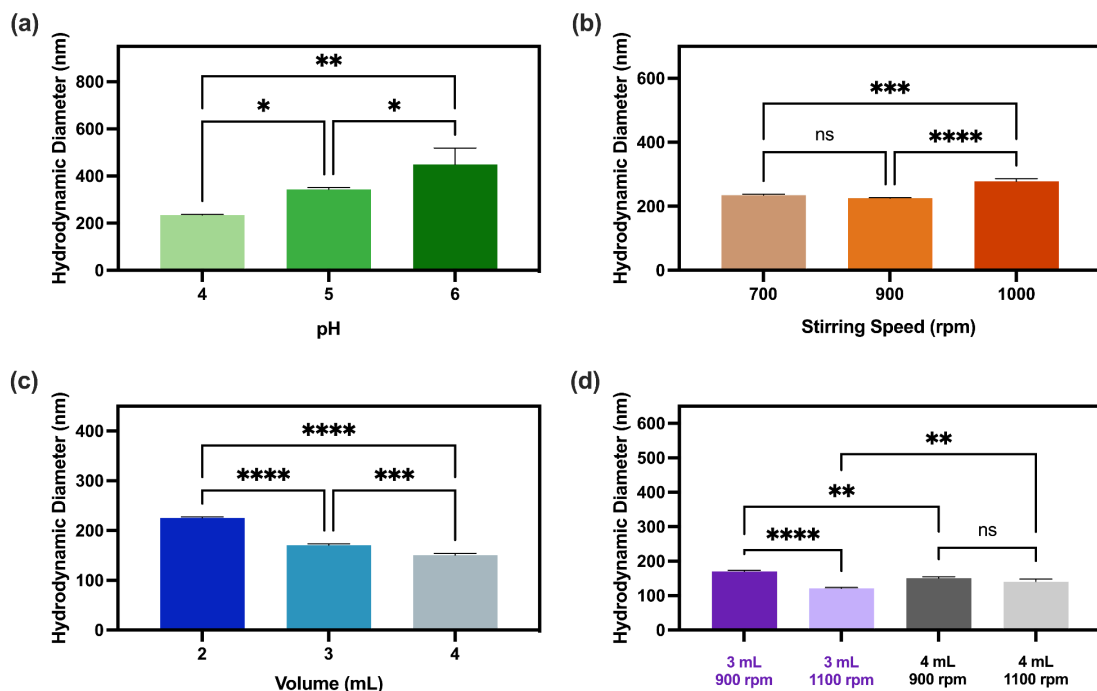


Figure 10. Pilot studies on the *in situ* formation of bacteria-responsive nanogels. (a) Influence of pH on bacteria-responsive nanogel size using a total reaction volume of 2 mL of 1× PBS with stirring at 900 rpm at 60°C. (b) Influence of stirring speed on bacteria-responsive nanogel size using a total reaction volume of 2 mL of 1× PBS (pH 4) at 60°C. (c) Influence of total reaction volume on bacteria-responsive nanogel size using 1× PBS (pH 4) with stirring at 900 rpm at 60°C. (d) Influence of stirring speed on bacteria-responsive nanogel size comparing two different total reaction volumes (3 and 4 mL) in 1× PBS (pH 4) at 60°C.

Further optimization of the colloidal stability of these nanogels would include investigating the influence of surfactants or identifying a surface coating method. Following optimization of nanogel stability, incorporation of antibiotics (free or chemically tethered using our β -lactamase-cleavable technology, detailed in the following section) and the bacteria-responsive release can be examined. Ultimately, the successful fabrication of nanogels using our novel β -lactamase-cleavable moiety shows promise for the development of antibacterial, bacteria-responsive nanogels for the treatment of infection.

Theoretical Strategies for Developing Bacteria-Responsive Nanoparticles and Surface Coatings

Bacteria-Responsive Antibiotic Conjugate

Rendering our bacteria-responsive biomaterials antibacterial will begin by synthesizing a β -lactamase-cleavable vancomycin conjugate by adapting synthetic approaches from our previous report.⁹⁴ In brief, this moiety will be synthesized from a cephalosporin precursor, which contains the β -lactam ring to be hydrolyzed, and flanked on one side by a maleimide and the other by vancomycin, which will undergo click chemistry with a thiol-terminated PEG backbone via Michael-type thiol-ene addition. The synthesis of the responsive vancomycin conjugate, mal- β L-vancomycin, will be accomplished using protocols adapted from Yu, C. et al.⁹⁴ and Umstätter, F. et al.⁹⁵ First, 3-maleimidopropionic acid (1.0 mmol) and 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (ACLE; 0.05 mmol) will be dissolved in 20 mL of dichloromethane (DCM) at room temperature. N,N-diisopropylethylamine (DIPEA; 3.1 mmol) will subsequently be added to deprotonate the carboxylic acid on 3-maleimidopropionic acid and this reaction will be stirred for 15 min under an inert atmosphere. Next, 2.1 mmol of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) will be added to activate the carboxylate group to form an amide bond with the primary amine on ACLE, leading to the formation of compound **1**, shown in **Figure 11** below. Compound **1** will be purified using flash chromatography (silica gel; 50-100% ethyl acetate (EtAc) in DCM) and dried under rotary evaporation. Compound **1** (0.07 mmol) will be dissolved in 1 mL

of DCM at 0°C. Triethylamine (TEA; 0.14 mmol) will be added slowly over 20 min under an inert atmosphere which will serve to deprotonate the carboxylic acid on vancomycin. 4-methylmorpholine (NMM; 0.1 mmol) and vancomycin (0.1 mmol) will then sequentially be added. NMM will replace the 3'-C chlorine on compound **1** to create a positively charged leaving group for nucleophilic substitution by the carboxylate on vancomycin, forming mal-βL-vancomycin. This reaction will continue for 1 h under inert gas, be purified via flash chromatography (silica gel, 1-2% methanol (MeOH) in DCM), then dried under rotary evaporation. Proton and carbon nuclear magnetic resonance (¹H and ¹³C NMR), Fourier-transform infrared spectroscopy (FTIR), and liquid chromatography-mass spectrometry (LC-MS) will be utilized to confirm successful synthesis and purity⁹⁶ of mal-βL-vancomycin.

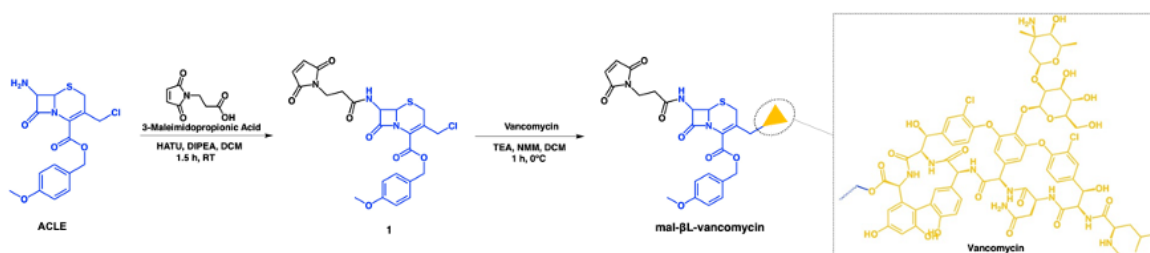


Figure 11. Synthesis of a bacteria-responsive vancomycin conjugate.

Bacteria-Responsive Nanoparticles

Synthesis of PEGylated mal-βL-vancomycin. The responsive vancomycin conjugate, mal-βL-vancomycin, will be functionalized with PEG chains of varying molecular weights on vancomycin's terminal secondary amine group using an adapted protocol from Umstätter, F. *et al.*⁹⁵ To begin, sulfonic acid-terminated PEG (PEG-SO₃H) will be dissolved in 1× PBS and the pH will be adjusted to reach mild alkaline conditions (pH

8-9). Mal- β L-vancomycin will be dissolved in DMSO at 0.5 molar equivalence to PEG-SO₃H, then slowly added to the PEG-SO₃H solution with vigorous stirring for 3 h. The resulting solution will be freeze-dried, redissolved in water, purified via preparative HPLC using a gradient elution,⁹⁵ and analyzed using LC-MS, NMR, and FTIR.

Synthesis of thiolated silica nanoparticles (SiNPs). SiNPs functionalized with thiol groups on the surface will undergo click-chemistry with PEGylated mal- β L-vancomycin to form antibacterial, bacteria-responsive nanoparticles. The thiolated SiNPs will be synthesized using a modified, base-catalyzed Stöber method.⁹⁷ The procedure for synthesizing thiolated SiNPs, adapted from Fernandes, R. *et al.*,⁹⁸ begins with diluting a stock ammonia solution to 0.3 mol L⁻¹ in absolute ethanol then heated to 70°C with slow stirring (~400 rpm). The silica precursor, (3-mercaptopropyl)-trimethoxysilane, is then added to the heated solution of ammonia in ethanol to a final concentration of 0.3 mol L⁻¹ and stirred for 90 min. The particles will be extracted by centrifugation at 15,000 rpm for 1 h, washed in absolute ethanol three times, then re-suspended in deionized water. Dynamic light scattering (DLS) will be used to measure the hydrodynamic diameter of the particles along with the size distribution, or polydispersity index. ζ -potential will be used to measure the electrokinetic potential of SiNPs in a colloidal suspension, providing an estimated surface charge. Size of the SiNPs will also be confirmed using transmission electron microscopy (TEM). To ensure the SiNPs are functionalized with thiols at the surface, environmental scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy (eSEM-EDS) can be used. eSEM-EDS is both a microscopy and elemental analysis technique to identify the atoms present in a sample. The degree of

thiol-functionalization can be determined by performing an Ellman's assay⁹⁹ to inform conjugation of our responsive vancomycin conjugate. This assay uses 5,5'-dithio-bis-(2-nitrobenzoic acid) which readily reacts with free sulfhydryl (thiol) groups to produce a colorimetric change which can be quantified via spectroscopy in the ultraviolet-visible light region (300-700 nm). Comparing the absorbance values to a standard curve of a known sulfhydryl-containing compound (i.e., cysteine)⁹⁹ will provide the estimated sulfhydryl concentration in a specific volume of our SiNPs.

PEGylated mal-βL-vancomycin SiNPs. The thiolated SiNPs from above will be conjugated with PEGylated mal-βL-vancomycin to create antibacterial, bacteria-responsive nanoparticles. First, the concentration of sulfhydryl/thiol groups will be determined by Ellman's assay, described previously. The responsive vancomycin conjugate will be dissolved in DMSO and added to the suspension of thiolated SiNPs in a 1:1 stoichiometric ratio under stirring for 15-30 min. As mentioned previously, the responsive vancomycin conjugate will undergo Michael-type addition with the SiNPs and form a covalent bond between the thiol on the SiNPs and maleimide on the conjugate. The nanoparticles will be purified by centrifugation (14,000 rpm, 1 h) and washed with deionized water three times to ensure any free PEGylated mal-βL-vancomycin is removed. Successful conjugation will be confirmed using similar methods described in the previous section.

Bacteria-Responsive Antibacterial Surface Coatings

We will develop antibacterial bacteria-responsive coatings through sol-gel coating with mercaptosilanes and subsequent tethering of the PEGylated vancomycin conjugate. Sol-gel coating offers many advantages to other coatings, such as polymeric films, due to their highly tunable physical and chemical properties.¹⁰⁰ Sol-gel coatings are fabricated using a polycondensation reaction with silica precursors,¹⁰¹ which can be modified to have a range of functional groups. The mechanical strength of the sol-gel coating is also highly tunable through addition of metal ions that complex between neighboring silanes to increase crystallinity of the coating.¹⁰² Additionally, this process occurs in one reaction vessel, making sol-gel coating on an implant substrate a simple process. We will investigate sol-gel coating of medical grade stainless steel and titanium alloy substrates and conjugation of our PEGylated vancomycin conjugate.

Sol-gel coating of metal implant substrates. The substrates mentioned above will be smoothed using 1200-grit silicon carbide polishing paper then ultrasonically washed with deionized water then acetone to remove free surface particles.¹⁰³ Hydrochloric acid (0.1 M) will be used to remove the passivating layer at the surface of the substrates to aid in adhesion of the coating.¹⁰³ The sol-gel coating process will begin by adding the polished metal substrates to a solution of absolute ethanol and water at volume ratios of 2:1, respectively. (3-mercaptopropyl)trimethoxysilane (MPTS) will then be added to achieve a 1:4:8 volumetric ratio of MPTS:water:ethanol.¹⁰⁴ The solution will be stirred for 5-8 h to ensure all components have reacted to form the sol-gel coating on the substrate, washed three times with deionized water, then dried at room temperature.¹⁰⁴

The coatings will be aged (allowed to set at room temperature) for 7-14 days. Following aging, PEGylated mal- β L-vancomycin will be dissolved in DMSO (at a 1:1 stoichiometric ratio with MPTS) then subsequently added to water followed by submerging the sol-gel coated substrate. This solution will be gently shaken at room temperature for 1-2 h to ensure the responsive vancomycin conjugate has reacted with the free thiols on the coating, washed three times in deionized water, and dried at room temperature. Characterization of the surface coatings will begin with small-angle X-ray scattering (SAXS) to verify substrates have been coated and to evaluate the homogeneity of coating.¹⁰⁴ SAXS also indirectly provides an estimate distribution of pore size of the sol-gel coatings to provide further information of homogeneity. We aim to have very homogenous, yet porous, sol-gel coatings to increase surface area and the number of thiols to react with our responsive vancomycin conjugate to effectively eradicate pathogenic bacteria. Atomic force microscopy (AFM) can provide additional information on the microstructure and topology of the coatings.¹⁰⁵ eSEM-EDS will be utilized to confirm successful conjugation of our responsive vancomycin conjugate;¹⁰⁶ more specifically, uncoated, sol-gel coated, and responsive-sol-gel coated substrates will be analyzed by eSEM-EDS and their elemental compositions will be compared to confirm successful conjugation.

For both of these proposed bacteria-responsive biomaterials/drug delivery systems, further characterization and validation studies in terms of their degradation and antibiotic release profiles, antibacterial efficacy, and cytocompatibility will need to be investigated. Methods from this thesis can be adapted to design experiments that validate the

aforementioned bacteria-responsive and antibacterial nature of these novel materials *in vitro*. With the promising results obtained through our robust hydrogel and initial nanogel work, we hypothesize the proposed bacteria-responsive, antibacterial nanoparticles and surface coatings will be achievable and will expand our pipeline of novel bacteria-specific infection treatments.

Appendices

Appendix A: Supporting Information - β -Lactamase-Responsive Hydrogel Drug
Delivery Platform for Bacteria-Triggered Cargo Release
*Authors: Dahlia Alkekhia, **Cassi LaRose**, and Anita Shukla*

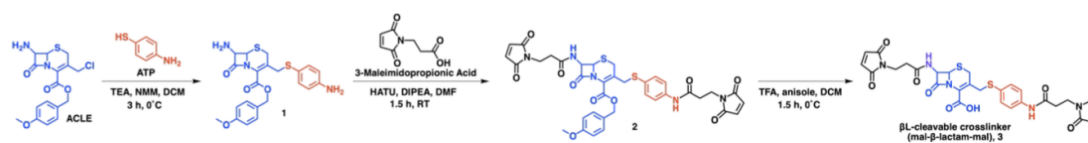


Figure S1: Synthesis route of the β L-cleavable crosslinker, mal- β -lactam-mal.*

*ACLE: 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride; ATP: 4-aminothiophenol; TEA: triethylamine; NMM: N-methylmorpholine; DCM: dichloromethane; HATU: 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; DIPEA: N,N-diisopropylethylamine; DMF: dimethylformamide; TFA: trifluoroacetic acid; β L: β -lactamase.

