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14. ABSTRACT

This research aims to address the needs of far forward Wounded Warriors in the era of prolonged field care (PFC) by developing a field-polymerizable hydrogel that can be readily applied in austere environments that enables sustained release of antibiotics, analgesics, and hemostatic agents over a five-day period. Following the optimization of our hydrogel system, we have analyzed therapeutic release in vitro and verified the presence of our therapeutics in the system using high-performance liquid chromatography and mass spectrometry. We have transitioned to in vivo studies using mouse models.

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1. INTRODUCTION:

This research aims to address the needs of far forward Wounded Warriors in the era of prolonged field care (PFC) by developing a field-polymerizable hydrogel that can be readily applied in austere environments that enables sustained release of antibiotics, analgesics, and hemostatic agents over a five-day period. This biochemical tool will be readily deployed in remote environments to improve antimicrobial, hemostatic, and pain outcomes at the point of injury in the setting of delayed evacuation.

2. KEYWORDS:

- Polymer Hydrogel
- Wound Sealant
- Antimicrobial
- Prolonged Field Care
- Wound Decontamination
- Extremity Blast Wounds

3. ACCOMPLISHMENTS:

Specific Aim 1 – Extend antibiotic, analgesic and hemostatic agent release profile through optimizing crosslink density of the hydrogel wound dressing

Major Task 1 – Synthesis of PEG-diacrylate macromers and hydrogels

Subtask 1 – Synthesis of PEG-diacrylate macromers of varying molecular weights, fabrication, and characterization of hydrogels. **Proposed Timeline: Months 1-12**

Percentage completion: 100%

Subtask 2 – Fabrication of hydrogels entrapping therapeutics, and characterization of release kinetics. **Proposed Timeline: Months 9-15**

Percentage completion: 100%

Subtask 3 – Leak pressure testing of hydrogels to ensure wound sealing. **Proposed Timeline: Months 6-12**

Percentage completion: 100%

Subtask 4 – Characterization the chemical and physical stability of the components under various environmental conditions. **Proposed Timeline: Months 9-12**

Percentage completion: 100%

Major Task 2 – In vitro assessment of released therapeutics

Subtask 1 – Molecular characterization of released therapeutics. Released drugs will be characterized by mass spectrometry, NMR, and/or other techniques to verify chemical structure. **Proposed Timeline: Months 12-18**

Percentage completion: 100%

Subtask 2- Efficacy of released antibiotics: we will use antibiotics released from hydrogels against E. coli (tobramycin) or S. Aureus (vancomycin) in a microdilution broth assay using standard protocols. **Proposed Timeline: Months 12-18**

Percentage completion: 100%

Subtask 3- Characterization of mechanical and thermal stability of components and hydrogels. **Proposed Timeline: Months 18-24**

Percentage completion: 100%

Specific Aim 2 – In vivo evaluation of PEG-diacrylate hydrogels in a) small and b) large animal models

Major Task 3 – In vivo assessment of wound dressing in mice

Subtask 1 – In vivo efficacy of wound sealant/dressing to prevent implant infection: Using the mouse model of infection after open wound, we will use non-invasive bioluminescence optical imaging (BLI) to longitudinally assess the efficacy of the wound dressing in eradicating bacteria from a contaminated tissue bed. **Proposed Timeline: Months 18-24**

Percentage completion: 100%

Subtask 2 – Immune response, toxicity: Assessing endpoints of neutrophil and macrophage recruitment (fluorescence imaging), renal function, and histology of kidney, we will assess local and systemic toxicity of the wound dressing delivery of TA, bupivacaine, and antibiotics (tobramycin or vancomycin). **Proposed Timeline: Months 24**

Percentage completion: 100%

Major Task 4 – In vivo assessment of wound dressing in goats

Subtask 1 – *In vivo* efficacy of wound dressing to prevent implant infection: Using the goat model of infection after open wound, we will use non-invasive bioluminescence optical imaging (BLI) to longitudinally assess the efficacy of the wound dressing in eradicating bacteria from a contaminated tissue bed. **Proposed Timeline: Months 36-48**

Percentage completion: 10%

Subtask 2 – Immune response, toxicity: Assessing endpoints of neutrophil and macrophage recruitment (fluorescence imaging), renal function, and histology of kidney, we will assess local and systemic toxicity of the wound dressing delivery of TA, bupivacaine and antibiotics (tobramycin or vancomycin). **Proposed Timeline: Months 36-48**

Percentage completion: 0%

Subtask 3 – All surviving bacteria will be cultured and assayed for antibiotic resistance through serial dilution techniques and whole genome sequencing. **Proposed Timeline: Months 36-48**

Percentage completion: 0%

Subtask 4 – Using a modification of the murine clotting assay we will assess the hemostasis achieved by the wound dressing. **Proposed Timeline: Months 36-48**

Percentage completion: 0%

Subtask 5 – Trained veterinary staff will evaluate animal discomfort quantitatively.
Proposed Timeline: Months 36-48

Percentage completion: 0%

Hydrogel Synthesis:

PEG-diacrylate (PEGDA) macromers of varying molecular weights were synthesized from PEG (M_n 575, 700, 2000, 3500, 4600) and used to fabricate hydrogels with different properties. Hydrogels were evaluated

through observation of gelation and by characterizing mechanical properties with mass swelling ratios and elastic moduli. PEGDA 3350 was found to rapidly polymerize into hydrogels with ideal mechanical properties and extended therapeutic release profiles; as such, it was selected as the optimal macromer for the hydrogel system. We have improved the polymerization time of our hydrogels by optimizing the methods used for PEGDA synthesis. The 300µL hydrogels used for *in vivo* studies have an approximate polymerization time of 2 minutes.

