**INTRODUCTION**

Bacterial infections are a critical health threat of our time.\(^1,2\) The standard approach to treat bacterial infections is through systemic administration of antibiotics. The choice of the antibiotic for a given infection is based on the minimum inhibitory concentration (MIC) profile of the targeted bacteria.\(^3\) The MIC is defined as the minimum concentration of an antibiotic that will inhibit bacterial growth *in vitro* during an overnight incubation.\(^4\) Historically, the MIC correlates with a specific dosing regimen (magnitude and frequency). Ideally, a supratherapeutic dose would be used to ensure complete elimination of the infection and to prevent emergence of antibiotic-resistant bacterial strains. However, high doses of systemic antibiotic therapy cause adverse side-effects including musculoskeletal, hepatic, and renal toxicity.\(^5\)–\(^7\) There is a growing need for better antibiotics. We believe that improved drug delivery methods that supply high doses of antibiotics specifically to the area of infection would fulfill this need.

An example that underscores the aforementioned challenges is the eradication of implant-associated infections (IAIs).\(^8\) IAIs due to bacterial biofilms formed on the surface of soft tissues and medical devices are more resistant to conventional antibiotics than planktonic bacteria. *Staphylococcus aureus* is one of the most frequent germs causing biofilm-associated infections on the medical indwelling devices such as contact lenses, catheters, endotracheal tubes, mechanical heart valves, pacemakers, and prosthetic implants.\(^9\)–\(^12\) Among *S. aureus* isolates the methicillin-resistant (MRSA) strains remain the most frequent multidrug-resistant hospital-associated infection.

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**ABSTRACT:** Systemic administration of antibiotics can cause severe side-effects such as liver and kidney toxicity, destruction of healthy gut bacteria, as well as multidrug resistance. Here, we present a bio-orthogonal chemistry-based strategy toward local prodrug concentration and activation. The strategy is based on the inverse electron-demand Diels–Alder chemistry between trans-cyclooctene and tetrazine and involves a biomaterial that can concentrate and activate multiple doses of systemic antibiotic therapy prodrugs at a local site. We demonstrate that a biomaterial, consisting of alginate hydrogel modified with tetrazine, is efficient at activating multiple doses of prodrugs of vancomycin and daptomycin *in vitro* as well as *in vivo*. These results support a drug delivery process that is independent of endogenous environmental markers. This approach is expected to improve therapeutic efficacy with decreased side-effects of antibiotics against bacterial infections. The platform has a wide scope of possible applications such as wound healing, and cancer and immunotherapy.
Microorganisms like *S. aureus* adhere to an extracellular polymeric matrix and develop into organized, complex communities with structural and functional heterogeneity, known as biofilms. Depletion of metabolic substances and accumulation of waste products causes biofilm bacteria to enter into a slow-growing state, rendering them more resistant to antibiotic drugs than their planktonic counterparts. More-\[\ldots\]

Figure 1. A bio-orthogonal chemistry-based strategy for concentration and activation of systemically administered antibiotic prodrugs. (A) Tz-modified alginate gel (TAG) is injected into the infected area. (B) An antibiotic, covalently modified with a TCO (prodrug), is given to the patient. (C) When the prodrug and the TAG come in contact, the IEDDA reaction enhances the amount of antibiotic present near the infected site. (D) The resulting cycloaddition product spontaneously isomerizes, releasing an equivalent of carbon dioxide and most importantly the active antibiotic. 

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standard of care treatment. In this report, we demonstrate that the local drug activation approach is indeed reloadable, and its versatility can be utilized to tackle planktonic and biofilm infections.