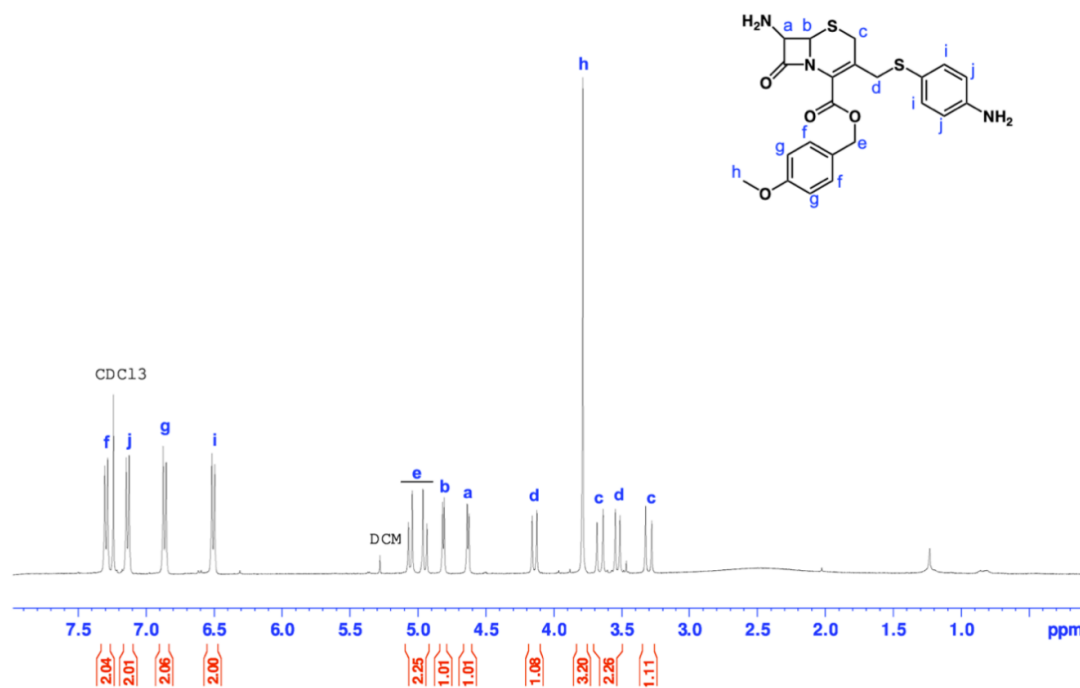


Figure S2: ^1H -NMR spectrum of compound **1** (CDCl₃, 400 MHz).

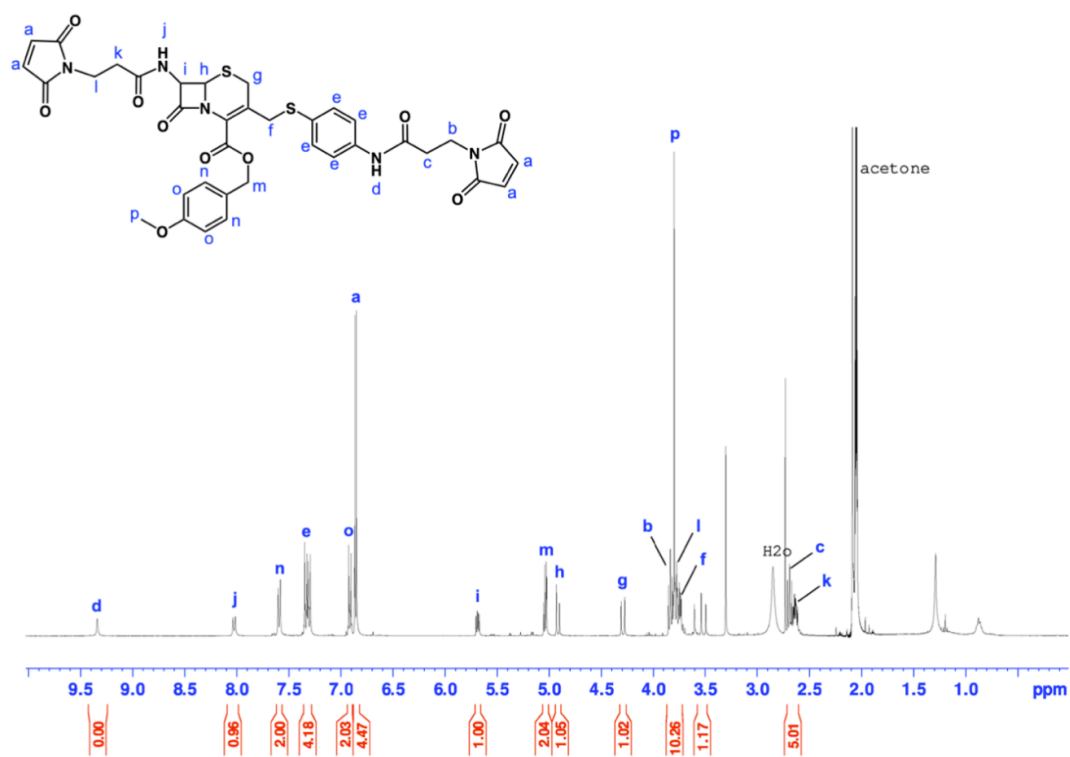


Figure S3: ^1H -NMR spectrum of compound 2 (acetone-d_6 , 400 MHz).

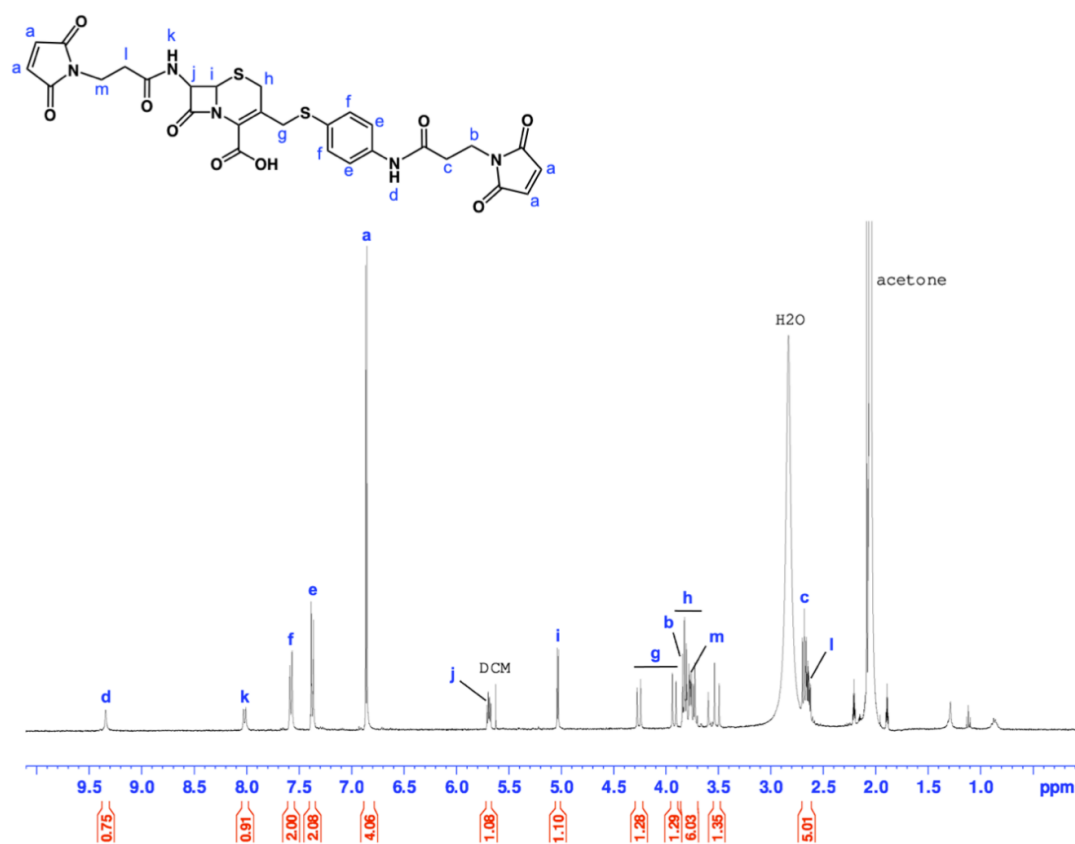


Figure S4: ^1H -NMR spectrum of the responsive crosslinker, mal- β -lactam-mal (compound 3) (acetone-d_6 , 400 MHz).

Appendix B: Peer-reviewed Publication - β -Lactamase-Responsive Hydrogel Drug
Delivery Platform for Bacteria-Triggered Cargo Release
*Authors: Dahlia Alkekhia, **Cassi LaRose**, and Anita Shukla*

β -Lactamase-Responsive Hydrogel Drug Delivery Platform for Bacteria-Triggered Cargo Release

Dahlia Alkekhia, Cassi LaRose, and Anita Shukla*

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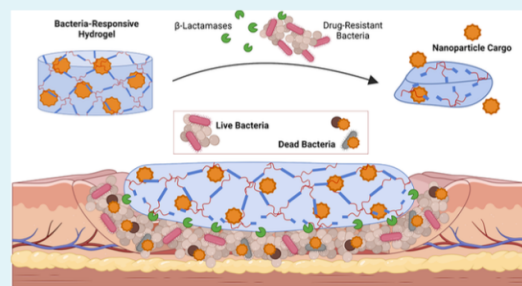
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ABSTRACT: Antibiotic resistance is a growing public health threat that complicates the treatment of infections. β -Lactamase enzymes, which hydrolyze the β -lactam ring present in many common antibiotics, are a major cause of this resistance and are produced by a broad range of bacterial pathogens. Here, we developed hydrogels that degrade specifically in the presence of β -lactamases and β -lactamase-producing bacteria as a platform for bacteria-triggered drug delivery. A maleimide-functionalized β -lactamase-cleavable cephalosporin was used as a crosslinker in the fabrication of hydrogels through end-crosslinked polymerization with multiarm thiol-terminated poly(ethylene glycol) macromers via Michael-type addition. We demonstrated that only hydrogels containing the responsive crosslinker were degraded by β -lactamases and β -lactamase-producing bacteria *in vitro* and in an *ex vivo* porcine skin infection model. Fluorescent polystyrene nanoparticles, encapsulated in the hydrogels as model cargo, were released at rates that closely tracked hydrogel wet mass loss, confirming β -lactamase-triggered controlled cargo release. Nonresponsive hydrogels, lacking the β -lactam crosslinker, remained stable in the presence of β -lactamases and β -lactamase-producing bacteria and exhibited no change in mass or nanoparticle release. Furthermore, the responsive hydrogels remained stable in non- β -lactamase enzymes, including collagenases and lipases. These hydrogels have the potential to be used as a bacteria-triggered drug delivery system to control unnecessary exposure to encapsulated antimicrobials, which can provide effective infection treatment without exacerbating resistance.

KEYWORDS: β -lactamases, hydrogels, bacteria-responsive materials, stimuli-responsive drug delivery, bacteria, enzyme-degradable hydrogels



1. INTRODUCTION

Antibiotic resistance is a global health threat that is making it increasingly difficult to treat bacterial infections. A 2019 report by the United States Centers for Disease Control and Prevention estimated that over 2.8 million infections and over 35,000 deaths are caused by antibiotic-resistant microbes annually in the U.S. alone. Overuse and misuse of antibiotics are implicated in increasing antibiotic resistance and toxicity.¹ As antibiotic resistance continues to rise and the development of new therapeutics remains stagnant, responsive local drug delivery systems have the potential to provide effective infection treatment while controlling exposure to antibiotics, helping limit the development of resistance.^{2,3} In this work, we have developed a stimuli-responsive local drug delivery system that responds to a family of enzymes uniquely produced by bacteria, the β -lactamases.

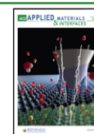
Various antibacterial drug delivery systems have been developed that respond to either exogenous or endogenous stimuli.⁴ Commonly explored external triggers include temperature, light, and magnetic fields. Alternatively, biomaterials that respond to the bacterial microenvironment have been explored

as self-defensive drug delivery systems. Among the most common examples are pH-responsive biomaterials that typically respond to the local decrease in pH caused by lactic and acetic acid-producing bacteria.^{5,6} Other examples include materials that respond to bacterial toxins⁷ and enzymes, such as lipases,^{8,9} proteases,¹⁰ phosphatases,¹¹ phospholipases,¹² and hyaluronidases.^{13–15} However, many of these triggers are not bacteria specific; acidic microenvironments are found naturally within the body or as a result of noninfectious diseases or injury, including wounds and tumors, and some bacterial enzymes have mammalian analogues that cleave similar substrates (e.g., hyaluronidases). In contrast, β -lactamases are produced only by bacteria and are therefore a promising bacteria-specific stimuli that can be adopted for use in bacteria-

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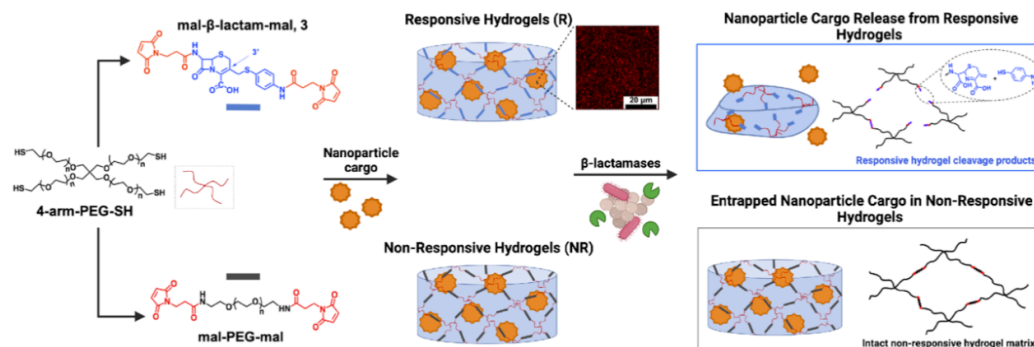
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Scheme 1. Schematic Representation of β -Lactamase-Responsive (R) Hydrogel Fabrication via Thiol–ene Michael-Type Addition, Cleavage of the Hydrogel Backbone Due to β -Lactam Hydrolysis That Enables Release of Nanoparticle Cargo, and Fabrication and Stability of Nonresponsive (NR) Hydrogels



responsive drug delivery. β -lactamases are one of the leading causes of antibiotic resistance.¹⁶ These enzymes, which are produced by many Gram-positive and Gram-negative bacteria, catalyze the hydrolysis of the β -lactam ring present in β -lactam antibiotics (e.g., penicillins, cephalosporins, and carbapenems) and inactivate them. Hydrolysis of the amide bond in the β -lactam ring of cephalosporins causes the spontaneous release of the 3'-position leaving group. This property has been exploited in the development of β -lactamase-responsive prodrugs,¹⁷ for the release of nitric oxide¹⁸ and antibiotics,^{19,20} and for the detection of β -lactamase-producing bacteria in conjunction with various probe molecules.^{21–29} To date, there have been only a handful of studies describing β -lactamase-responsive polymeric biomaterials. This existing work includes nanoparticle drug delivery systems,³⁰ amphiphilic polymers for β -lactamase-triggered hydrogelation as a detection mechanism,³¹ self-healing β -lactamase-degradable supramolecular hydrogels,³² and chromogenic β -lactam-polymer conjugates for bacteria detection.³³

In this work, we report the first responsive hydrogel drug delivery system that selectively releases encapsulated cargo in the presence of β -lactamases. Hydrogels have been used extensively for a variety of biomedical applications, including the treatment of wounds.³⁴ They can hydrate wounds, enhance autolytic debridement, absorb exudate, allow gaseous exchange, and fill deep and irregular wounds, which are all important factors in wound healing. Hydrogels can also be loaded with antibacterial agents to prevent infections, which can delay wound healing and potentially lead to sepsis. Here, we synthesized a β -lactamase-labile crosslinker that was used in a thiol–ene Michael-type addition with multiarm thiol-terminated poly(ethylene glycol) (PEG) macromers to form bacteria-degradable PEG hydrogels (Scheme 1). PEG-based hydrogels are inherently hydrophilic, bioinert, antiprotein-fouling, and nondegradable by mammalian enzymes, with minimal toxicity and inflammation observed *in vivo*.^{35–37} Responsive PEG hydrogels have been fabricated by incorporating enzyme-cleavable moieties, typically peptides, into the matrix for protein delivery and tissue engineering applications.^{38–41} When the responsive PEG hydrogels developed in this work are exposed to β -lactamases, the β -lactam ring in the hydrogel backbone is hydrolyzed, which leads to matrix cleavage and macroscopic degradation of the hydrogel. We demonstrated the selective degradation of these β -lactamase-

responsive hydrogels and release of encapsulated cargo in the presence of β -lactamases and β -lactamase-producing bacteria. We also investigated how hydrogel composition and environmental factors such as β -lactamase concentration and source influence hydrogel degradation and cargo release kinetics. The bacteria-responsive hydrogels developed in this work have the potential to be used as a controlled-release prophylactic technology (e.g., wound dressing) that will enable a truly controlled release of encapsulated therapeutics in the presence of bacteria at a local site and limit exposure to antimicrobial agents, thereby suppressing resistance.