In Vitro Therapeutic Release:

We successfully transitioned from bupivacaine to lidocaine and were able to detect both in a similar manner. Therapeutic release of lidocaine/bupivacaine and vancomycin could be detected using a UV-Vis spectrophotometer. We developed a system using Fe(III)Cl to quantify tobramycin and tranexamic acid concentrations, in addition to a Cu(II)-based assay that can detect tobramycin using a UV-Vis spectrophotometer at high concentrations, which may be helpful in quantifying release from hydrogels delivering a combination of therapeutics. We have characterized vancomycin, lidocaine, tranexamic acid, and tobramycin release kinetics from hydrogels using PEGDA 3350. Vancomycin and lidocaine diffused out of the hydrogel over the span of 2-3.75 days, tobramycin released within 1 day, and tranexamic acid, the hemostatic agent, experienced burst release from the hydrogel within 2 hours. We have developed HPLC protocols to quantify drug release, using vancomycin and lidocaine for preliminary studies. We will then transition to quantifying the release of multiple drugs from a single hydrogel.

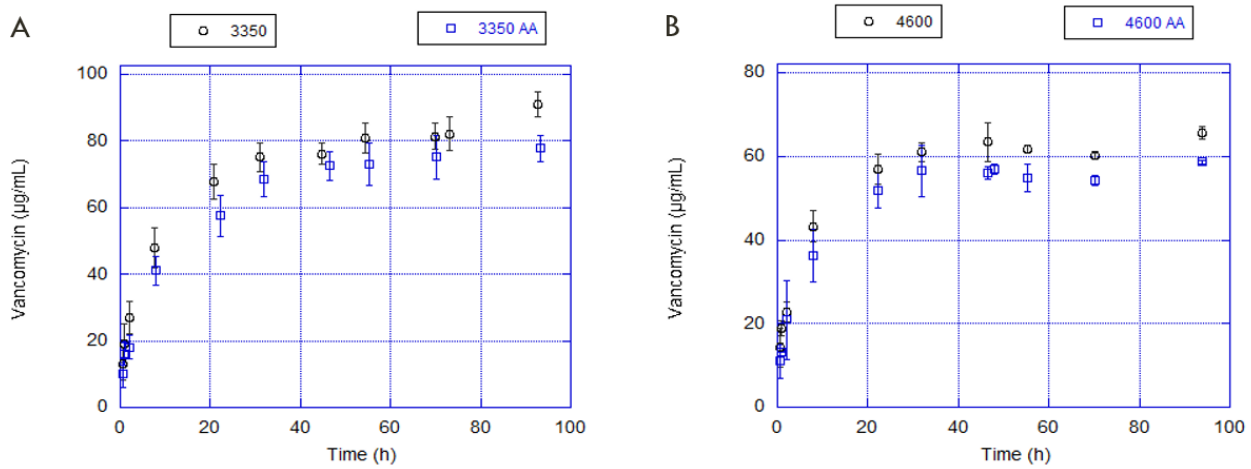


Figure 1. Kinetic release profiles of vancomycin from hydrogels synethsized using (A) PEGDA 3350 with (*blue*) and without (*black*) acrylic acid, and (B) PEGDA 4600 with (*blue*) and without (*black*) acrylic acid

In our attempts to further slow the release of therapeutics from the hydrogels, we incorporated charged acrylic acid to retard the diffusion of cationic drugs, primarily vancomycin and lidocaine. While hydrogels synthesized using lower molecular weight PEG-diacrylates (575 to 2000) displayed sustained release of vancomycin and lidocaine, higher molecular weight hydrogels showed only a slight improvement in drug release kinetics (Fig. 1).

We also explored incorporating sodium polyacrylate (PAA) into the hydrogels to slow the release of therapeutic agents. By adding PAA to the system in its solid, powdered form—as would be the case in the final platform—hydrogels incorporating these chains were successfully synthesized. The ratio of PEG to PAA can easily be tailored to produce hydrogels of different stiffness (elastic modulus), therefore different

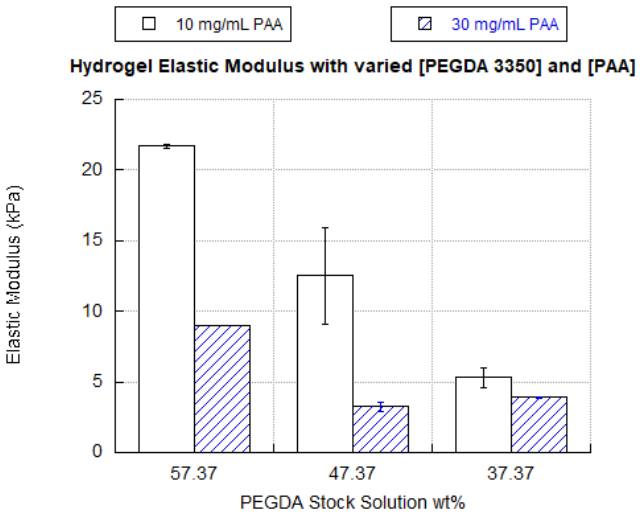


Figure 2. The decrease in hydrogel elastic modulus with the addition of sodium polyacrylate (PAA) can be counteracted by increasing the concentration of PEGDA added to system.

therapeutic release rates (Fig. 2). Preliminary testing shows that adding 2 mg/mL of sodium polyacrylate to the hydrogel system may enable sustained vancomycin release over a 7-day period (Fig. 3).