To illustrate our approach, we chose two FDA-approved antibiotics, vancomycin and daptomycin that have widespread clinical significance in the treatment of S. aureus infections, including MRSA strains, and whose versatility is limited by harmful side-effects. Vancomycin is a tricyclic glycopeptide antibiotic that is indicated to fight severe infections caused by Gram-positive bacteria. It has a well-understood mechanism of action involving inhibition of bacterial cell wall biosynthesis. Vancomycin is often used as a drug of “last resort” against microorganisms that proved resistant against other antimicrobial agents.32 Vancomycin is also used in the treatment of planktonic bacteria in the surrounding of the device-related infections.29,30 Wider indication of vancomycin is limited due to its adverse effects like hypotension and tachycardia, phlebitis, nephrotoxicity, and ototoxicity.31 Meanwhile, daptomycin is a cyclic lipopeptide antibiotic. Its activity is dependent on the presence of Ca2+ that facilitates oligomerization and insertion into bacterial membranes. Oligomers of daptomycin are thought to form toxic pores inside of bacterial membranes causing membrane depolarization. Daptomycin has a narrow therapeutic window. It is approved at a dose of 4 mg/kg for bloodstream infections and at a dose of 6 mg/kg for S. aureus bloodstream infection.33 Daptomycin has recently been reported to be effective against biofilms and therefore is a promising option for IAI treatment.34 However, during phase 1 clinical trials, higher doses of daptomycin such as 8 mg/kg led to unacceptable adverse effects involving the musculoskeletal system with accompanying increases in creatine phosphokinase levels.35 Moreover, its applications to joint infections have been limited due to limited biodistribution and the aforementioned side-effect profile.36 In this work we will show that systemically administered produgs of vancomycin and daptomycin can be converted into active antibiotics at the site of bacterial infection using bio-orthogonal chemistry.

## RESULTS AND DISCUSSION

Our local prodrug activation approach is based on the inverse electron-demand Diels–Alder (IEDDA) chemistry between trans-cyclooctene (TCO) and tetrazine (Tz). During the past decade, this chemistry has become increasingly popular for in vivo bioconjugation due to fast reaction kinetics and inertness of the two bio-orthogonal groups to endogenous biomolecules and bioprocesses.37,38 IEDDA chemistry has been shown to be virtually nontoxic and highly effective under physiological conditions.37 As illustrated in Figure 1, the strategy starts with the injection of a biocompatible tetrazine-modified alginate gel (TAG) at the site of an infection. A prodrug, synthesized by covalent modification of a clinically tested antibiotic with a releasable TCO moiety, is injected intravenously (Figure 1B). When the prodrug reaches the site of the hydrogel, the bio-orthogonal agents (TCO and Tz) react with each other through the IEDDA chemistry, concentrating the therapeutic payload at the site of an infection (Figure 1C).37,38 Finally, the resulting intermediate isomerizes spontaneously releasing the active antibiotic from the hydrogel to perform its therapeutic function locally (Figure 1D). The multivalency of Tz on the hydrogel’s surface allows catching of multiple doses of systemically administered TCO-modified compounds.

To evaluate the strategy, TAG was prepared by modifying an ultrapure medium viscosity (>200 mPa s) sodium alginate, containing a minimum of 60% guluronate monomer units, with the Tz (Figure S1). Based on 1H NMR analysis, TAG contained about 400 nmol of Tz per mg of the material (~8% of alginate monomers modified). The extent of Tz loading reflects the maximum amount of produgs that can theoretically be activated. To determine if Tz incorporation altered the flow properties of alginate, the viscosities of 2.5% w/v aqueous solutions of TAG and unmodified gel (UG) were measured. In general, aqueous polysaccharide solutions are known to behave as non-Newtonian fluids, such that their dynamic viscosities decrease in response to an increase in the applied shear rate. Consistently, both TAG and UG displayed such a shear-thinning or pseudoplastic behavior (Figure S2). Furthermore, zeta (ζ) potential, a surrogate measurement for surface charge, at 1 mg/mL dilution in distilled water at pH 7.0 was found to be −78.6 ± 5.2 mV for TAG and −75.6 ± 5.7 mV for UG. These values suggest that the low extent of modification with Tz did not affect the inherent surface charge of the alginate gel. Additionally, the high magnitude of ζ (> ±30 mV) of these gels indicates that they are stable in water and do not aggregate, as consistent with the literature.