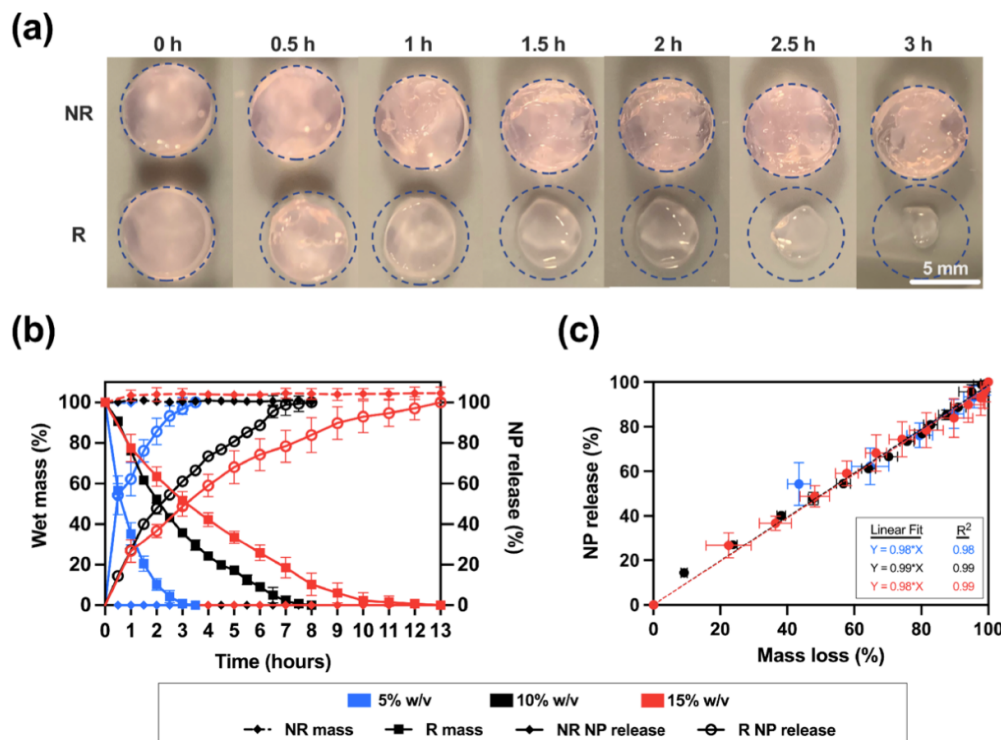
2. RESULTS AND DISCUSSION

2.1. Synthesis and Characterization of β -Lactamase-Responsive Hydrogels. We have developed a responsive hydrogel drug delivery platform for the on-demand release of encapsulated cargo upon β -lactamase-triggered hydrogel degradation (Scheme 1). In order to fabricate β -lactamase-degradable hydrogels, we first synthesized a maleimide-functionalized β -lactamase-cleavable cephalosporin (Figure S1, compound 3). The 4-aminothiophenol (ATP) at the 3'-C site of the 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (ACLE) β -lactam core (Figure S1, compound 1) serves as a leaving group that is expelled in the presence of β -lactamases. The terminal amines on either end of this compound were functionalized with maleimide moieties by conjugating 3-maleimidopropionic acid via amidation, forming compound 2 (Figure S1). After functionalization with maleimides, the carboxylic acid on the β -lactam, which is an enzyme–substrate recognition site for some β -lactamases,⁴² was deprotected by the removal of the *p*-methoxybenzyl protecting group, yielding the β -lactamase-responsive crosslinker, mal- β -lactam-mal (Figure S1, compound 3). Proton nuclear magnetic resonance (¹H NMR) (Figure S2–S4) and high resolution mass spectrometry (HRMS) electrospray ionization (ESI) confirmed successful synthesis of this crosslinker.

Compound 3 was then utilized in the thiol–ene-mediated crosslinking of 4-arm-PEG-thiol macromers, thus incorporating the β -lactamase-responsive moiety into the backbone of the responsive hydrogel network. Control nonresponsive hydrogels were instead fabricated using a maleimide-functionalized PEG crosslinker, mal-PEG-mal (Scheme 1). Hydrogels were fabricated at three different polymer concentrations, 5, 10,

Table 1. Elastic Modulus, Equilibrium Swelling Ratio, and Theoretical Mesh Size of Responsive (R) and Nonresponsive (NR) Hydrogels of Different Polymer Densities, with (+) and without (−) Encapsulated 100 nm Nanoparticles

formulation		elastic modulus (kPa)		equilibrium swelling ratio, Q_e		mesh size (nm)	
PEG % (w/v)	nanoparticles ($\pm$)	R	NR	R	NR	R	NR
5	+	6.6 $\pm$ 0.8	9.6 $\pm$ 0.7	31.8 $\pm$ 1.7	28.3 $\pm$ 1.1	6.2 $\pm$ 0.1	5.9 $\pm$ 0.1
	−	6.0 $\pm$ 0.5	7.8 $\pm$ 0.8	32.3 $\pm$ 2.3	28.7 $\pm$ 1.7	6.2 $\pm$ 0.2	5.9 $\pm$ 0.2
10	+	7.4 $\pm$ 1.5	18.7 $\pm$ 1.0	26.0 $\pm$ 1.2	22.5 $\pm$ 2.9	5.8 $\pm$ 0.1	5.4 $\pm$ 0.3
	−	9.5 $\pm$ 0.3	20.1 $\pm$ 3.5	25.6 $\pm$ 1.7	22.5 $\pm$ 1.5	5.8 $\pm$ 0.2	5.4 $\pm$ 0.2
15	+	17.0 $\pm$ 1.0	34.8 $\pm$ 4.2	22.0 $\pm$ 0.6	16.9 $\pm$ 0.5	5.5 $\pm$ 0.1	4.8 $\pm$ 0.1
	−	26.1 $\pm$ 5.0	35.8 $\pm$ 1.0	23.1 $\pm$ 1.4	16.8 $\pm$ 0.8	5.6 $\pm$ 0.1	4.8 $\pm$ 0.1

**Figure 1.** β -lactamase-triggered hydrogel degradation and nanoparticle (NP) release. (a) Digital images over time of 10% (w/v) nonresponsive (NR) and responsive (R) 100 nm NP-loaded hydrogels incubated with 50 units (U)/mL of β -lactamases from *P. aeruginosa*. The dashed circular overlay represents the original hydrogel circumference. Images are representative of three repeats. (b) Wet mass and NP release over time for 100 nm NP-loaded 5, 10, and 15% (w/v) R and NR hydrogels incubated with 30 U/mL of β -lactamases from *P. aeruginosa*. Wet mass is normalized to the initial mass of the swollen hydrogels before incubation with β -lactamases. NP release is normalized to the NP fluorescence signal measured in the incubation solution after complete hydrogel degradation. (c) Normalized NP release as a function of normalized wet mass loss of 100 nm NP-loaded 5, 10, and 15% (w/v) R hydrogels incubated with 30 U/mL of β -lactamases from *P. aeruginosa*, with each data point representing one time point. The linear fit and coefficient of determination, R^2 , are shown for each formulation in blue [5% (w/v)], black [10% (w/v)], and red [15% (w/v)]. Data are presented as mean $\pm$ standard deviation; $n = 4$.

and 15% (w/v), with or without fluorescent polystyrene nanoparticles to assess controlled release behavior. This model cargo is representative of therapeutic antibacterial nanoparticles, which could be encapsulated in the hydrogel drug delivery platform. We empirically determined the reaction conditions for hydrogel fabrication for the two types of hydrogels individually in order to yield similar reaction times between formulations. We found that responsive hydrogels fabricated at room temperature in 1 $\times$ phosphate buffered saline (PBS) (pH 7) were polymerized within $\sim$ 10 min, while the nonresponsive hydrogels required 0.1 $\times$ PBS (pH 3) to

achieve a similar gelation time. These differences are potentially due to variations in thiol reactivity in both formulations. The carboxylic acid of compound 3, which is negatively charged at responsive hydrogel fabrication conditions (i.e., pH 7), potentially increases the pK_a of the thiols on the 4-arm-PEG-thiol macromers during polymerization, thus decreasing their reactivity. The influence of charged species on thiol reactivity has previously been reported as an approach to tune thiol-ene reaction rates.^{43,44} Increasing the pH for responsive hydrogel polymerization increases the concentration of the reactive thiolates and enables polymer-

ization to proceed more rapidly compared with lower pH conditions.

Upon hydrogel fabrication, we measured the hydrogel crosslinking efficiency, elastic modulus, and equilibrium swelling ratio, and we also estimated the hydrogel mesh size (Figure S5a–d and Table 1). The crosslinking efficiency was found to be ~99% for all formulations. Consistent with previous work, a decrease in PEG concentration led to an increase in swelling and mesh size and a decrease in stiffness.^{45–47} Although differences in reaction times for responsive and nonresponsive formulations were minimized by optimizing gelation buffer concentration and pH, the responsive hydrogels typically exhibited greater swelling ratios and mesh sizes and decreased stiffness compared with nonresponsive hydrogels. The deprotonated carboxylate of the β -lactam in the responsive hydrogels at neutral pH may lead to charge repulsion, thus expanding the network, and can also enable hydrogen bonding with water molecules to contribute to increased water uptake.^{48,49} For hydrogels fabricated with nanoparticles, the concentration of nanoparticles was selected to obtain a similar gelation time as for formulations lacking nanoparticles. In comparing hydrogels with nanoparticles to those without nanoparticles for each polymer concentration, the swelling ratio and average mesh sizes remained largely unchanged, with a decrease in elastic modulus observed only for 15% (w/v) responsive hydrogels with nanoparticles. Scanning electron microscopy images (Figure S6) of 10% (w/v) responsive and nonresponsive hydrogels with and without nanoparticles exhibited no major differences; the presence of nanoparticles was not discernible compared with other surface features and potential drying artifacts.

We also evaluated the rehydration ratio for 5 and 15% (w/v) hydrogels to investigate the ability of dried hydrogels to regain their initial swollen mass when incubated in 1× PBS. As shown in Figure S5e, none of the formulations were able to regain their original postfabrication wet mass and there were no notable differences between hydrogels of a given formulation fabricated with or without nanoparticles. Both responsive and nonresponsive 5% (w/v) hydrogel formulations, with and without encapsulated nanoparticles, reached ~50% of their initial postfabrication wet mass after rehydration. All 15% (w/v) formulations were able to re-swell to ~70–75% of their initial postfabrication wet mass. During hydrogel drying, the micro- and nanostructure of the hydrogel is likely affected by factors such as pore collapse, which can affect rehydration.⁵⁰

2.2. Hydrogel Degradation and Nanoparticle Release in the Presence of β -Lactamases. In order to investigate the behavior of hydrogels in β -lactamases, 10% (w/v) responsive and nonresponsive hydrogels formulated with 100 nm polystyrene nanoparticles were incubated in β -lactamases isolated from *Pseudomonas aeruginosa*. *P. aeruginosa* is a common pathogenic, Gram-negative bacteria that is known to cause a range of acute and chronic infections and to produce multiple classes of β -lactamases.⁵¹ Hydrogels were monitored visually over time, and Figure 1a shows representative images of these hydrogels. Nonresponsive hydrogels did not appear to change visually over three hours of incubation, while responsive hydrogels underwent a clear change in morphology and a decrease in size during this time period, suggesting a β -lactamase-triggered degradation of these hydrogels.

Degradation rates of enzyme-responsive hydrogels depend on the concentration of the enzyme-labile moiety in the

hydrogel matrix and polymer crosslinking density, along with enzyme concentration and enzyme–substrate cleavage kinetics. We next began probing these effects by examining the response of hydrogels fabricated with varying polymer concentrations or in varying concentrations of β -lactamases. Along with examining the hydrogel mass over time as an indicator of degradation, nanoparticle release was also quantified. First, we compared responsive and nonresponsive 5, 10, and 15% (w/v) hydrogels incubated in β -lactamases from *P. aeruginosa* (Figure 1b). All nonresponsive formulations remained stable in these conditions and exhibited no significant change in mass or release of nanoparticles (with a nanoparticle detection limit of 0.3 μ g/mL). These results were expected due to the lack of a β -lactamase-cleavable backbone and the much smaller average mesh size of these hydrogels compared with the nanoparticle diameter of 100 nm leading to physical entrapment. In contrast, all responsive hydrogels exhibited mass loss over time, with complete degradation in ~3.5, 8, and 13 h for 5, 10, and 15% (w/v) hydrogels, respectively (Figure 1b). An overall decrease in the rate of mass loss was observed over time for these formulations, suggesting a surface degradation mechanism as opposed to bulk degradation.^{52,53} Surface erosion is common in enzymatically degradable PEG hydrogels where the enzyme hydrodynamic diameter is on the same order of magnitude as the hydrogel mesh size, which prevents or slows enzyme penetration and diffusion into the hydrogel.³⁸ Many β -lactamases have been characterized as having dimensions that are comparable with the estimated mesh sizes of the hydrogels developed in this work at ~5–6 nm.⁵⁴

We further examined the degradation kinetics of these responsive hydrogels by fitting the hydrogel mass time course using eqs 1–7.

$$m(t) = \rho \pi r(t)^2 h(t) \quad (1)$$

$$r(t) = r_o - kt \quad (2)$$

$$h(t) = h_o - kt \quad (3)$$

$$X = \left(\frac{m_o}{\rho \pi r_{\text{mold}}^2 h_{\text{mold}}} \right)^{1/3} \quad (4)$$

$$r_o = X r_{\text{mold}} \quad (5)$$

$$h_o = X h_{\text{mold}} \quad (6)$$

$$m(t) = \rho \pi [r_o^2 h_o - k r_o (2 h_o + r_o) t + k^2 (2 r_o + h_o) t^2 - k^3 t^3] \quad (7)$$

Here, the mass, $m(t)$, of the cylindrical hydrogels at any time, t , is a function of the hydrogel density, ρ ; radius, $r(t)$; and height, $h(t)$. Eqs 2 and 3 describe the linear change in hydrogel dimensions over time due to surface erosion, assuming a rate constant, k . Here, r_o and h_o are the initial dimensions of the swollen hydrogel. These initial dimensions were estimated by calculating a swelling factor, X , on the basis of the initial hydrogel mass measured, m_o , and the known radius and height of the mold used to fabricate the hydrogels, r_{mold} and h_{mold} , respectively (eqs 4–6). By substituting eqs 2 and 3 in eq 1, eq 7 was obtained to describe the hydrogel mass. Fitting hydrogel mass over time using this equation, we obtained k for 5, 10, and 15% (w/v) responsive hydrogels (Figure S7). As expected, because of the greater crosslinking density of the higher polymer concentration hydrogels, a slower degradation rate