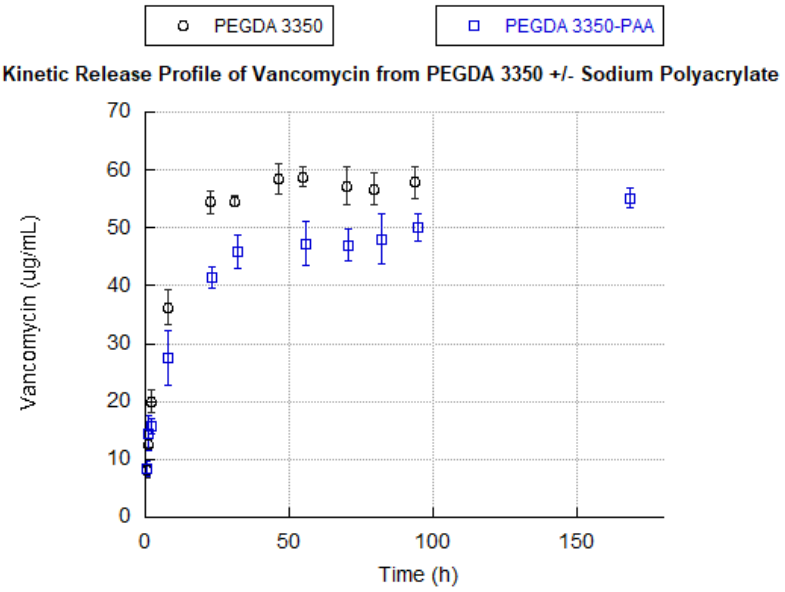


Figure 3. Adding 2 mg/mL PAA to the hydrogel system (*blue*) enables sustained release of vancomycin over 7 days, while the hydrogels without PAA (*black*) released vancomycin over approximately 2 days.

Elution experiments:

We have successfully optimized a method for the detection of the multiple drugs from our hydrogel system using HPLC. This method was adopted for the first *in vivo* preliminary studies. We used this to determine the amount of vancomycin remaining in the hydrogels after a 5-day implantation using mouse models. Analysis showed an average of 97.4% release of vancomycin *in vivo* (Fig. 4). We are currently transitioning into multi-drug studies where the current HPLC protocol will be used to quantify the release of multiple drugs from *in vitro* and *in vivo* models respectively.

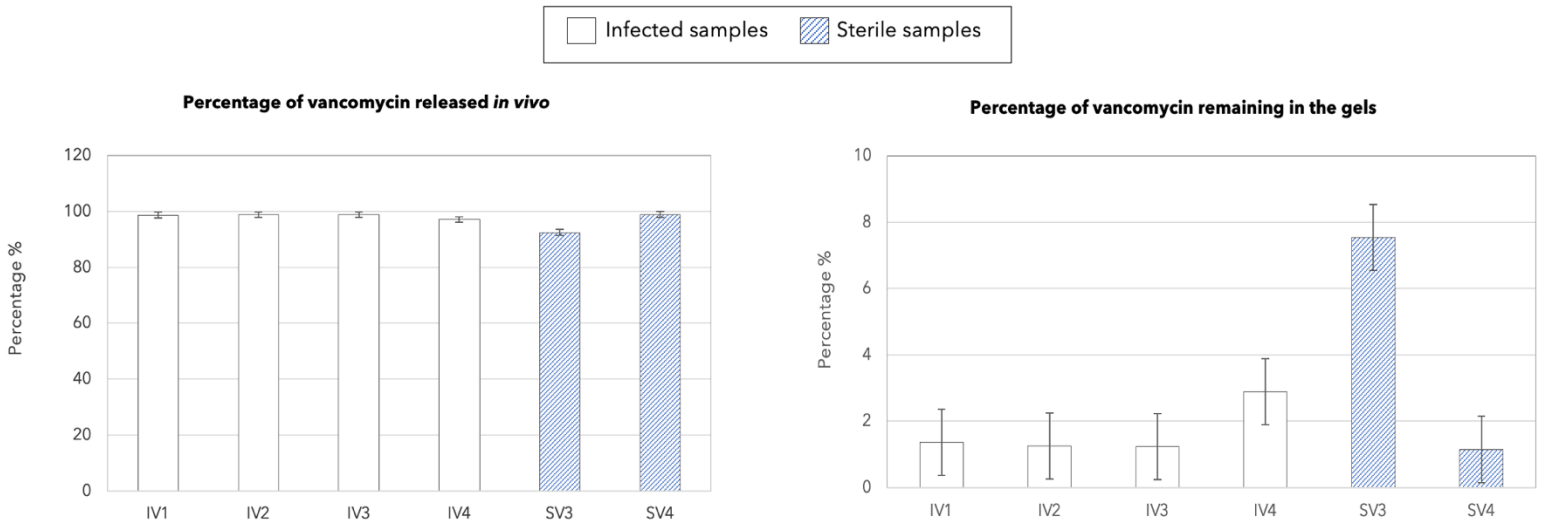


Figure 4. Amount of vancomycin released *in vivo* after a 5-day implantation. (A) Percentage released *in vivo*. (B) Percentage of drug remaining in the hydrogel system. IV – Gels implanted in the presence of *S. aureus.* SV – Gels implanted without *S. aureus.*

Multi-drug studies & assessment of released therapeutics:

To ensure that multi-drug loading does not affect the release of individual therapeutics, we studied the release of vancomycin from our hydrogel network containing all therapeutics using HPLC. Vancomycin and lidocaine only were detected and separated via our HPLC method; therefore, tranexamic acid and tobramycin will require colorimetric assays to be detected, as previously described (Fig. 5). Total vancomycin release was observed after 48 hours and mass spectrometry was used to ensure the efficacy of the separation method (Fig. 6). These data confirmed that multi-drug loading does not affect the release kinetics of individual therapeutics.