The produgs of vancomycin and daptomycin, shown in Scheme 1, were synthesized by covalent modification of the parent antibiotics with the releasable TCO moiety. The detailed syntheses are illustrated in Figures S9 and S16.
Vancomycin was modified at the N-terminus which is involved in binding the C-terminal portion of bacterial cell wall precursor peptides ending in the sequence Lys–d-Ala–d-Ala. This binding sequesters substrates necessary for construction of the bacterial cell wall, eventually causing cell death. Extensive studies by Williams have shown that acylation of the N-terminus of vancomycin causes a 17-fold decrease in antibiotic activity. Daptomycin was converted into a prodrug by modifying the most reactive, ornithine residue (Orn6). The precise role of this residue in daptomycin’s mechanism of action is not fully understood. However, Marahiel reported that a prodrug formed by acylation of the ornithine residue is 4 times less active. Due to its well-known reactivity and documented significance, Orn6 was chosen as the site of attachment of the releasable TCO group.

In Vitro Prodrug Activation. TAG can efficiently activate multiple doses of prodrugs of vancomycin and daptomycin, TCO–Vanco and TCO–Dapto, under simulated physiological conditions. When 25 mg of TAG and 5 nmol of either prodrug were mixed in phosphate buffered saline for 2 h at room temperature, over 99% of the compounds detected in the supernatant were activated vancomycin and daptomycin as shown by LC-MS and HPLC analyses (Figure 2A). Subsequent measurements over a 24 h period detected only the release of the corresponding antibiotic. Calibration of the acquired spectra revealed that TCO–Vanco and TCO–Dapto are rapidly captured by TAG and that over 80% of the activated antibiotics are released within 24 h (Figure 2B). To test the ability of TAG to be reloaded, the hydrogel was treated with second and third doses of 5 nmol of TCO–Vanco and TCO–Dapto 24 and 48 h after the first dose. As illustrated in Figure 2, TAG is capable of efficiently “catching” multiple doses of prodrugs and subsequently releasing their corresponding activated antibiotics. The kinetics of drug release after the second and the third doses followed essentially the same trend as those seen after the first dose.

Microcalorimetric Analysis of Inhibition of Growth of Planktonic Bacteria. Isothermal microcalorimetry experiments using two laboratory strains of S. aureus (MSSA and MRSA) confirmed efficient in vitro activation of the antibiotic prodrugs by TAG. The experiments were based on the known principle that metabolically active (live and reproducing) bacteria produce heat that can be measured using an isothermal calorimeter. Heat generated by MSSA and MRSA bacteria when treated with different concentrations of TCO–Vanco or TCO–Dapto in the presence of either TAG or UG was plotted as a function of time (Figures S4–S7). Analogue experiments using the parent antibiotics were carried out as a positive control (Figure S9). An in-depth

Figure 2. In vitro activation of TCO–Vanco and TCO–Dapto by TAG. (A) HPLC analysis of in vitro activation of TCO–Vanco. A 25 mg portion of TAG was treated with the 5 nmol doses of TCO–Vanco at 0, 24, and 48 h. (B) Cumulative release of vancomycin after mixing TAG with the three doses of TCO–Vanco. (C) LC-MS analysis of in vitro activation of TCO–Dapto. A 25 mg portion of TAG was treated with the 5 nmol doses of TCO–Dapto at 0, 24, and 48 h. (D) Cumulative release of daptomycin after mixing TAG with the three doses of TCO–Dapto.

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data analysis is described in Tables S1 and S2. A threshold of 4 μW for total heat production was selected as a marker of bacterial growth, and the time required for bacteria heat to reach that threshold was measured as "time to detection" (TTD). For example, bacteria without any antibiotic treatment were the fastest to reach the heat threshold and hence had a low TTD (Figure 3). Conversely, higher TTD values indicate a delay in heat production due to bacterial susceptibility to antibiotics. Inability to reach the 4 μW threshold within 24 h corresponds to 99% inhibition of bacterial growth. The TTD was assigned to be nondetectable (ND), and the minimum antibiotic dosage required to achieve 99% inhibition of bacterial growth was considered as the minimum heat inhibitory concentration (MHIC), the term which is analogous to MIC.

Figure 3 shows TTD values corresponding to MRSA and MSSA bacteria treated with different concentrations of TCO−Vanco and TCO−Dapto in the presence of TAG or UG. Statistics was done with two-way ANOVA followed by multiple comparisons with Sidak’s post-test. An overall p-value less than 0.05 was accepted as significant. Adjusted p-values are indicated for individual comparisons as •• p < 0.05, ••• p < 0.005, # p < 0.001, ○ p < 0.0001. Error bars represent standard error over mean (SEM) of n = 3–6. The minimum dosage at which TTD became nondetectable (ND) is marked as the MHIC. Blue and red correspond to MHIC of prodrug with TAG and UG, respectively.