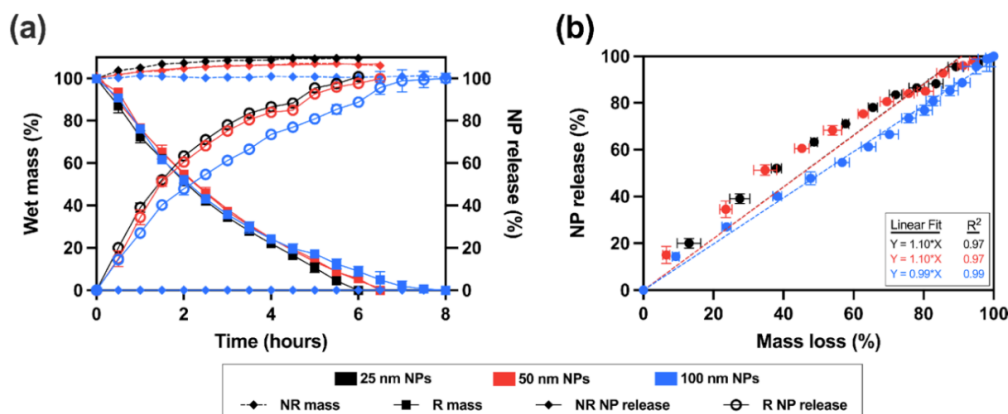


Figure 2. Effect of encapsulated nanoparticle (NP) diameter on β -lactamase-triggered hydrogel degradation and NP release. (a) Normalized wet mass and NP release over time from 10% (w/v) responsive (R) and nonresponsive (NR) NP-loaded hydrogels incubated in solutions of 30 U/mL of β -lactamases from *P. aeruginosa*. Wet mass is normalized to the initial mass of the swollen hydrogels before incubation with β -lactamases. NP release is normalized to the NP fluorescence signal measured in the incubation solution after complete hydrogel degradation. (b) Normalized NP release as a function of normalized wet mass loss of 25, 50, and 100 nm NP-loaded 10% (w/v) R hydrogels with each data point representing one time point. The linear fit and coefficient of determination, R^2 , are shown for each formulation in black (25 nm NPs), red (50 nm NPs), and blue (100 nm NPs). Data are presented as mean $\pm$ standard deviation; $n = 4$.

was noted with increasing polymer concentration, with k ranging from 0.83 h^{-1} to 0.23 h^{-1} for 5 to 15% (w/v) hydrogels, respectively.

Surface erosion typically enables a more controlled release of encapsulated therapeutics compared with bulk degradation, where release in the latter case can be affected by increased hydrogel swelling over time.^{38,55} All responsive hydrogel formulations exhibited a highly linear correlation between release of the encapsulated 100 nm nanoparticles and hydrogel mass loss in β -lactamases from *P. aeruginosa* (Figure 1c). We also investigated the behavior of 10% (w/v) responsive and nonresponsive hydrogels formulated with smaller 25 and 50 nm nanoparticles in β -lactamases from *P. aeruginosa* (Figure 2a). As with the 100 nm nanoparticle-loaded hydrogels, the nonresponsive hydrogels with smaller nanoparticles also exhibited no release of the encapsulated nanoparticles. Nanoparticle release from the responsive hydrogels followed hydrogel degradation, while exhibiting slightly accelerated release kinetics compared with 100 nm nanoparticle-loaded responsive hydrogels (Figure 2b). This increase is likely due simply to the ease of release for smaller particles once surface erosion has begun. Together, these results confirmed the ability to achieve an on-demand, β -lactamase-triggered release of encapsulated cargo (over a broad range of sizes) from these hydrogel drug delivery systems.

We further tested the potential for a controlled, on-demand release, by investigating the feasibility of achieving an on-off response from the responsive hydrogel formulations. This situation represents a scenario in which degradation and cargo release would cease if an infection was cleared but would have the potential to restart if an infection recurred. Hydrogel mass and nanoparticle release were monitored for 10% (w/v) hydrogels with 100 nm nanoparticles that were exposed to alternating incubation steps in β -lactamases from *P. aeruginosa* and 1X PBS every hour (Figure S8). Nonresponsive hydrogels remained stable throughout these incubation steps and exhibited no change in mass or nanoparticle release, as expected. Responsive hydrogels exhibited the desired on-off

response when the enzyme was introduced and removed, respectively, which was most easily observed at the earlier time points.

2.2.1. Effect of β -Lactamase Concentration and Source on Hydrogel Response. Having explored the impact of changes to the hydrogel formulation on β -lactamase-triggered hydrogel degradation and nanoparticle release, we proceeded to examine the impact of β -lactamase concentration and origin on hydrogel behavior. For these studies, we used 10% (w/v) responsive and nonresponsive hydrogels formulated with 100 nm nanoparticles. Figure 3a shows the effect on the hydrogels of increasing the concentration of β -lactamases from *P. aeruginosa*. Nonresponsive hydrogels remained stable, as expected, and exhibited no mass loss or nanoparticle release. For responsive hydrogels, a more rapid degradation of the responsive hydrogels was observed with 50 U/mL versus 30 U/mL of β -lactamase leading to complete degradation in 3.5 h versus 8 h, respectively. In both enzyme concentrations, nanoparticle release and hydrogel degradation exhibited a strong linear correlation (Figure 3b).

We observed that the bacterial source of the β -lactamases had a significant impact on hydrogel degradation rate. There are four major classes of β -lactamases, and at the time of writing, there have already been ~ 7400 different β -lactamases reported.⁵⁶ Bacteria produce varying levels of different β -lactamases in a dynamic manner, which can affect the hydrolysis kinetics of the β -lactamase-cleavable crosslinker in the responsive hydrogel backbone. Specifically, we investigated the hydrogel response in β -lactamases from Gram-positive *Bacillus cereus* and Gram-negative *Enterobacter cloacae*. While nonresponsive hydrogels remained stable in these enzymes, responsive hydrogels incubated in β -lactamases isolated from *B. cereus* and *E. cloacae* required drastically differing concentrations of enzyme to achieve hydrogel degradation on the time scale of hours, which is comparable with what was observed for β -lactamases isolated from *P. aeruginosa* at 30 U/mL (Figure 3c). We observed that hydrogels incubated in 1 U/mL of β -lactamases from *E. cloacae* exhibited a rapid, linear

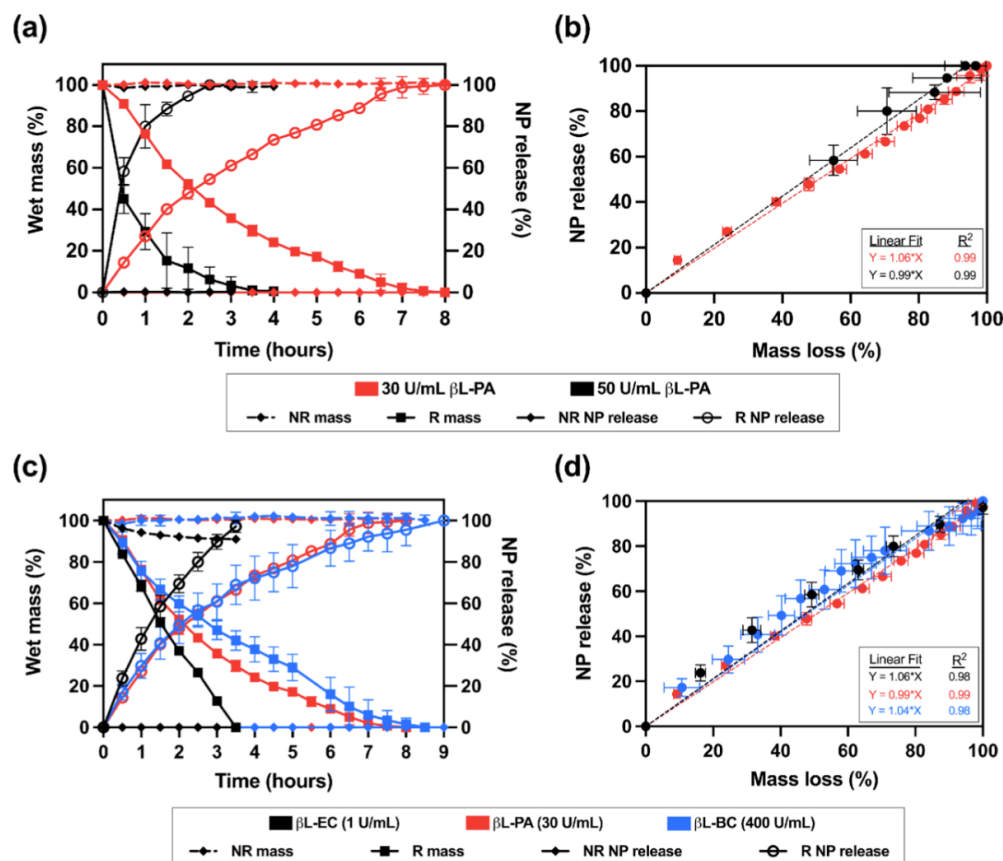


Figure 3. Effect of enzyme concentration and bacterial source on β -lactamase-triggered hydrogel degradation and nanoparticle (NP) release. (a) Wet mass and NP release of 100 nm NP-loaded 10% (w/v) responsive (R) and nonresponsive (NR) hydrogels incubated with 30 U/mL or 50 U/mL of β -lactamases from *P. aeruginosa* (β L-PA). Wet mass is normalized to the initial mass of the swollen hydrogels before incubation with β -lactamases. NP release is normalized to the NP fluorescence signal measured in the incubation solution after complete hydrogel degradation. (b) Normalized NP release as a function of normalized wet mass loss of 100 nm NP-loaded 10% (w/v) R hydrogels incubated with 30 U/mL or 50 U/mL of β L-PA, with each data point representing one time point. The linear fit and coefficient of determination, R^2 , are shown for each enzyme concentration in red (30 U/mL) and black (50 U/mL). (c) Wet mass and NP release of 100 nm NP-loaded 10% (w/v) R and NR hydrogels incubated with either 1 U/mL of β -lactamases from *E. cloacae* (β L-EC), 30 U/mL of β L-PA, or 400 U/mL of β -lactamases from *B. cereus* (β L-BC). (d) Normalized NP release as a function of normalized wet mass loss of 100 nm NP-loaded 10% (w/v) R hydrogels incubated with 1 U/mL of β L-EC, 30 U/mL of β L-PA, or 400 U/mL of β L-BC, with each data point representing one time point. The linear fit and coefficient of determination, R^2 , are shown for each enzyme source in black (β L-EC), red (β L-PA), and blue (β L-BC). Data are presented as mean $\pm$ standard deviation; $n = 4$.

degradation over ~ 3.5 h, while hydrogels incubated in 400 U/mL of β -lactamases from *B. cereus* degraded over ~ 8.5 h. Nanoparticle release was enabled by responsive hydrogel degradation, with the release kinetics following closely with hydrogel mass loss (Figure 3d). Responsive hydrogels formulated with 25 and 50 nm nanoparticles exposed to 1 U/mL of β -lactamases from *E. cloacae* exhibited comparable degradation and nanoparticle release kinetics as the formulations containing 100 nm nanoparticles (Figure S9). We found that eq 7 did a good job of fitting the mass time course for the 100 nm nanoparticle-loaded responsive hydrogels in β -lactamases from *P. aeruginosa* and *B. cereus* at the tested concentrations, although not quite as well as what was observed for responsive hydrogels in β -lactamases from *E. cloacae* (Figure S10), which suggests that these hydrogels may not undergo an entirely surface-erosion-based degradation.

The differences between the hydrogel responses observed may be due to differences in the types of β -lactamases produced by the three bacterial species, as well as the specific strains that were utilized. β -Lactamases exhibit varying specificity and activity toward different β -lactam containing compounds.⁵⁷ For example, some β -lactamases have a larger or more flexible substrate binding pocket compared with other classes of β -lactamases and can better accommodate polymers or bulky moieties near the β -lactam ring.^{25,29,58}

2.2.2. Cytocompatibility of β -Lactamase-Degraded Responsive Hydrogels. We investigated cytocompatibility of the responsive hydrogels by exploring the effect of the degraded hydrogel products on the metabolic activity of murine fibroblasts (NIH 3T3) and human umbilical vein endothelial cells (HUVEC), along with potential hemolysis of human red blood cells (HRBCs). These cells were selected as

Table 2. Bacteria Strains Tested with Responsive and Nonresponsive Hydrogels

Bacteria	Strain	Gram	β -lactamase	Nitrocefin Disk Assay*
<i>S. aureus</i>	ATCC® 25923™	Positive	No	
<i>B. cereus</i>	ATCC® 13061™	Positive	Yes	
<i>P. aeruginosa</i>	ATCC® 27853™	Negative	Yes	
<i>E. cloacae</i>	ATCC® 13047™	Negative	Yes	
<i>K. pneumoniae</i>	Clinical isolate 3834	Negative	Yes	
<i>K. pneumoniae</i>	Clinical isolate 1603	Negative	Yes	
Water	—	—	—	

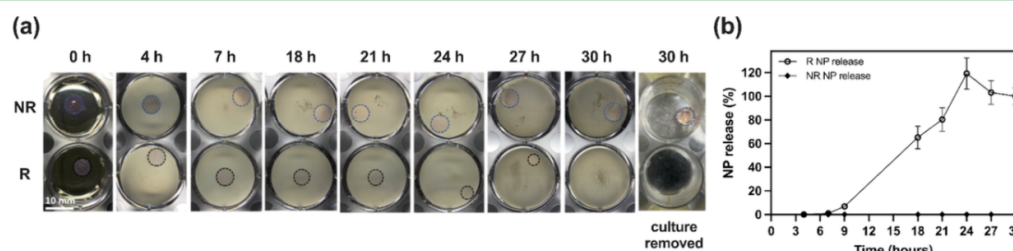
*Red color indicates β -lactamase production.

Figure 4. Hydrogel degradation in β -lactamase-producing *B. cereus* cultures. (a) Representative digital images of 100 nm nanoparticle (NP)-loaded 10% (w/v) responsive (R) and nonresponsive (NR) hydrogels incubated with *B. cereus* cultures over time. The dashed circular overlay outlines the hydrogel circumference. Images are representative of three repeats. Note that the media at 0 h appears translucent as the initial bacteria density of 1×10^6 colony forming unit (CFU)/mL does not visually appear turbid. The last image at 30 h was taken following culture removal to enable a clearer view of the wells. (b) Normalized NP release from R and NR hydrogels incubated in *B. cereus* cultures. NPs were only detected in *B. cereus* cultures incubated with R hydrogels. Data is presented as mean $\pm$ standard deviation; $n = 3$.

representative of mammalian cells that would be encountered in wounds where these hydrogels may eventually be applied. Responsive hydrogels [15% (w/v)] were completely degraded in β -lactamases from *P. aeruginosa*, *B. cereus*, or *E. cloacae*. The mammalian cells were incubated with these degradation products, along with 4-arm-PEG-thiol macromer, responsive crosslinker (mal- β -lactam-mal, compound 3), β -lactamases, and the appropriate positive and negative controls. A CCK-8 assay indicated no significant difference in HUVEC or NIH 3T3 viability for any of the hydrogel degradation products compared with untreated controls (Figure S11a); the polymer backbone, crosslinker, and β -lactamases also had no impact on cell viability (Figure S11b). As expected, HRBC hemolysis was not observed for any of the hydrogel degradation products (Figure S11c). Overall, these results indicate that the responsive hydrogel degradation products are compatible with important mammalian cell types.