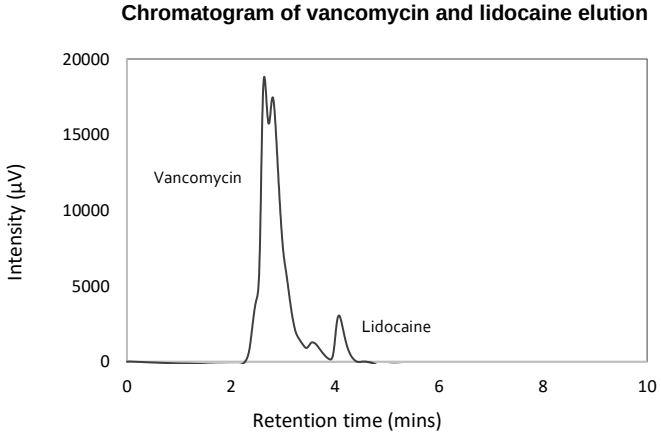


Figure 5. Detection and separation of vancomycin and lidocaine using HPLC. Retention times: Vancomycin; 2.6 – 3 mins. Lidocaine; 3.7 – 4.2 mins.

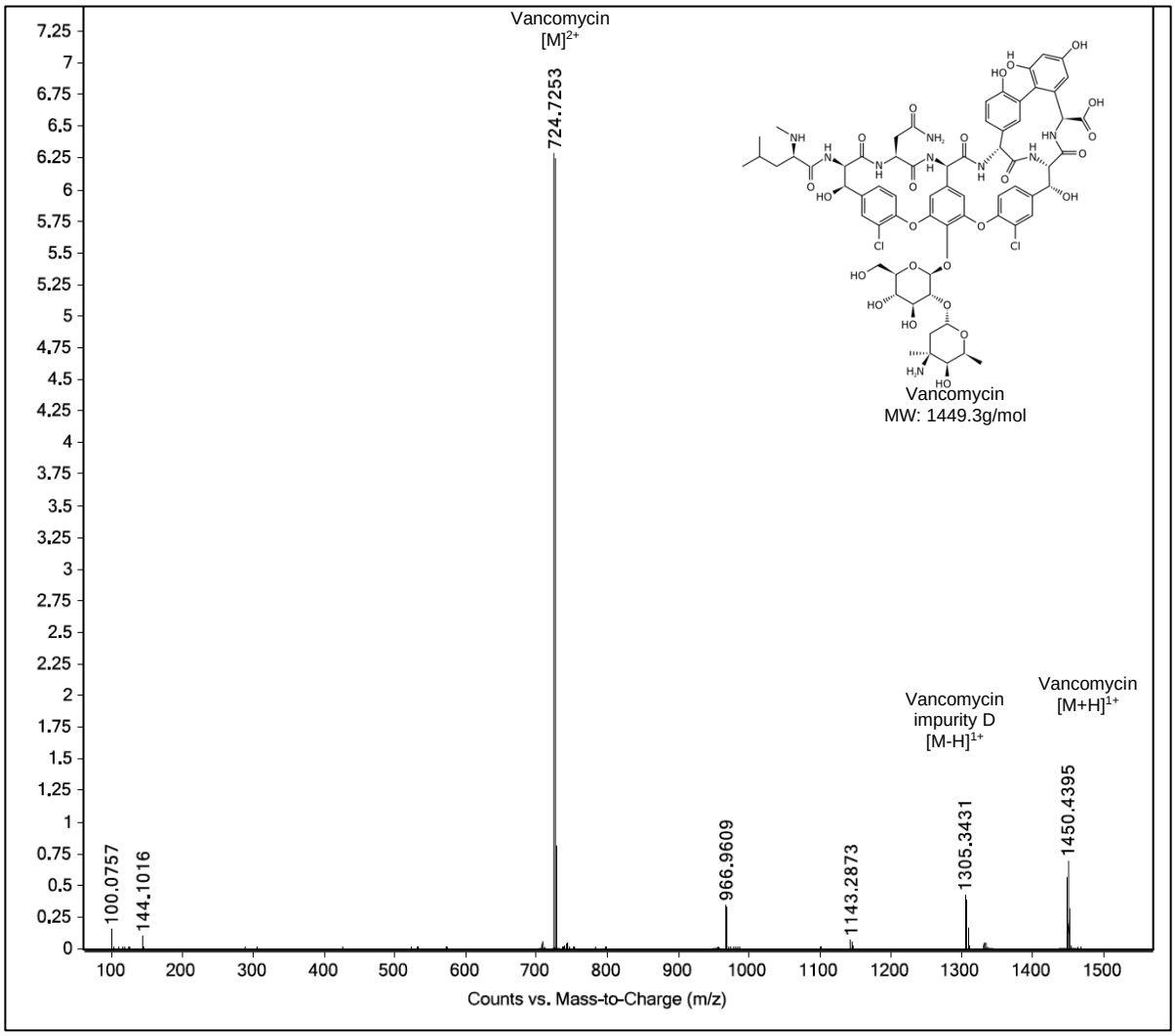


Figure 6: Mass spectrum (electron ionization) of vancomycin, isolated from a hydrogel system containing all therapeutics. *Sample collected post-HPLC separation*.