Figure 3 illustrates that bacterial treatment in the presence of TAG resulted in significantly higher TTD values, relative to UG. For example, MRSA treated with 2 μg/mL TCO−Vanco and TAG showed TTD of 15 h, while the same treatment with UG had a TTD of 9 h (Figure 3B). The difference was even more dramatic when MRSA was treated with 0.5 μg/mL TCO−Dapto. TTD of 23 h was observed with TAG, while the same treatment with UG had a TTD of 4 h (Figure 3D). Analogous trends were observed with MSSA treated with TCO−Vanco and TCO−Dapto. The MHIC values derived from these data are listed in Table 1. The MHIC values indicate that the antibiotic prodrugs have higher antibiotic activity when activated in the presence of TAG.

Microcalorimetric Analysis of Inhibition of Growth of Biofilm Bacteria. Among S. aureus strains, MRSA remains the most prevalent microorganism associated with hospital infections.23 We tested the ability of our produrg activation system to eradicate MRSA biofilms grown on porous glass beads. First the beads were immersed in either TAG or UG and then incubated with different concentrations of daptomycin or TCO−Dapto. TCO−Vanco and vancomycin were not tested as the antibiotic has limited efficacy against S.
Table 1. Minimum Heat Inhibitory Concentration (MHIC)
Measured from the Total Heat Produced by Bacteria at 24 h

|                | MHIC for MSSA [μg/mL] | activity improvement [%] |
|----------------|------------------------|--------------------------|
|                | TAG | UG   | (UG − TAG)/UG           |
| TCO−Vanco      | 3   | 4    | 23%                     |
| TCO−Dapto      | 1   | 3.5  | 71%                     |

|                | MHIC for MRSA [μg/mL] | activity improvement [%] |
|----------------|------------------------|--------------------------|
|                | TAG | UG   | (UG − TAG)/UG           |
| TCO−Vanco      | 2.5 | 3    | 16%                     |
| TCO−Dapto      | 1   | 3    | 67%                     |

After antibiotic treatment, the beads were sonicated, and the sonication fluid was plated on agar plates. The colony-forming units (CFUs) were counted after 24 h. The results are shown in Figure 4 as CFU/mL. The lowest antibiotic concentration that inhibited colony formation on the agar plate was defined as the minimum biofilm eradication concentration (MBEC). 44 TCO−Dapto is significantly more effective at eliminating MRSA biofilms in the presence of TAG. MBEC was achieved at 64 μg/mL for TCO−Dapto with the tetrazine-modified gel, which is comparable to the standard daptomycin treatment. Meanwhile in the presence of UG, the TCO−Dapto was unable to eradicate biofilms even at concentrations of 2.56 μg/mL.

Elimination of *S. aureus* Infection in Mice. The hypothesis that TAG is reloadable and capable of concentrating and activating antibiotic prodrugs at a location of choice was first tested using a fluorescent model system. TAG or UG (negative control) were implanted at the dorsum of mice, while TCO−TAMRA was administered systemically. Figure S8 shows that TAG was able to enhance the local delivery of the fluorescent payload over days, after multiple doses. The areas injected with UG or without a gel injection (negative controls) did not have an equivalent enhancement of fluorescence, confirming that the bio-orthogonal reaction is the reason for the enhancement. Moreover, the fact that the fluorescence decreased to background levels in all cases confirmed that the cargo was not trapped indefinitely but rather was concentrated and activated (released from the material).

To study the *in vivo* behavior of TAG, we synthesized a gel to contain Cyanine S.5 dye (0.732 nmol/mg) in addition to Tz. We found that TAG injected in the thigh muscle remains at the site of the injection for at least 3 months (Figure 5). Fluorescence was observed throughout the study, and representative images of mice immediately postinjection, on days 7 and 90, are shown (Figure 5A). The fluorescence signal observed on day 7 showed a gradual decrease over time up to 58% on day 90 (Figure 5B). Postnecropsy relative fluorescence biodistribution showed Cy5.5 signal in the lymph nodes and liver, indicating that TAG is likely cleared from the body, with a preference for lymphatic and hepatic routes over renal clearance (Figure 5C). Histopathological analysis of the gel site showed the presence of infiltrating macrophages without any associated fibrosis or cell death, indicating a mild inflammatory response only (Figure 5D). Together, these results suggest that TAG undergoes gradual biodegradation, persists at the injection site for at least 90 days, and does not induce a fibrotic inflammatory response. These findings suggest that TAG is suitable for extended treatment durations.