2.3. Hydrogel Stability in Non- β -Lactamase Enzymes.

The β -lactamase specificity of the responsive hydrogels was further examined by exposing 10% (w/v) responsive and nonresponsive hydrogels formulated with 100 nm nanoparticles to other enzymes that are commonly observed in wound environments where these hydrogels may eventually be applied to treat potential infections. Specifically, we investigated incubation in collagenases and lipases. Collagenases are produced by bacteria like *P. aeruginosa*^{59–61} as a virulence factor and also by mammalian cells during tissue remodeling and wound healing.^{62,63} Lipases are also produced by

pathogenic bacteria such as *P. aeruginosa*^{8,9,64} and *Staphylococcus aureus*⁶⁵ as virulence factors that play a role in metabolizing lipids. When incubated with lipases or collagenases for 7 days, neither responsive nor nonresponsive hydrogels exhibited a decrease in wet mass (Figure S12), suggesting that our responsive hydrogels exhibit a β -lactamase-specific degradation. Interestingly, while nonresponsive hydrogels exhibited no change in their wet mass over this time, the wet mass of the responsive hydrogel formulations gradually increased. This behavior was also observed in PBS buffer controls that were included (Figure S12). The observed swelling was likely due to increasing network expansion and hydrogen bonding driven by the β -lactam moieties in the responsive hydrogels (Figure S5). Despite the swelling of the responsive hydrogels, no nanoparticle release was detected from these formulations or from the nonresponsive hydrogels in any of the non- β -lactamase test conditions over 7 days at 37 °C, thereby indicating no uncontrolled release. The responsive hydrogels maintained their responsiveness when later incubated in β -lactamases from *P. aeruginosa* (data not shown), indicating the stability of the β -lactam crosslinker under these conditions. Altogether, these results demonstrate the potential of this platform to prevent uncontrolled release and achieve enzyme-triggered release of antibacterial agents.

2.4. Hydrogel Degradation and Nanoparticle Release in the Presence of Bacteria. Having established the β -lactamase-responsive degradation of these hydrogels, we next examined whether this triggered hydrogel degradation is also

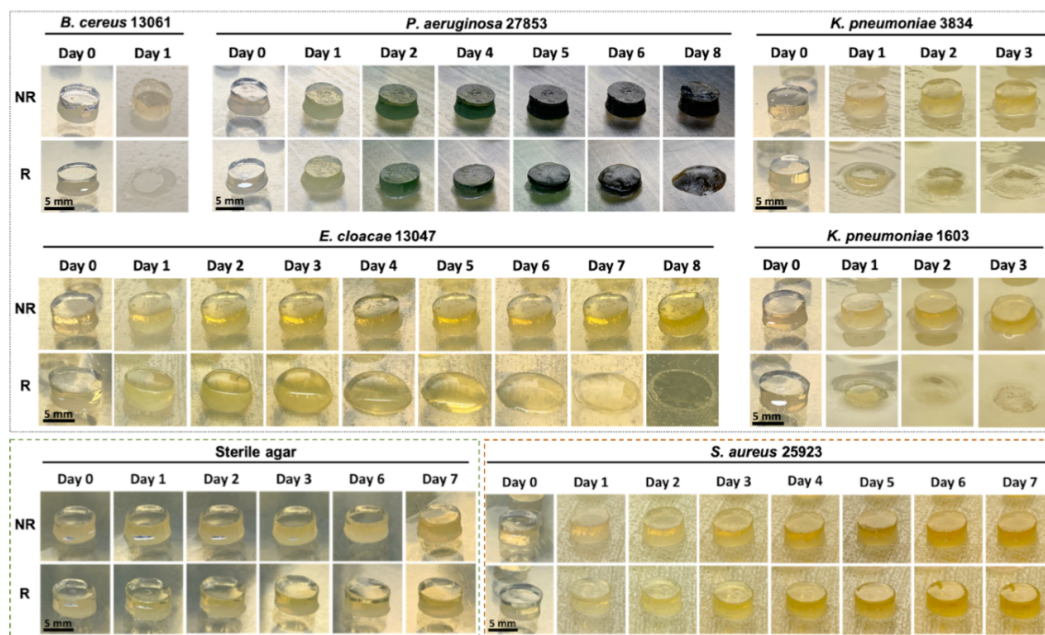


Figure 5. Responsive hydrogel degradation by β -lactamase-producing bacteria on agar. Digital images over time of 10% (w/v) nonresponsive (NR) and responsive (R) hydrogels incubated on agar inoculated with β -lactamase-producing *B. cereus* 13061, *P. aeruginosa* 27853, *E. cloacae* 13047, *K. pneumoniae* 3834, and *K. pneumoniae* 1603; non- β -lactamase-producing *S. aureus* 25923; and a sterile agar control at 37 °C. Images are representative of three repeats.

observed in the presence of β -lactamase-producing bacteria. Both responsive and nonresponsive 10% (w/v) hydrogels were incubated with a range of Gram-positive and Gram-negative bacteria, including several ESKAPE pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* spp.). The highly virulent ESKAPE pathogens are particularly concerning because of the prevalence of antibiotic resistance in these species. Specifically, we examined strains of *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, *E. cloacae*, and *B. cereus* (a non-ESKAPE pathogen). We first categorized the specific strains selected as either β -lactamase-producing or non- β -lactamase-producing by examining nitrocefin hydrolysis and nitrocefin disk color change upon exposure to these bacteria (Table 2). Our results agreed with previous reports confirming β -lactamase-production by *B. cereus* 13061, *P. aeruginosa* 27853, *E. cloacae* 13047, and *K. pneumoniae* 1603 and 3834 and lack of β -lactamase-production by *S. aureus* 25923.

Hydrogels formulated with 100 nm nanoparticles were then examined when incubated with *B. cereus* because of the ease of monitoring nanoparticle release in these cultures (i.e., lack of interfering fluorescence signals). The responsive hydrogels were found to fully degrade in *B. cereus* cultures over 30 h, as demonstrated by their decreasing size (Figure 4a) and accumulation of nanoparticles released into the culture over time (Figure 4b). In contrast, no changes were detected in nonresponsive hydrogels incubated with *B. cereus*, and no nanoparticle release was observed (Figure 4). These results further confirmed the degradation and triggered cargo release achieved by the responsive hydrogels in β -lactamase-producing bacteria.

Next, we investigated β -lactamase-triggered degradation of hydrogels incubated with bacteria cultured on agar (Figure 5). Similar to the results observed for hydrogels incubated with *B. cereus* bacterial suspensions, responsive hydrogels degraded on agar inoculated with *B. cereus*, while nonresponsive hydrogels did not. Responsive hydrogels also underwent degradation over time with all other β -lactamase-producing bacteria examined, including the *K. pneumoniae* clinical isolates, while nonresponsive hydrogels remained stable and visibly unchanged on the agar during this time. Responsive and nonresponsive hydrogels incubated on the non- β -lactamase-producing *S. aureus* 25923 behaved identically and remained stable and unchanged on the bacteria inoculated agar over 7 days. These results matched controls of hydrogels incubated on sterile, non-bacteria-inoculated agar over 7 days. The time scales of degradation observed for responsive hydrogels on agar inoculated with the β -lactamase-producing strains varied widely, ranging from a day for *B. cereus* to 2–3 days for both *K. pneumoniae* clinical isolates to ~8 days for *P. aeruginosa* and *E. cloacae* (Figure 5). Note, the green-blue color seen in the hydrogels incubated on *P. aeruginosa* is likely the pyocyanin and pyoverdine metabolites secreted by *P. aeruginosa*,^{66,67} which can change the color of media and agar and here also seems to diffuse into the hydrogels. There are many factors that may contribute to the differences in degradation time observed, including differences in β -lactamase composition and concentration produced by these strains, which may also be influenced by differences in bacterial growth rates in the specific media and growth conditions used. Overall, these results indicate that the β -lactamase-responsive hydrogels retain their enzyme-responsive degradation capability in the presence of a broad range of β -lactamase-producing bacteria.

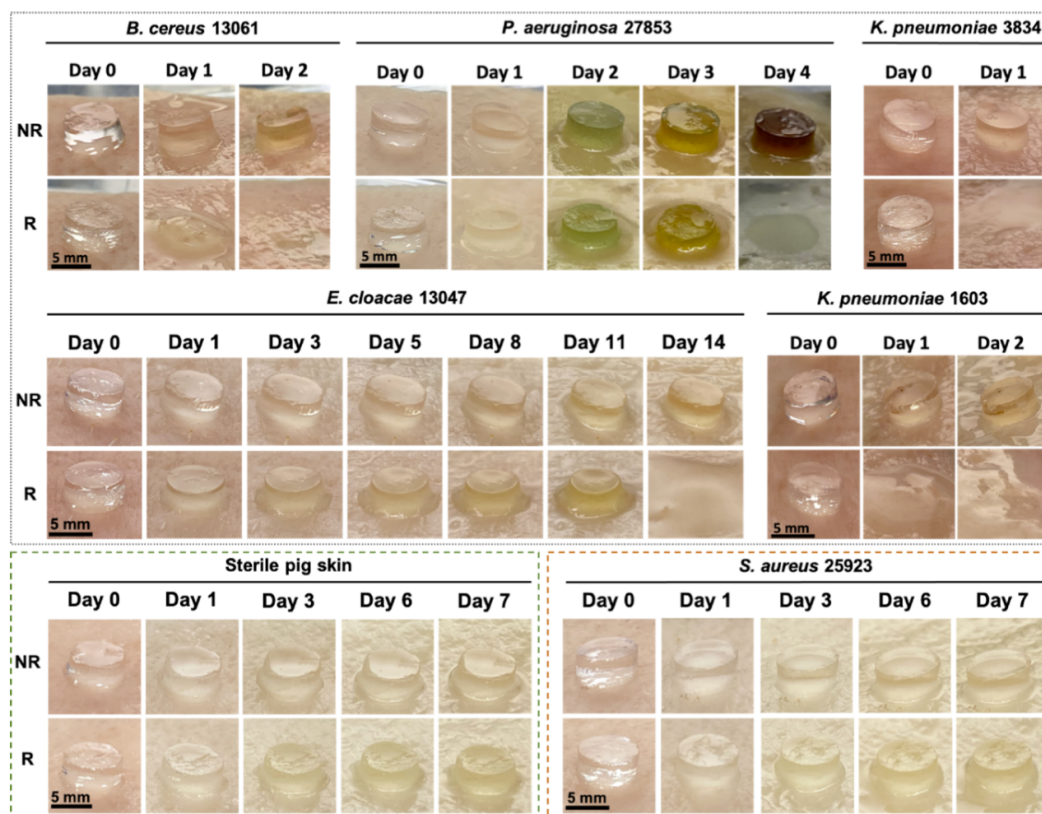


Figure 6. Responsive hydrogel degradation by β -lactamase-producing bacteria on *ex vivo* porcine skin. Digital images over time of 10% (w/v) nonresponsive (NR) and responsive (R) hydrogels incubated on porcine *ex vivo* skin tissue inoculated with β -lactamase-producing *B. cereus* 13061, *P. aeruginosa* 27853, *E. cloacae* 13047, *K. pneumoniae* 3834, *K. pneumoniae* 1603; non- β -lactamase-producing *S. aureus* 25923; and a sterile agar control at 37 °C. Images are representative of three repeats.

and that non- β -lactamase-cleavable hydrogels remain stable in these conditions.

2.4.1. Hydrogel Response in an Ex Vivo Infection Model.

After demonstrating the robust β -lactamase response of the hydrogels developed here *in vitro*, we investigated the translational potential of the hydrogels using model infections of *ex vivo* porcine skin tissue. *Ex vivo* porcine skin inoculated with bacteria is commonly used as a model to study wound infections that closely mimic superficial tissue infections.⁶⁸ As shown in Figure 6, again, only the responsive hydrogels degraded when incubated on porcine skin inoculated with the β -lactamase-producing *B. cereus*, *P. aeruginosa*, *K. pneumoniae*, and *E. cloacae*, while the nonresponsive hydrogels remained stable. As with the agar studies, responsive and nonresponsive hydrogels remained stable and visibly unchanged on *S. aureus* 25923 inoculated and sterile porcine tissue. These results further confirmed the selective degradability of the hydrogels by β -lactamase-producing bacteria. Interestingly, degradation induced by *P. aeruginosa* was faster on the skin (~4 days) than on agar (~8 days). A potential reason for this more rapid degradation by *P. aeruginosa* may be a higher bacteria growth rate on skin compared with agar, which has been observed in an *ex vivo* human skin model infected with *P. aeruginosa* PAO1,⁶¹ potentially resulting in a higher concentration of β -

lactamases. *K. pneumoniae* inoculated samples also demonstrated a faster hydrogel degradation on pig skin (1–2 days) compared with agar (~3 days), while *E. cloacae* demonstrated slower degradation on pig skin (~14 days) than on agar (~8 days). Future *in vivo* investigation of these materials will enable further understanding of the differences in hydrogel degradation rates on the basis of the type of bacteria and β -lactamases present and also factors such as host immune response and wound healing.

3. CONCLUSIONS

In this work, a β -lactamase-cleavable crosslinker was synthesized and used to fabricate β -lactamase-degradable hydrogels. These responsive hydrogels are the first hydrogel drug delivery platform demonstrated to achieve a β -lactamase-controlled release of encapsulated cargo. Responsive degradation of the hydrogels was observed in the presence of β -lactamases isolated from several different bacteria as well as β -lactamase-producing bacteria (including several ESKAPE species) both *in vitro* and in an *ex vivo* porcine skin infection model. In the conditions investigated, it was observed that nanoparticle cargo release from responsive hydrogels was enabled only by β -lactamase-triggered hydrogel degradation. Hydrogels incubated in other common enzymes found in

wounds, namely collagenases and lipases, did not degrade or release nanoparticles, demonstrating selectivity for β -lactamases. This bacterial enzyme-responsive hydrogel has the potential to be used for the on-demand delivery of encapsulated antimicrobial nanoparticles. A broad range of nanoparticle cargo that is larger than the average hydrogel mesh size could be included, such as liposomes, micelles, nanogels, silver nanoparticles, and other polymeric drug carriers (e.g., drug-loaded or drug-coated nanoparticles and cationic nanoparticles). The hydrogel would thus prevent uncontrolled release of the encapsulated therapeutics and ensure triggered release only upon matrix cleavage by β -lactamases. Our results have demonstrated that a wide range of cargo sizes (25 to 100 nm) can achieve a triggered release using this hydrogel system. These hydrogels hold great promise for use as prophylactic biomaterial, such as a wound dressing that can deliver encapsulated therapeutics on-demand when triggered by the presence of pathogenic, β -lactamase-producing bacteria. The mechanism of action of this drug delivery system will allow for reduced unnecessary exposure to encapsulated therapeutics and effective local delivery, which can reduce off-site toxicity and lower susceptibility for antibiotic resistance development.

4. METHODS

4.1. Synthesis and Characterization of β -Lactamase-Cleavable Crosslinker. Figure S1 provides the reaction scheme for the synthesis of the β -lactamase-responsive crosslinker, mal- β -lactam-mal (compound 3), used in the fabrication of β -lactamase-responsive hydrogels. Details of the syntheses for compounds 1, 2, and 3 are provided in the Supporting Information.