Evaluation of systemic drug concentrations:

To study the performance of our hydrogel system in relation to the traditional antibiotic system, serum samples were retro-orbitally obtained from mouse models after polymicrobial efficacy experiments (POD5). The drug components present in these samples were extracted, and vancomycin was separated using our optimized HPLC method. The samples obtained post-chromatography were analyzed via mass spectrometry to determine the presence/ absence of vancomycin. We detected very low concentrations of vancomycin present in both hydrogel and antibiotic-related samples. However, the hydrogel samples showed a higher indication of the presence of vancomycin.

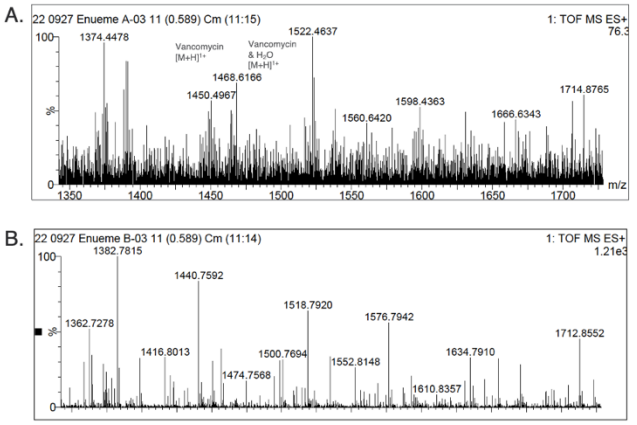


Figure 7. Mass spectrum of hydrogel serum samples (A) and antibiotic powder serum samples (B). *Low spectrum signals indicate low concentrations.*

Stability Studies:

We stored the hydrogel components at an elevated temperature (110-115 °F) to characterize the physical and chemical stability of each. Samples were placed in an Eppendorf tube with no additional interventions (i.e. Parafilm around the cap), and placed in a pre-heated oven. Samples were characterized at 1 day, 1 week, 1 month, and 3 months using ^{1}H NMR and solubility and gelation studies. No degradation of PEGDA was observed while comparing the ^{1}H NMRs at each time point. Additionally, all samples were readily solubilized in water, indicating that they had not polymerized while stored at an elevated temperature. While the PEGDA 3350 and APS demonstrate a high degree of stability, the system's catalyst, TEMED, had completely evaporated within 1 week. This problem may be addressed by i) exploring alternative amine catalysts, ii) eliminating TEMED by increasing the amount of amine-containing drugs in gels, or iii) modifying final packaging with an improved seal.

Leak Pressure Testing:

We are currently developing the leak pressure testing set-up. Our system will consist of PTFE tubing connected to a syringe pump. The tubing, with a puncture through one side to emulate a blast wound, will be housed within a halved, rigid cylindrical casing filled with collagen gel on the side opposite the puncture. Our hydrogel system will be injected into the puncture site, allowed to solidify, and wrapped with a gauze bandage. The flow rate of the syringe pump will be gradually increased until a leak is observed.

In-Vivo Dosing:

We performed an in vivo experiment using our murine open fracture model. First, a titanium k-wire was place in a retrograde fashion into the right distal femur of the mouse in order to model a foreign body from a blast wound. Then, a lateral thigh incision was made, the femur was exposed and a lateral cortical defect over the distal femur was made using a rongeur to model a fracture. For each experimental group, an inoculum of 1x 10^5 and 1 x 10^7 of a bioluminescent strain of *S. aureus* (Xen36) was placed directly into the fracture site. Following inoculation, the hydrogel was placed into the wound. The experimental groups included: sterile control (2 mice), hydrogel only (infected control) (2 mice), hydrogel + 4 mg of vancomycin (8 mice). Outcomes included in vivo bioluminescence as a marker of bacterial burden on post-operative days (POD) 1-4, as well as soft tissue and metal foreign body colony forming units (CFUs) following sacrifice on POD 4.

The mean bioluminescence of the groups which received hydrogel + vancomycin was as low as the sterile control group at all time points. Conversely, the mean bioluminescence of the groups that received hydrogel only was significantly elevated at all time points (Fig. 5).

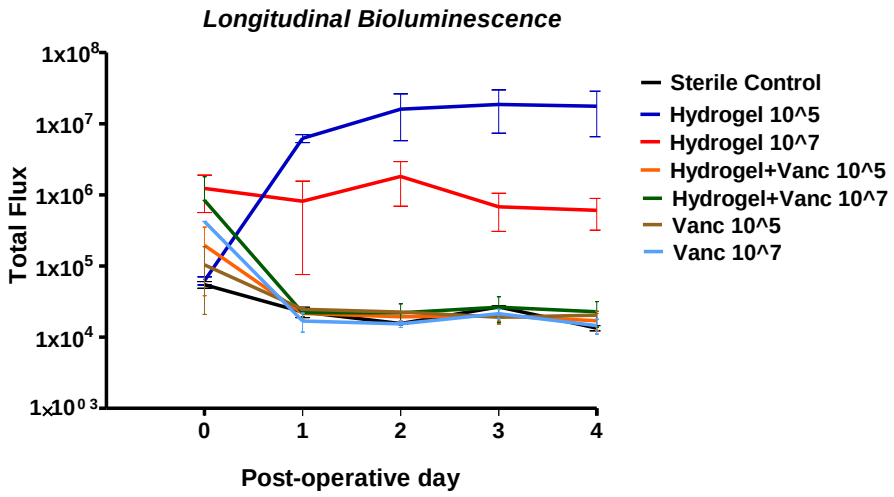


Figure 8. In vivo bioluminescent signal over time representing bacterial burden.