The total therapeutic benefit of the platform comes from two components. On one hand we have the activation component. This can be determined by the difference of activity (MHIC) of the prodrug with TAG or UG as shown in Table 1. On the other hand, it is challenging to determine the benefits of the concentration step *in vitro* as it is hard to model the amount of prodrug that would be concentrated at the area labeled with TAG. In this study we used an *in vivo* efficacy study using a bioluminescent MRSA strain 45 and TCO−Vanco, a prodrug that has a limited benefit from the activation factor as determined by the difference between the MHIC against MRSA when used with TAG and UG of only 16% (Table 1). Fluorescently labeled TAG and UG were synthesized to contain ~0.5% Cy5 loading (w/w), in addition to tetrazine in the case of TAG. Bioluminescent MRSA was injected along with the Cy5-modified hydrogels into the thighs of neutropenic mice. Mice were subsequently administered a systemic dose of TCO−Vanco. Figure 6A shows that the bioluminescent bacteria was completely eradicated within 24 h of receiving TCO−Vanco. Analogous treatments involving UG or saline in combination with TCO−Vanco were unsuccessful at clearing the infection. Upon acquiring the *in vivo* images, mice were sacrificed, and bacterial load of the harvested thigh
tissue was assessed in terms of colony-forming units (CFU) obtained per gram of the harvested tissue. As illustrated in Figure 6B, treatment with TAG and TCO−Vanco resulted in a 800-fold reduction of bacterial load relative to UG and TCO−Vanco. The difference of almost 3 orders of magnitude in eliminating bacteria surpasses the 16% improvement by the activation that was observed in vitro (Table 1). These results suggest that TAG is very efficient at concentrating prodrugs from the plasma circulation at the desired location.

This is consistent with the unexpected results seen in our previous work with chemotherapeutic prodrugs, where Tz-mediated activation of 10 daily prodrug doses led to a sustained remission of tumors.27 Future studies will evaluate directly the pharmacokinetics of these antibiotic prodrugs as well as other prodrugs to quantify the effect directly.

**CONCLUSION**

Herein we have described a hydrogel-based reloadable platform that utilizes bio-orthogonal IEDDA chemistry to achieve local activation of systemically administered antibiotic prodrugs. The local drug activation platform was shown to be capable of activating multiple doses of prodrugs of vancomycin and daptomycin in vitro. Isothermal microcalorimetry experiments showed that the prodrugs activated by TAG were significantly more potent at inhibiting bacterial growth than the corresponding negative controls using unmodified hydrogel. In vivo experiments showed that treatment of TAG and TCO−Vanco is more effective than UG and TCO−Vanco, in eradicating luminescent MRSA infections.

A number of elements of the local drug activation platform require further optimization. First, the prodrug of daptomycin requires structural modification, as TCO−Dapto was found to have poor aqueous solubility, which limited its in vivo testing. We plan to report our efforts in this area shortly. In addition, we envision that other prodrug modifications would additionally attenuate the antibacterial activity of the resulting prodrug.
as daptomycin’s mode of action heavily relies on its hydrophobicity for incorporation into bacterial membranes. This will be advantageous, as it will allow administration of higher systemic dosages of prodrugs toward local activation in the area of interest. These strategies will undoubtedly be pursued in subsequent studies.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00344.

Synthesis and characterization of TAG and the prodrugs of vancomycin and daptomycin; description of microcalorimetry and kinetic experiments; and detailed analysis of microcalorimetry data (PDF)

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Notes
The authors declare the following competing financial interest(s): Jose M. Mejia Oneto is the founder of Shasqi, Inc. Nathan A. Yee and Sangeetha Srinivasan are employed by Shasqi, Inc. Its core technology is based on the research described herein.

Safety statement: the MRSA and MSSA bacteria described in the experimental data.

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