4.2. Hydrogel Fabrication. Responsive and nonresponsive hydrogels were formed using the β -lactamase-cleavable crosslinker, mal- β -lactam-mal (compound 3), and a noncleavable crosslinker, mal-PEG-mal, respectively. The appropriate crosslinker was dissolved in dimethyl sulfoxide (DMSO). 4-arm-PEG-thiol was dissolved in 1× PBS (pH 7) or in 0.1× PBS (pH 3) for responsive or nonresponsive hydrogel fabrication, respectively. These buffer concentrations and pH values were selected to achieve similar gelation times between the two hydrogel types. Hydrogels of varying polymer density [5, 10, and 15% (w/v)] were formed by mixing the crosslinker and PEG at a 1:1 maleimide/thiol stoichiometric ratio with the resulting hydrogel containing 25% (w/w) DMSO in PBS. The prepolymer mixture was rapidly vortexed, and 50 μ L was transferred into a 2 mm (height) $\times$ 5 mm (diameter) cylindrical polydimethylsiloxane (PDMS) mold. Gelation occurred within $\sim$ 10 min; however, samples were left for 1.5 h at 37 $^{\circ}$ C to ensure complete gelation throughout the full hydrogel volume. For nanoparticle-loaded hydrogel studies, fluorescent polystyrene nanoparticles of the desired size were added to the prepolymer PEG solution at a final concentration of 0.08% (w/v). Hydrogel crosslinking efficiency, swelling, rehydration, and mechanical properties assessment methods are described in Supporting Information.

4.3. Examining Hydrogel Response in Enzyme Solutions. The hydrogel degradation response and nanoparticle release in enzyme solutions were investigated by fabricating 5, 10, and/or 15% (w/v) responsive and nonresponsive hydrogels with 100 nm polystyrene nanoparticles and then swelling the hydrogels in 2 mL of 1× PBS (pH 7.4) at 37 $^{\circ}$ C with shaking (100 rpm) for $\sim$ 16–18 h to remove DMSO (and potential surface bound nanoparticles).

These swollen hydrogels were incubated with β -lactamases in 1 mL of 1× PBS (pH 7.4) at 37 $^{\circ}$ C with shaking (100 rpm). For all β -lactamases tested, activity was reported by the vendor with one unit (U) defined as hydrolyzing 1.0 μ mol of benzylpenicillin per min at pH 7.0 at 25 $^{\circ}$ C. At predetermined time points, hydrogels were removed from the β -lactamase solution and weighed. Simultaneously, 150 μ L of the incubation solution was removed and nanoparticle fluorescence

(excitation 580 nm, emission 607 nm) was measured. This fluorescence signal was compared with standard curves of just nanoparticles in enzyme solution to determine released nanoparticle concentration from the hydrogels. These incubation solutions and hydrogels were placed back into the β -lactamase solution for continued monitoring of degradation and release.

Hydrogels [10% (w/v) fabricated with 100 nm polystyrene nanoparticles] were similarly examined for potential nonspecific degradation in collagenases and lipases. Collagenase solutions were formulated at 0.1 and 1 mg/mL in 1× PBS (pH 7.4) with 1 mM of calcium chloride (note, 1 mg contains 229 collagen digestion units (CDUs), where 1 CDU liberates peptides from collagen from bovine achilles tendon equivalent to 1.0 μ mol of leucine in 5 h at pH 7.4 at 37 $^{\circ}$ C in the presence of calcium). Lipase solutions were formulated at 1.4 mg/mL in 1× PBS (pH 7.4) (note, 1 mg contains 35 units, where 1 unit liberates 1 μ mol of oleic acid per min at pH 8.0 and 40 $^{\circ}$ C). Potential contamination was prevented by adding 0.02% (w/v) sodium azide to these enzyme solutions, which were refreshed every 24 h over the course of 168 h.

4.4. Examining Hydrogel Response to Bacteria. Hydrogel response was examined upon incubation of 10% (w/v) hydrogels with β -lactamase-producing *B. cereus* 13061, *P. aeruginosa* 27853, *E. cloacae* 13047, *K. pneumoniae* 1603 and 3834 and non- β -lactamase-producing *S. aureus* 25923. All bacteria were utilized in their logarithmic growth phase in tryptic soy broth (TSB), as determined by optical density measurements at 600 nm. For all assays, responsive and non-responsive hydrogels were swollen after fabrication, as done for the hydrogel response studies in enzyme solutions, and then sterilized via ultraviolet light in a Nuaire Class II Type A2 biosafety cabinet for 15 min at RT. Details of hydrogel response assessment methods in bacterial growth media, on agar, and on porcine skin are provided in the Supporting Information.

4.5. Statistical Analysis. Results are reported as mean $\pm$ standard deviation whenever appropriate. All experiments were repeated three times with three samples at minimum. Statistical significance was calculated using unpaired, two-tailed Student's *t* test or one- or two-way analysis of variance (ANOVA; $\alpha = 0.05$) with Tukey's posthoc analysis using GraphPad PRISM software. A value of $p < 0.05$ was considered statistically significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. MATLAB was used for data fitting and obtaining goodness of fit.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsami.2c02614>.

Experimental sections including materials, instrumentation, and β -lactamase-responsive crosslinker synthesis scheme and details; 1 H NMR of all compounds; methods of assessment and results of hydrogel morphology and physical properties; methods of determining hydrogel response in bacterial media, on nutrient agar, and on porcine skin; methods of evaluating cytocompatibility and results; surface degradation modeling; on–off behavior of responsive hydrogels; and stability of hydrogels in common wound enzymes (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Llor, C.; Bjerrum, L. Antimicrobial Resistance: Risk Associated with Antibiotic Overuse and Initiatives to Reduce the Problem. *Ther. Adv. Drug Saf.* **2014**, *5*, 229–241.
- (2) Brooks, B. D.; Brooks, A. E. Therapeutic Strategies to Combat Antibiotic Resistance. *Adv. Drug Delivery Rev.* **2014**, *78*, 14–27.
- (3) Aslam, B.; Wang, W.; Arshad, M. I.; Khurshid, M.; Muzammil, S.; Rasool, M. H.; Nisar, M. A.; Alvi, R. F.; Aslam, M. A.; Qamar, M. U.; Salamat, M. K. F.; Baloch, Z. Antibiotic Resistance: A Rundown of a Global Crisis. *Infect. Drug Resist.* **2018**, *11*, 1645–1658.
- (4) Deussenbery, C.; Wang, Y.; Shukla, A. Recent Innovations in Bacterial Infection Detection and Treatment. *ACS Infect. Dis.* **2021**, *7*, 695–720.
- (5) Zhuk, I.; Jariwala, F.; Attygalle, A. B.; Wu, Y.; Libera, M. R.; Sukhishvili, S. a. Self-Defensive Layer-by-Layer Films with Bacteria-Triggered Antibiotic Release. *ACS Nano* **2014**, *8*, 7733–7745.
- (6) Haidari, H.; Kopecki, Z.; Sutton, A. T.; Garg, S.; Cowin, A. J.; Vasilev, K. pHResponsive “Smart” Hydrogel for Controlled Delivery of Silver Nanoparticles to Infected Wounds. *Antibiotics* **2021**, *10*, 49.
- (7) Pornpattananakul, D.; Zhang, L.; Olson, S.; Aryal, S.; Obonyo, M.; Vecchio, K.; Huang, C.-M.; Zhang, L. Bacterial Toxin-Triggered Drug Release from Gold Nanoparticle-Stabilized Liposomes for the Treatment of Bacterial Infection. *J. Am. Chem. Soc.* **2011**, *133*, 4132–4139.
- (8) Chen, M.; Xie, S.; Wei, J.; Song, X.; Ding, Z.; Li, X. Antibacterial Micelles with Vancomycin-Mediated Targeting and pH/Lipase-Triggered Release of Antibiotics. *ACS Appl. Mater. Interfaces* **2018**, *10*, 36814–36823.
- (9) Singh, H.; Li, W.; Kazemian, M. R.; Yang, R.; Yang, C.; Logsetty, S.; Liu, S. Lipase-Responsive Electrospun Theranostic Wound Dressing for Simultaneous Recognition and Treatment of Wound Infection. *ACS Appl. Bio Mater.* **2019**, *2*, 2028–2036.
- (10) Johnson, C. T.; Wroe, J. A.; Agarwal, R.; Martin, K. E.; Guldberg, R. E.; Donlan, R. M.; Westblade, L. F.; Garcia, A. J. Hydrogel Delivery of Lysozyme Eliminates Orthopedic Implant Infection by *Staphylococcus aureus* and Supports Fracture Healing. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, E4960–E4969.
- (11) Kang, E. B.; Mazrad, Z. A. I.; Robby, A. I.; In, I.; Park, S. Y. Alkaline Phosphatase-Responsive Fluorescent Polymer Probe Coated Surface for Colorimetric Bacteria Detection. *Eur. Polym. J.* **2018**, *105*, 217–225.
- (12) Xiong, M.-H.; Li, Y.-J.; Bao, Y.; Yang, X.-Z.; Hu, B.; Wang, J. Bacteria-Responsive Multifunctional Nanogel for Targeted Antibiotic Delivery. *Adv. Mater.* **2012**, *24*, 6175–6180.
- (13) Alkekhaia, D.; Shukla, A. Influence of Poly-L-Lysine Molecular-Weight on Antibacterial Efficacy in Polymer Multilayer Films. *J. Biomed. Mater. Res. Part A* **2019**, *107*, 1324–1339.
- (14) Francesko, A.; Fernandes, M. M.; Ivanova, K.; Amorim, S.; Reis, R. L.; Pashkuleva, I.; Mendoza, E.; Pfeifer, A.; Heinze, T.; Tzanov, T. Bacteria-Responsive Multilayer Coatings Comprising Polycationic Nanospheres for Bacteria Biofilm Prevention on Urinary Catheters. *Acta Biomater* **2016**, *33*, 203–212.
- (15) Cado, G.; Aslam, R.; Séon, L.; Garnier, T.; Fabre, R.; Parat, A.; Chassepot, A.; Voegel, J.-C.; Senger, B.; Schneider, F.; Frère, Y.; Jierry, L.; Schaaf, P.; Kerdjoudj, H.; Metz- Boutigue, M.-H.; Boulmedais, F. Self-Defensive Biomaterial Coating Against Bacteria and Yeasts: Polysaccharide Multilayer Film with Embedded Antimicrobial Peptide. *Adv. Funct. Mater.* **2013**, *23*, 4801–4809.
- (16) Bush, K. Past and Present Perspectives on β -Lactamases. *Antimicrob. Agents Chemother.* **2018**, *62*, 01076–18.
- (17) Perez-Inestrosa, E.; Suau, R.; Montanez, M. I.; Rodriguez, R.; Mayorga, C.; Torres, M. J.; Blanca, M. Cephalosporin Chemical Reactivity and Its Immunological Implications. *Curr. Opin. Allergy Clin. Immunol.* **2005**, *5*, 323–330.
- (18) Yepuri, N. R.; Barraud, N.; Mohammadi, N. S.; Kardak, B. G.; Kjelleberg, S.; Rice, S. A.; Kelso, M. J. Synthesis of Cephalosporin-3-Diazoniumdiolates: Biofilm Dispersing NO-Donor Prodrugs Activated by β -lactamase. *Chem. Commun.* **2013**, *49*, 4791.
- (19) Evans, L. E.; Krishna, A.; Ma, Y.; Webb, T. E.; Marshall, D. C.; Tooke, C. L.; Spencer, J.; Clarke, T. B.; Armstrong, A.; Edwards, A. M. Exploitation of Antibiotic Resistance as a Novel Drug Target: Development of a β -Lactamase-Activated Antibacterial Prodrug. *J. Med. Chem.* **2019**, *62*, 4411–4425.
- (20) Liu, R.; Miller, P. A.; Vakulenko, S. B.; Stewart, N. K.; Boggess, W. C.; Miller, M. J. A Synthetic Dual Drug Sideromycin Induces Gram-Negative Bacteria to Commit Suicide with a Gram-Positive Antibiotic. *J. Med. Chem.* **2018**, *61*, 3845–3854.
- (21) Shao, Q.; Xing, B. Enzyme Responsive Luminescent Ruthenium(II) Cephalosporin Probe for Intracellular Imaging and Photoinactivation of Antibiotics Resistant Bacteria. *Chem. Commun.* **2012**, *48*, 1739–1741.
- (22) Van Berkel, S. S.; Brem, J.; Rydzik, A. M.; Salimraj, R.; Cain, R.; Verma, A.; Owens, R. J.; Fishwick, C. W.; Spencer, J.; Schofield, C. J. Assay Platform for Clinically Relevant Metallo- β -lactamases. *J. Med. Chem.* **2013**, *56*, 6945–6953.
- (23) Cheng, Y.; Xie, H.; Sule, P.; Hassounah, H.; Graviss, E. A.; Kong, Y.; Cirillo, J. D.; Rao, J. Fluorogenic Probes with Substitutions at the 2 and 7 Positions of Cephalosporin are Highly BlaC-Specific for Rapid *Mycobacterium tuberculosis* Detection. *Angew. Chemie Int. Ed.* **2014**, *53*, 9360–9364.
- (24) Xiao, J.-M.; Feng, L.; Zhou, L.-S.; Gao, H.-Z.; Zhang, Y.-L.; Yang, K.-W. Novel Fluorescent Cephalosporins: Synthesis, Antimicrobial Activity and Photodynamic Inactivation of Antibiotic Resistant Bacteria. *Eur. J. Med. Chem.* **2013**, *59*, 150–159.
- (25) Xie, H.; Mire, J.; Kong, Y.; Chang, M.; Hassounah, H. A.; Thornton, C. N.; Sacchetti, J. C.; Cirillo, J. D.; Rao, J. Rapid Point-of-Care Detection of the Tuberculosis Pathogen Using a BlaC-Specific Fluorogenic Probe. *Nat. Chem.* **2012**, *4*, 802–809.
- (26) Cheng, Y.; Xie, J.; Lee, K.-H.; Gaur, R. L.; Song, A.; Dai, T.; Ren, H.; Wu, J.; Sun, Z.; Banaei, N.; Akin, D.; Rao, J. Rapid and Specific Labeling of Single Live *Mycobacterium tuberculosis* with a

Dual-Targeting Fluorogenic Probe. *Sci. Transl. Med.* **2018**, *10*, No. eaar4470.

(27) Kong, Y.; Yao, H.; Ren, H.; Subbian, S.; Cirillo, S. L. G.; Sacchetti, J. C.; Rao, J.; Cirillo, J. D. Imaging Tuberculosis with Endogenous β -Lactamase Reporter Enzyme Fluorescence in Live Mice. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 12239–12244.

(28) Chen, Y.; Xianyu, Y.; Wu, J.; Zheng, W.; Rao, J.; Jiang, X. Point-of-Care Detection of β -Lactamase in Milk with a Universal Fluorogenic Probe. *Anal. Chem.* **2016**, *88*, 5605–5609.

(29) Chan, H. L.; Lyu, L.; Aw, J.; Zhang, W.; Li, J.; Yang, H.-H.; Hayashi, H.; Chiba, S.; Xing, B. Unique Fluorescent Imaging Probe for Bacterial Surface Localization and Resistant Enzyme Imaging. *ACS Chem. Biol.* **2018**, *13*, 1890–1896.

(30) Li, Y.; Liu, G.; Wang, X.; Hu, J.; Liu, S. Enzyme-Responsive Polymeric Vesicles for Bacterial-Strain-Selective Delivery of Antimicrobial Agents. *Angew. Chemie Int. Ed.* **2016**, *55*, 1760–1764.

(31) Yang, Z.; Ho, P.-L.; Liang, G.; Chow, K. H.; Wang, Q.; Cao, Y.; Guo, Z.; Xu, B. Using β -Lactamase to Trigger Supramolecular Hydrogelation. *J. Am. Chem. Soc.* **2007**, *129*, 266–267.

(32) Yu, C.; Alkekha, D.; Shukla, A. β -Lactamase Responsive Supramolecular Hydrogels with Host-Guest Self-Healing Capability. *ACS Appl. Polym. Mater.* **2020**, *2*, 55–65.

(33) Alkekha, D.; Safford, H.; Shukla, S.; Hopson, R.; Shukla, A. β -Lactamase Triggered Visual Detection of Bacteria Using Cephalosporin Functionalized Biomaterials. *Chem. Commun.* **2020**, *56*, 11098–11101.