For POD 4 soft tissue CFUs, the hydrogel + vanc group had lower mean CFUs compared to the infected control group (Fig. 6-A). Similarly, the POD 4 metal foreign body CFUs were lower for the hydrogel + vancomycin group as compared to the hydrogel only group (Fig. 6-B).

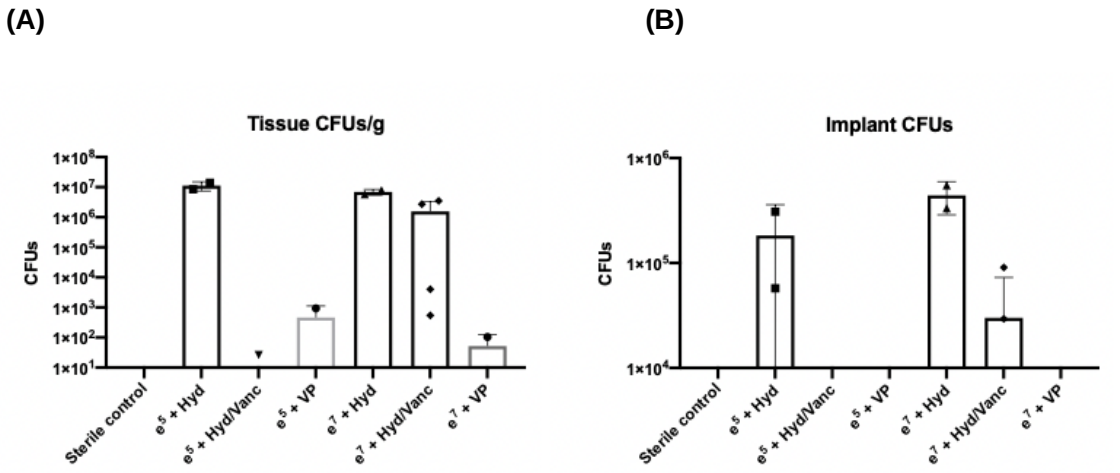


Figure 9. The average bacterial colony forming units (CFU) from the surrounding tissues (A) and implants (B).

In-Vivo Efficacy:

After the aforementioned dosing experiment, we proceeded with an inoculum of 1e5 CFUs S. *aureus* (Xen36). In addition, to provide limb stability to experimental mice, our titanium implant was lengthened by 4mm. In this experiment, we test the efficacy of hydrogel + 4mg vancomycin against the application of 4mg intrawound vancomycin powder. The aforementioned surgical procedures were performed. The experimental groups included: sterile control (2 mice), hydrogel only (infected control) (2 mice), hydrogel + 4 mg of vancomycin (8 mice) and 4mg intrawound vancomycin (8 mice). Outcomes included *in vivo* bioluminescence as a marker of bacterial burden on post-operative days (POD) 1-5, as well as soft tissue and implant colony forming units (CFUs) following sacrifice on POD 5 (Fig. 7).

There was not a significant difference in the mean bioluminescence between hydrogel + 4mg vancomycin and 4mg vancomycin powder. In addition, the bioluminescent curve for both of these treatment groups were as low as sterile control. The bioluminescent curve for the hydrogel only group was significantly elevated at all time points.

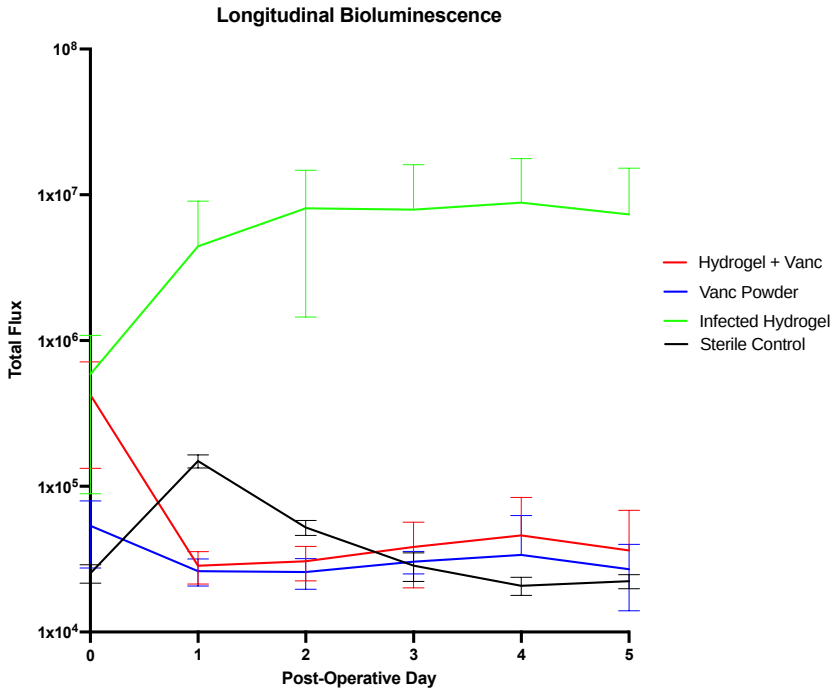
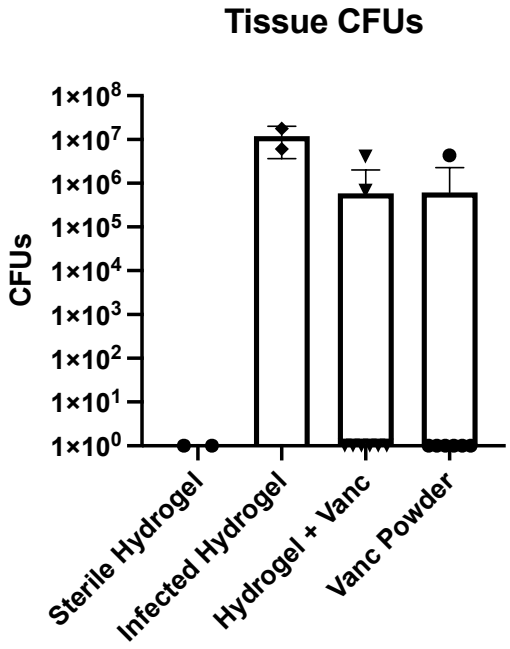


Figure 10. In vivo bioluminescent signal over time representing bacterial burden.

On POD5, tissue and implants were collected for CFUs. Both hydrogel + 4mg vancomycin and 4mg vancomycin powder had significantly lower tissue and implant CFUs compared to the infected hydrogel group. There was no significant difference in tissue or implant CFUs between the hydrogel + 4mg vancomycin and 4mg vancomycin powder groups. In addition, hydrogel + 4mg vancomycin prevented 6/8 tissue and implant infections. On the other hand, vancomycin powder prevented 5/8 tissue infections and 7/8 implant infections (Fig. 8).

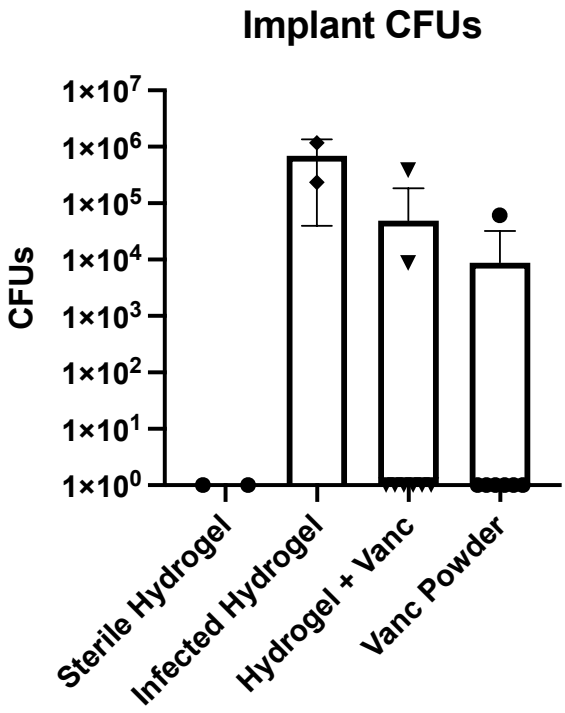
A).



B).

Sample	Tissue
Sterile Hydrogel	0/2
Infected Hydrogel	2/2
Hydrogel + Vanc	2/8
Vanc Powder	3/8

C). D).



Sample	Implant
Sterile Hydrogel	0/2
Infected Hydrogel	2/2
Hydrogel + Vanc	2/8
Vanc Powder	1/8

Figure 11. Average tissue colony forming units (CFUs) (A) and binary tissue CFU growth (B). Average implant colony forming units (CFUs) (C) and binary implant CFU growth (D).

In-Vivo Vancomycin Elution Kinetics:

In this experiment, we want to determine the rate of vancomycin elution in our small animal model and see if the acidity of an infected wound environment affects the rate of drug elution. We performed an in vivo experiment using our murine open fracture model. First, a titanium k-wire was place in a retrograde fashion into the right distal femur of the mouse in order to model a foreign body from a blast wound. Then, a lateral thigh incision was made, the femur was exposed and a lateral cortical defect over the distal femur was made using a rongeur to model a fracture. For each experimental group, an inoculum of 1x 10^5 of a bioluminescent strain of *S. aureus* (Xen36) or sterile saline was placed directly into the fracture site. A biopsy punch was used to create identical cylinders of hydrogel containing 4mg of vancomycin and no drug. Following inoculation, the hydrogel was placed into the wound. The experimental groups included: sterile hydrogel (4 mice), sterile hydrogel + 4mg vancomycin (4 mice), infected hydrogel (6 mice) and infected hydrogel + 4 mg of vancomycin (6 mice). High performance liquid chromatography (HPLC) was used to calculate the concentration of vancomycin remaining in each hydrogel on POD5.

Please see figure 4 above for HPLC data.

In-Vivo Development of a Polymicrobial Model of Infection:

In this series of 2 bacterial dosing experiments, we wanted to establish a polymicrobial model using MSSA (Xen36) and Pseudomonas (Xen41) to test the efficacy of multi-antibiotic loaded hydrogel. The aforementioned surgical procedures were performed. Experiment 1 groups included sterile control (1), E5(Xen36)E5(Xen41) (3), E5(Xen36)E4(Xen41) (3), and E5(Xen36)E3(Xen41) (3). Experiment 2 groups included sterile control (2), E3(Xen36)E2(Xen41) + Hydrogel (3), and E4(Xen36)E2(Xen41) + Hydrogel (3). Outcomes included *in-vivo* bioluminescence as a marker of bacterial burden on POD 1-5, as well as soft tissue and implant CFUs following sacrifice on POD 5. This experiment allowed us to refine selective media protocols to isolate either MSSA (TSB + 7.5%NaCL) or Pseudomonas (LB + 1ug/mL Vancomycin) for counting.