(34) Caló, E.; Khutoryanskiy, V. V. Biomedical Applications of Hydrogels: A Review of Patents and Commercial Products. *Eur. Polym. J.* **2015**, *65*, 252–267.

(35) García, A. J. PEG-Maleimide Hydrogels for Protein and Cell Delivery in Regenerative Medicine. *Ann. Biomed. Eng.* **2014**, *42*, 312–322.

(36) Kamoun, E. A.; Kenawy, E.-R. S.; Chen, X. A Review on Polymeric Hydrogel Membranes for Wound Dressing Applications: PVA-Based Hydrogel Dressings. *J. Adv. Res.* **2017**, *8*, 217–233.

(37) Wang, Z.; Scheres, L.; Xia, H.; Zuilhof, H. Developments and Challenges in Self-Healing Antifouling Materials. *Adv. Funct. Mater.* **2020**, *30*, 1908098.

(38) Aimetti, A. A.; Machen, A. J.; Anseth, K. S. Poly(ethylene glycol) Hydrogels Formed by Thiol-Ene Photopolymerization for Enzyme-Responsive Protein Delivery. *Biomaterials* **2009**, *30*, 6048–6054.

(39) Knipe, J. M.; Chen, F.; Peppas, N. A. Enzymatic Biodegradation of Hydrogels for Protein Delivery Targeted to the Small Intestine. *Biomacromolecules* **2015**, *16*, 962–972.

(40) Lutolf, M.; Raebler, G.; Zisch, A.; Tirelli, N.; Hubbell, J. Cell-Responsive Synthetic Hydrogels. *Adv. Mater.* **2003**, *15*, 888–892.

(41) Lutolf, M. P.; Lauer-Fields, J. L.; Schmoekel, H. G.; Metters, A. T.; Weber, F. E.; Fields, G. B.; Hubbell, J. A. Synthetic Matrix Metalloproteinase-Sensitive Hydrogels for the Conduction of Tissue Regeneration: Engineering Cell-Invasion Characteristics. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 5413–5418.

(42) Morandi, F.; Caselli, E.; Morandi, S.; Focia, P. J.; Blázquez, J.; Shoichet, B. K.; Prati, F. Nanomolar Inhibitors of AmpC β -Lactamase. *J. Am. Chem. Soc.* **2003**, *125*, 685–695.

(43) Jansen, L. E.; Negrón-Piñero, L. J.; Galarza, S.; Peyton, S. R. Control of Thiol-Maleimide Reaction Kinetics in PEG Hydrogel Networks. *Acta Biomater.* **2018**, *70*, 120–128.

(44) Darling, N. J.; Hung, Y.-S.; Sharma, S.; Segura, T. Controlling the Kinetics of Thiol-Maleimide Michael-Type Addition Gelation Kinetics for the Generation of Homogenous Poly(ethylene glycol) Hydrogels. *Biomaterials* **2016**, *101*, 199–206.

(45) Cruise, G. M.; Scharp, D. S.; Hubbell, J. A. Characterization of Permeability and Network Structure of Interfacially Photopolymerized Poly(ethylene glycol) Diacrylate Hydrogels. *Biomaterials* **1998**, *19*, 1287–1294.

(46) Lutolf, M. P.; Hubbell, J. A. Synthesis and Physicochemical Characterization of End-Linked Poly(ethylene glycol)-co-Peptide

Hydrogels Formed by Michael-Type Addition. *Biomacromolecules* **2003**, *4*, 713–722.

(47) Macdougall, L. J.; Pérez-Madrugal, M. M.; Arno, M. C.; Dove, A. P. Nonswelling Thiol-Yne Cross-Linked Hydrogel Materials as Cytocompatible Soft Tissue Scaffolds. *Biomacromolecules* **2018**, *19*, 1378–1388.

(48) Betancourt, T.; Pardo, J.; Soo, K.; Peppas, N. A. Characterization of pH-Responsive Hydrogels of Poly(itaconic acid-g-ethylene glycol) Prepared by UV-Initiated Free Radical Polymerization as Biomaterials for Oral Delivery of Bioactive Agents. *J. Biomed. Mater. Res. Part A* **2009**, *93A*, 175–188.

(49) Avila-Salas, F.; Rodríguez Nuñez, Y.; Marican, A.; Castro, R.; Villaseñor, J.; Santos, L.; Wehinger, S.; Durán-Lara, E. Rational Development of a Novel Hydrogel as a pH-Sensitive Controlled Release System for Nifedipine. *Polymers* **2018**, *10*, 806.

(50) Ahmed, V.; Kumar, J.; Kumar, M.; Chauhan, M. B.; Dahiya, P.; Chauhan, N. S. Functionalised Iron Nanoparticle-Penicillin G Conjugates: A Novel Strategy to Combat the Rapid Emergence of β -Lactamase Resistance Among Infectious Micro-Organism. *J. Exp. Nanosci.* **2015**, *10*, 718–728.

(51) Moradali, M. F.; Ghods, S.; Rehm, B. H. A. Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. *Front. Cell. Infect. Microbiol.* **2017**, *7*, 39.

(52) Kamaly, N.; Yameen, B.; Wu, J.; Farokhzad, O. C. Degradable Controlled-Release Polymers and Polymeric Nanoparticles: Mechanisms of Controlling Drug Release. *Chem. Rev.* **2016**, *116*, 2602–2663.

(53) Lin, C.-C.; Metters, A. T. Hydrogels in Controlled Release Formulations: Network Design and Mathematical Modeling. *Adv. Drug Delivery Rev.* **2006**, *58*, 1379–1408.

(54) Oefner, C.; D'Arcy, A.; Daly, J. J.; Gubernator, K.; Charnas, R. L.; Heinze, L.; Hubschwerlen, C.; Winkler, F. K. Refined Crystal Structure of β -Lactamase from *Citrobacter freundii* Indicates A Mechanism for β -Lactam Hydrolysis. *Nature* **1990**, *343*, 284–288.

(55) DuBose, J. W.; Cutshall, C.; Metters, A. T. Controlled Release of Tethered Molecules via Engineered Hydrogel Degradation: Model Development and Validation. *J. Biomed. Mater. Res. Part A* **2005**, *74A*, 104–116.

(56) Naas, T.; Oueslati, S.; Bonnin, R. A.; Dabos, M. L.; Zavala, A.; Dortet, L.; Retaillieu, P.; Iorga, B. I. β -Lactamase Database (BLDB) - Structure and Function. *J. Enzyme Inhib. Med. Chem.* **2017**, *32*, 917–919.

(57) Livermore, D. M. β -Lactamases in Laboratory and Clinical Resistance. *Clin. Microbiol. Rev.* **1995**, *8*, 557–584.

(58) Miller, L. M.; Herman, R.; Gyulev, I.; Krauss, T. F.; Thomas, G. H.; Duhme-Klair, A.-K. Synthesis and Biochemical Evaluation of Cephalosporin Analogues Equipped with Chemical Tethers. *RSC Adv.* **2020**, *10*, 36485–36494.

(59) Olivas, A. D.; Shogan, B. D.; Valuckaitis, V.; Zaborin, A.; Belogortseva, N.; Musch, M.; Meyer, F.; L. Trimble, W.; An, G.; Gilbert, J.; Zaborina, O.; Alverdy, J. C. Intestinal Tissues Induce an SNP Mutation in *Pseudomonas aeruginosa* That Enhances Its Virulence: Possible Role in Anastomotic Leak. *PLoS One* **2012**, *7*, No. e44326.

(60) Diener, B.; Carrick, L.; Berk, R. S. In Vivo Studies with Collagenase from *Pseudomonas aeruginosa*. *Infect. Immun.* **1973**, *7*, 212–217.

(61) Schaudinn, C.; Dittmann, C.; Jurisch, J.; Laue, M.; Günday-Türeli, N.; Blume-Peytavi, U.; Vogt, A.; Rancan, F. Development, Standardization and Testing of a Bacterial Wound Infection Model Based on Ex Vivo Human Skin. *PLoS One* **2017**, *12*, No. e0186946.

(62) Nwomeh, B. C.; Liang, H.-X.; Cohen, I.; Yager, D. R. MMP-8 Is the Predominant Collagenase in Healing Wounds and Nonhealing Ulcers. *J. Surg. Res.* **1999**, *81*, 189–195.

(63) Zhang, Y.-Z.; Ran, L.-Y.; Li, C.-Y.; Chen, X.-L. Diversity, Structures, and Collagen-Degrading Mechanisms of Bacterial Collagenolytic Proteases. *Appl. Environ. Microbiol.* **2015**, *81*, 6098–6107.

(64) Wilhelm, S.; Tommassen, J.; Jaeger, K.-E. A Novel Lipolytic Enzyme Located in the Outer Membrane of *Pseudomonas aeruginosa*. *J. Bacteriol.* **1999**, *181*, 6977–6986.

(65) Xiong, M.-H.; Bao, Y.; Yang, X.-Z.; Wang, Y.-C.; Sun, B.; Wang, J. Lipase-Sensitive Polymeric Triple-Layered Nanogel for "On-Demand" Drug Delivery. *J. Am. Chem. Soc.* **2012**, *134*, 4355–4362.

(66) Lau, G. W.; Ran, H.; Kong, F.; Hassett, D. J.; Mavrodi, D. *Pseudomonas aeruginosa* Pyocyanin Is Critical for Lung Infection in Mice. *Infect. Immun.* **2004**, *72*, 4275–4278.

(67) Lau, G. W.; Hassett, D. J.; Ran, H.; Kong, F. The Role of Pyocyanin in *Pseudomonas aeruginosa* Infection. *Trends Mol. Med.* **2004**, *10*, 599–606.

(68) Yang, Q.; Phillips, P. L.; Sampson, E. M.; Progulske-Fox, A.; Jin, S.; Antonelli, P.; Schultz, G. S. Development of a Novel Ex Vivo Porcine Skin Explant Model for the Assessment of Mature Bacterial Biofilms. *Wound Repair Regen* **2013**, *21*, 704–714.

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Bibliography

1. Podolsky, S. H. The evolving response to antibiotic resistance (1945–2018). *Palgrave Commun.* **4**, 1–8 (2018).
2. Ligon, B. L. Penicillin: Its Discovery and Early Development. *Semin. Pediatr. Infect. Dis.* **15**, 52–57 (2004).
3. Gaynes, R. The Discovery of Penicillin—New Insights After More Than 75 Years of Clinical Use. *Emerg. Infect. Dis.* **23**, 849–853 (2017).
4. Hutchings, M., Truman, A. & Wilkinson, B. Antibiotics: past, present and future. *Curr. Opin. Microbiol.* **51**, 72–80 (2019).
5. Davies, J. Where have All the Antibiotics Gone? *Can. J. Infect. Dis. Med. Microbiol.* **17**, 287–290 (2006).
6. Mitcheltree, M. J. *et al.* A synthetic antibiotic class overcoming bacterial multidrug resistance. *Nature* **599**, 507–512 (2021).
7. Varela, M. F. *et al.* Bacterial resistance to antimicrobial agents. *Antibiotics* **10**, (2021).
8. Bush, K. & Bradford, P. A. Epidemiology of β -lactamase-producing pathogens. *Clin. Microbiol. Rev.* **33**, (2020).
9. He, Y., Lei, J., Pan, X., Huang, X. & Zhao, Y. The hydrolytic water molecule of Class A β -lactamase relies on the acyl-enzyme intermediate ES* for proper coordination and catalysis. *Sci. Rep. 2020 101* **10**, 1–13 (2020).
10. Reygaert, W. C. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol.* **4**, 482 (2018).
11. Centers for Disease Control and Prevention. Biggest Threats and Data | Antibiotic/Antimicrobial Resistance | CDC. 2019 1
<https://www.cdc.gov/drugresistance/biggest-threats.html>%0A<https://www.cdc.gov/drug>

- resistance/biggest_threats.html (2018).
12. AMR Industry Alliance: 2020 Progress Report. 1–128 (2020).
 13. Aslam, B. *et al.* Antibiotic Resistance: A Rundown of a Global Crisis. *Infect Drug Resist* **11**, 1645–1658 (2018).
 14. Yeh, Y. C., Huang, T. H., Yang, S. C., Chen, C. C. & Fang, J. Y. Nano-Based Drug Delivery or Targeting to Eradicate Bacteria for Infection Mitigation: A Review of Recent Advances. *Front. Chem.* **8**, (2020).
 15. Negut, I., Grumezescu, V. & Grumezescu, A. M. Treatment strategies for infected wounds. *Molecules* **23**, 2392 (2018).
 16. Polat, G., Ugan, R. A., Cadirci, E. & Halici, Z. Sepsis and Septic Shock: Current Treatment Strategies and New Approaches. *Eurasian J. Med.* **49**, 53–58 (2017).
 17. Llor, C. & Bjerrum, L. Antimicrobial Resistance: Risk Associated with Antibiotic Overuse and Initiatives to Reduce the Problem. *Ther Adv Drug Saf* **5**, 229–241 (2014).
 18. Centers for Disease Control and Prevention. CDC: 1 in 3 antibiotic prescriptions unnecessary.
 19. World Health Organization (WHO). New report calls for urgent action to avert antimicrobial resistance crisis.
<https://www.who.int/news/item/29-04-2019-new-report-calls-for-urgent-action-to-avert-antimicrobial-resistance-crisis>.
 20. Park, H., Otte, A. & Park, K. Evolution of drug delivery systems: From 1950 to 2020 and beyond. *J. Controlled Release* **342**, 53–65 (2022).
 21. Khan, I., Saeed, K. & Khan, I. Nanoparticles: Properties, applications and toxicities. *Arab. J. Chem.* **12**, 908–931 (2019).
 22. Patra, J. K. *et al.* Nano based drug delivery systems: Recent developments and future prospects 10 Technology 1007 Nanotechnology 03 Chemical Sciences 0306

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23. Gao, W., Thamphiwatana, S., Angsantikul, P. & Zhang, L. Nanoparticle Approaches against Bacterial Infections. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **6**, 532–547 (2014).
 24. Yuk, S. A., Sanchez-Rodriguez, D. A., Tsifansky, M. D. & Yeo, Y. Recent advances in nanomedicine for sepsis treatment. *Ther. Deliv.* **9**, 435–450 (2018).
 25. Sabuj, M. Z. R. & Islam, N. Inhaled antibiotic-loaded polymeric nanoparticles for the management of lower respiratory tract infections. *Nanoscale Adv.* **3**, 4005–4018 (2021).
 26. Álvarez, E. *et al.* Nanoantibiotics Based in Mesoporous Silica Nanoparticles: New Formulations for Bacterial Infection Treatment. *Pharmaceutics* **13**, 2033 (2021).
 27. García-Fernández, A., Sancenón, F. & Martínez-Máñez, R. Mesoporous silica nanoparticles for pulmonary drug delivery. *Adv. Drug Deliv. Rev.* **177**, 113953 (2021).
 28. Wang, D. Y., van der Mei, H. C., Ren, Y., Busscher, H. J. & Shi, L. Lipid-Based Antimicrobial Delivery-Systems for the Treatment of Bacterial Infections. *Front. Chem.* **7**, 872 (2020).
 29. Surgical Site Infections.
 30. Pan, C., Zhou, Z. & Yu, X. Coatings as the useful drug delivery system for the prevention of implant-related infections. *J. Orthop. Surg. Res. 2018 131* **13**, 1–11 (2018).
 31. Pietrocola, G. *et al.* Colonization and Infection of Indwelling Medical Devices by *Staphylococcus aureus* with an Emphasis on Orthopedic Implants. *Int. J. Mol. Sci.* **23**, 5958 (2022).
 32. Negut, I., Bitá, B. & Groza, A. Polymeric Coatings and Antimicrobial Peptides as Efficient Systems for Treating Implantable Medical Devices Associated-Infections.

Polymers **14**, 1611 (2022).

33. Sen, C. K. Human Wound and Its Burden: Updated 2020 Compendium of Estimates. *Adv. Wound Care* **10**, 281–292 (2021).
34. Boateng, J. S., Matthews, K. H., Stevens, H. N. E. & Eccleston, G. M. Wound healing dressings and drug delivery systems: A review. *J. Pharm. Sci.* **97**, 2892–2923 (2008).
35. R  ther, L. & Voss, W. Hydrogel or ointment? Comparison of five different galenics regarding tissue breathability and transepidermal water loss. *Heliyon* **7**, e06071 (2021).
36. Canaparo, R. *et al.* Recent developments in antibacterial therapy: Focus on stimuli-responsive drug-delivery systems and therapeutic nanoparticles. *Molecules* **24**, (2019).
37. Zhao, Y. Z., Du, L. N., Lu, C. T., Jin, Y. G. & Ge, S. P. Potential and problems in ultrasound-responsive drug delivery systems. *Int. J. Nanomedicine* **8**, 1621–1633 (2013).
38. Li, X. *et al.* Recent developments in smart antibacterial surfaces to inhibit biofilm formation and bacterial infections. *J. Mater. Chem. B* **6**, 4274–4292 (2018).
39. Xiao, X., Zhao, W., Liang, J., Sauer, K. & Libera, M. Self-defensive antimicrobial biomaterial surfaces. *Colloids Surf. B Biointerfaces* **192**, 110989 (2020).
40. Zhuk, I. *et al.* Self-Defensive Layer-by-Layer Films with Bacteria-Triggered Antibiotic Release. *ACS Nano* **8**, 7733–7745 (2014).
41. Haidari, H. *et al.* pH-Responsive “Smart” Hydrogel for Controlled Delivery of Silver Nanoparticles to Infected Wounds. *Antibiotics* **10**, 49 (2021).
42. Pornpattananangkul, D. *et al.* Bacterial Toxin-Triggered Drug Release from Gold Nanoparticle-Stabilized Liposomes for the Treatment of Bacterial Infection. *J Am Chem Soc* **133**, 4132–4139 (2011).

43. Ahmed, W., Zhai, Z. & Gao, C. Adaptive antibacterial biomaterial surfaces and their applications. *Mater. Today Bio* **2**, 100017 (2019).
44. Smith, S. M. D-Lactic acid production as a monitor of the effectiveness of antimicrobial agents. *Antimicrob. Agents Chemother.* **35**, 237–241 (1991).
45. Dissemond, J., Witthoff, M., Brauns, T. C., Haberer, D. & Goos, M. pH-wert des milieus chronischer wunden: Untersuchungen im rahmen einer modernen wundtherapie. *Hautarzt* **54**, 959–965 (2003).
46. Boedtkjer, E. & Pedersen, S. F. The Acidic Tumor Microenvironment as a Driver of Cancer. *Annu. Rev. Physiol.* **82**, 103–126 (2020).
47. Alkekhia, D. & Shukla, A. Influence of Poly-L-Lysine Molecular Weight on Antibacterial Efficacy in Polymer Multilayer Films. *J Biomed Mater Res Part A* **107**, 1324–1339 (2019).
48. Francesko, A. *et al.* Bacteria-responsive multilayer coatings comprising polycationic nanospheres for bacteria biofilm prevention on urinary catheters. *Acta Biomater.* **33**, 203–212 (2016).
49. Cado, G. *et al.* Self-Defensive Biomaterial Coating Against Bacteria and Yeasts: Polysaccharide Multilayer Film with Embedded Antimicrobial Peptide. *Adv Funct Mater* **23**, 4801–4809 (2013).
50. Johnson, C. T. *et al.* Hydrogel delivery of lysostaphin eliminates orthopedic implant infection by *Staphylococcus aureus* and supports fracture healing. *Proc Natl Acad Sci* **115**, E4960–E4969 (2018).
51. Xiong, M. H. *et al.* Bacteria-responsive multifunctional nanogel for targeted antibiotic delivery. *Adv. Mater.* **24**, 6175–6180 (2012).
52. Chen, M. *et al.* Antibacterial Micelles with Vancomycin-Mediated Targeting and pH/Lipase-Triggered Release of Antibiotics. *ACS Appl Mater Interfaces* **10**, 36814–36823 (2018).

53. Wang, N. *et al.* HClO/CIO—Indicative Interpenetrating Polymer Network Hydrogels as Intelligent Bioactive Materials for Wound Healing. *ACS Appl. Bio Mater.* **3**, 37–44 (2020).
54. Ji, H. *et al.* Bacterial Hyaluronidase Self-Triggered Prodrug Release for Chemo-Photothermal Synergistic Treatment of Bacterial Infection. *Small* **12**, 6200–6206 (2016).
55. Bush, K. & Jacoby, G. A. Updated functional classification of β -lactamases. *Antimicrob. Agents Chemother.* **54**, 969–976 (2010).
56. Bush, K. Past and Present Perspectives on β -Lactamases. *Antimicrob Agents Chemother* **62**, e01076-18 (2018).
57. Bush, K. & Bradford, P. A. Interplay between β -lactamases and new β -lactamase inhibitors. *Nat. Rev. Microbiol.* **17**, 295–306 (2019).
58. O'Callaghan, C. H., Morris, A., Kirby, S. M. & Shingler, A. H. Novel method for detection of beta-lactamases by using a chromogenic cephalosporin substrate. *Antimicrob. Agents Chemother.* **1**, 283–288 (1972).
59. Perez-Inestrosa, E. *et al.* Cephalosporin chemical reactivity and its immunological implications. *Current Opinion in Allergy and Clinical Immunology* vol. 5 323–330 <https://journals.lww.com/co-allergy> (2005).
60. Evans, L. E. *et al.* Exploitation of Antibiotic Resistance as a Novel Drug Target: Development of a β -Lactamase-Activated Antibacterial Prodrug. *J. Med. Chem.* **62**, 4411–4425 (2019).
61. Liu, R. *et al.* A Synthetic Dual Drug Sideromycin Induces Gram-Negative Bacteria to Commit Suicide with a Gram-Positive Antibiotic. *J Med Chem* **61**, 3845–3854 (2018).
62. Alkekhia, D., Safford, H., Shukla, S., Hopson, R. & Shukla, A. β -lactamase triggered visual detection of bacteria using cephalosporin functionalized biomaterials.

- Chem. Commun. Camb. Engl.* **56**, 11098 (2020).
63. Li, J. & Mooney, D. J. Designing hydrogels for controlled drug delivery. *Nat. Rev. Mater.* **1**, 16071 (2016).
64. Zheng, X. *et al.* Exploiting a Bacterial Drug-Resistance Mechanism: A Light-Activated Construct for the Destruction of MRSA. *Angew Chem. Int Ed* **48**, 2148–2151 (2009).
65. Peppas, N. A., Hilt, J. Z., Khademhosseini, A. & Langer, R. Hydrogels in Biology and Medicine: From Molecular Principles to Bionanotechnology. *Adv Mater* **18**, 1345–1360 (2006).
66. Koetting, M. C., Peters, J. T., Steichen, S. D. & Peppas, N. A. Stimulus-Responsive Hydrogels: Theory, Modern Advances, and Applications. *Mater Sci Eng R Rep.* **93**, 1–49 (2015).
67. Canal, T. & Peppas, N. A. Correlation Between Mesh Size and Equilibrium Degree of Swelling of Polymeric Networks. *J Biomed Mater Res* **23**, 1183–1193 (1989).
68. Morandi, F. *et al.* Nanomolar Inhibitors of AmpC β -Lactamase. *J Am Chem Soc* **125**, 685–695 (2003).
69. Jansen, L. E., Negrón-Piñeiro, L. J., Galarza, S. & Peyton, S. R. Control of Thiol-Maleimide Reaction Kinetics in PEG Hydrogel Networks. *Acta Biomater* **70**, 120–128 (2018).
70. Darling, N. J., Hung, Y.-S., Sharma, S. & Segura, T. Controlling the Kinetics of Thiol-Maleimide Michael-Type Addition Gelation Kinetics for the Generation of Homogenous Poly(ethylene glycol) Hydrogels. *Biomaterials* **101**, 199–206 (2016).
71. Cruise, G. M., Scharp, D. S. & Hubbell, J. A. Characterization of Permeability and Network Structure of Interfacially Photopolymerized Poly(ethylene glycol) Diacrylate Hydrogels. *Biomaterials* **19**, 1287–1294 (1998).

72. Lutolf, M. P. & Hubbell, J. A. Synthesis and Physicochemical Characterization of End-Linked Poly(ethylene glycol)-co-Peptide Hydrogels Formed by Michael-Type Addition. *Biomacromolecules* **4**, 713–722 (2003).
73. Macdougall, L. J., Pérez-Madrigal, M. M., Arno, M. C. & Dove, A. P. Nonswelling Thiol–Yne Cross-Linked Hydrogel Materials as Cytocompatible Soft Tissue Scaffolds. *Biomacromolecules* **19**, 1378–1388 (2018).
74. Betancourt, T., Pardo, J., Soo, K. & Peppas, N. A. Characterization of pH-Responsive Hydrogels of Poly(itaconic acid-g-ethylene glycol) Prepared by UV-Initiated Free Radical Polymerization as Biomaterials for Oral Delivery of Bioactive Agents. *J Biomed Mater Res Part A* **9999A**, 175–188 (2009).
75. Avila-Salas, F. *et al.* Rational Development of a Novel Hydrogel as a pH-Sensitive Controlled Release System for Nifedipine. *Polymers* **10**, 806 (2018).
76. Annabi, N. *et al.* Controlling the Porosity and Microarchitecture of Hydrogels for Tissue Engineering. *Tissue Eng. Part B Rev.* **16**, 371–383 (2010).
77. Moradali, M. F., Ghods, S. & Rehm, B. H. A. *Pseudomonas aeruginosa* Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. *Front Cell Infect Microbiol* **7**, 39 (2017).
78. Kamaly, N., Yameen, B., Wu, J. & Farokhzad, O. C. Degradable Controlled-Release Polymers and Polymeric Nanoparticles: Mechanisms of Controlling Drug Release. *Chem Rev* **116**, 2602–2663 (2016).
79. Lin, C.-C. & Metters, A. T. Hydrogels in Controlled Release Formulations: Network Design and Mathematical Modeling. *Adv Drug Deliv Rev* **58**, 1379–1408 (2006).
80. Aimetti, A. A., Machen, A. J. & Anseth, K. S. Poly(ethylene glycol) Hydrogels Formed by Thiol-Ene Photopolymerization for Enzyme-Responsive Protein Delivery. *Biomaterials* **30**, 6048–6054 (2009).

81. Oefner, C. *et al.* Refined Crystal Structure of β -Lactamase from *Citrobacter freundii* Indicates A Mechanism for β -Lactam Hydrolysis. *Nature* **343**, 284–288 (1990).
82. Naas, T. *et al.* Beta-Lactamase Database (BLDB) – Structure and Function. *J Enzyme Inhib Med Chem* **32**, 917–919 (2017).
83. Livermore, D. M. β -Lactamases in Laboratory and Clinical Resistance. *Clin Microbiol Rev* **8**, 557–584 (1995).
84. Xie, H. *et al.* Rapid Point-of-Care Detection of the Tuberculosis Pathogen Using a BlaC-Specific Fluorogenic Probe. *Nat Chem* **4**, 802–809 (2012).
85. Chan, H. L. *et al.* Unique Fluorescent Imaging Probe for Bacterial Surface Localization and Resistant Enzyme Imaging. *ACS Chem Biol* **13**, 1890–1896 (2018).
86. Miller, L. M. *et al.* Synthesis and biochemical evaluation of cephalosporin analogues equipped with chemical tethers. *RSC Adv.* **10**, 36485–36494 (2020).
87. Lau, G. W., Ran, H., Kong, F., Hassett, D. J. & Mavrodi, D. *Pseudomonas aeruginosa* Pyocyanin Is Critical for Lung Infection in Mice. *Infect Immun* **72**, 4275–4278 (2004).
88. Lau, G. W., Hassett, D. J., Ran, H. & Kong, F. The Role of Pyocyanin in *Pseudomonas aeruginosa* Infection. *Trends Mol Med* **10**, 599–606 (2004).
89. Yang, Q. *et al.* Development of a Novel Ex Vivo Porcine Skin Explant Model for the Assessment of Mature Bacterial Biofilms. *Wound Repair Regen* **21**, 704–714 (2013).
90. Schaudinn, C. *et al.* Development, Standardization and Testing of a Bacterial Wound Infection Model Based on Ex Vivo Human Skin. *PLOS ONE* **12**, e0186946 (2017).
91. Keskin, D. *et al.* Nanogels: A novel approach in antimicrobial delivery systems and antimicrobial coatings. *Bioact. Mater.* **6**, 3634–3657 (2021).

92. Phan, H. T. & Haes, A. J. What Does Nanoparticle Stability Mean? *J. Phys. Chem. C Nanomater. Interfaces* **123**, 16495–16507 (2019).
93. Hoshyar, N., Gray, S., Han, H. & Bao, G. The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction. *Nanomed.* **11**, 673–692 (2016).
94. Yu, C., Alkekhia, D. & Shukla, A. β -Lactamase Responsive Supramolecular Hydrogels with Host–Guest Self-Healing Capability. *ACS Appl. Polym. Mater.* **2**, 55–65 (2020).
95. Umstätter, F. *et al.* Vancomycin Resistance Is Overcome by Conjugation of Polycationic Peptides. *Angew. Chem. - Int. Ed.* **59**, 8823–8827 (2020).
96. Zheng, X. *et al.* Exploiting a bacterial drug-resistance mechanism: A light-activated construct for the destruction of MRSA. *Angew. Chem. - Int. Ed.* **48**, 2148–2151 (2009).
97. Selvarajan, V., Obuobi, S. & Ee, P. L. R. Silica Nanoparticles—A Versatile Tool for the Treatment of Bacterial Infections. *Front. Chem.* **8**, 602 (2020).
98. Fernandes, R. S., Raimundo, I. M. & Pimentel, M. F. Revising the synthesis of Stöber silica nanoparticles: A multivariate assessment study on the effects of reaction parameters on the particle size. *Colloids Surf. Physicochem. Eng. Asp.* **577**, 1–7 (2019).
99. Bret, L., Bhat, R. & Grover, G. Ellman 's assay for in-solution quantification of sulfhydryl groups. (2018).
100. Tranquillo, E. & Bollino, F. Surface modifications for implants lifetime extension: An overview of sol-gel coatings. *Coatings* **10**, 589 (2020).
101. Livage, J. & Sanchez, C. Sol-gel chemistry. *J. Non-Cryst. Solids* **145**, 11–19 (1992).
102. Zeumault, A. & Volkman, S. K. Probing the Kinetics of Crystallite Growth in Sol-Gel Derived Metal-Oxides Using Nanocalorimetry. *Cryst. Growth Des.* **20**,

1590–1597 (2020).

103. Soule, L. D. *et al.* Sol-Gel-Derived Bioactive and Antibacterial Multi-Component Thin Films by the Spin-Coating Technique. *ACS Biomater. Sci. Eng.* **6**, 5549–5562 (2020).
104. Reiser, J. T., Ryan, J. V. & Wall, N. A. Sol-Gel Synthesis and Characterization of Gels with Compositions Relevant to Hydrated Glass Alteration Layers. *ACS Omega* **4**, 16257–16269 (2019).
105. Kuznetsova, T. *et al.* Surface Microstructure of Mo(C)N Coatings Investigated by AFM. *J. Mater. Eng. Perform.* **25**, 5450–5459 (2016).
106. Li, Y. *et al.* Preparation of sol-gel derived anticorrosive coating on Q235 carbon steel substrate with long-term corrosion prevention durability. *Materials* **12**, (2019).