In experiment 1, all 3 mice that received E5 Pseudomonas expired on POD1. 2 of 3 mice receiving E4 Pseudomonas expired on POD1 and POD2, respectively. All 3 mice receiving E3 Pseudomonas survived the experiment (Fig. 9-A). In surviving mice, we obtained substantial CFUs from each respective organism indicating a robust infection (Fig. 9-B&C).

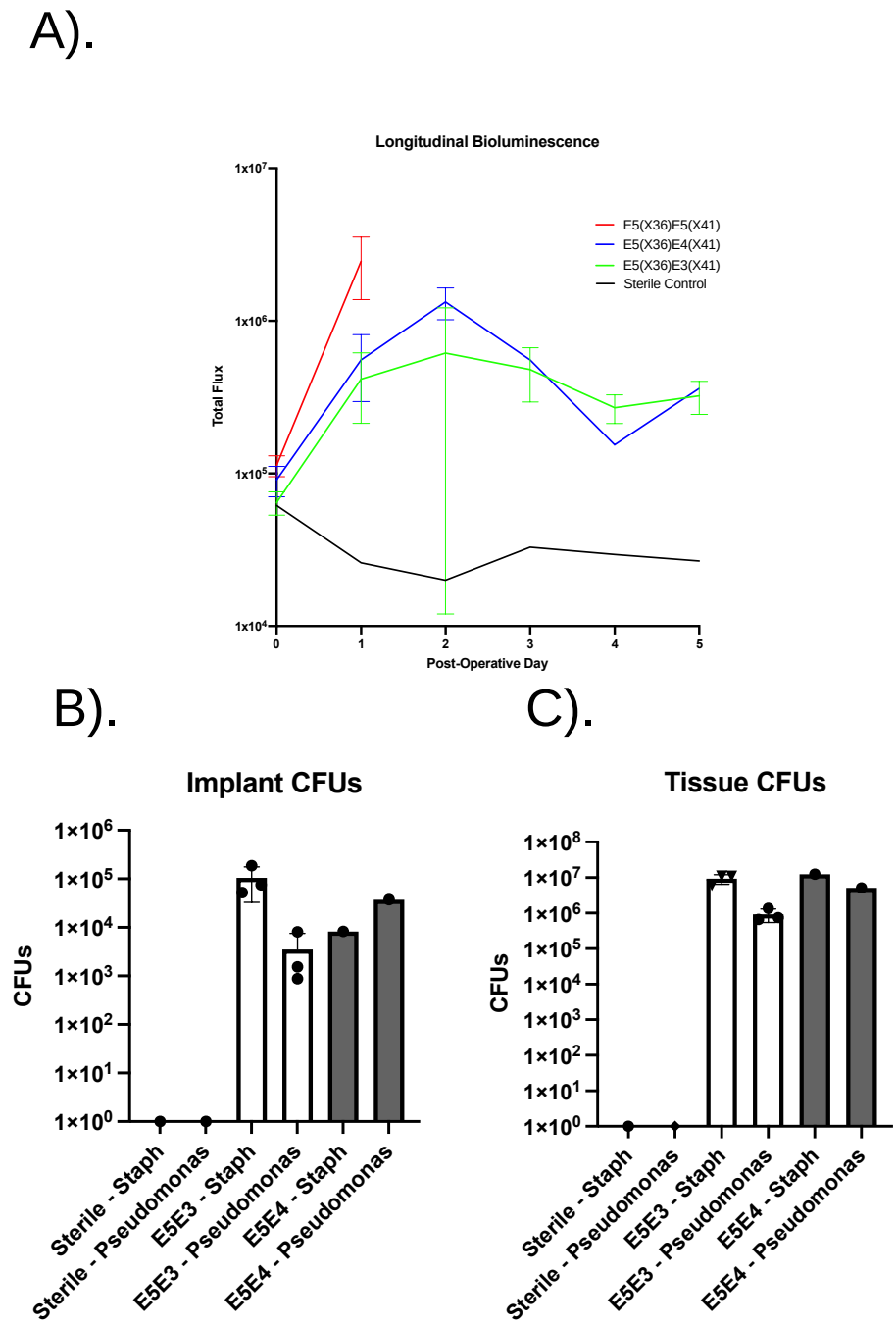


Figure 12. In vivo bioluminescent signal over time representing bacterial burden (A). Average implant colony forming units (CFUs) (B). Average tissue colony forming units (CFUs) (C).

E5 and E4 doses of Pseudomonas are lethal to mice in combination with E5 MSSA. Given the aggressive nature of Pseudomonas and its tendency to cause sepsis in this surgical model, we wondered if the addition of non-antibiotically loaded hydrogel will amplify infection in animals.

In experiment 2, 1 of 4 mice in the E3(Xen36)E2(Xen41) group expired on POD3. No mice expired in the E4(Xen36)E2(Xen41) group (Fig. 10-A). Despite lowering initial inoculum for both organisms, we were able to establish a robust infection (Fig. 10-B&C) with comparable CFUs to experiment 1 (Fig. 9-B&C).

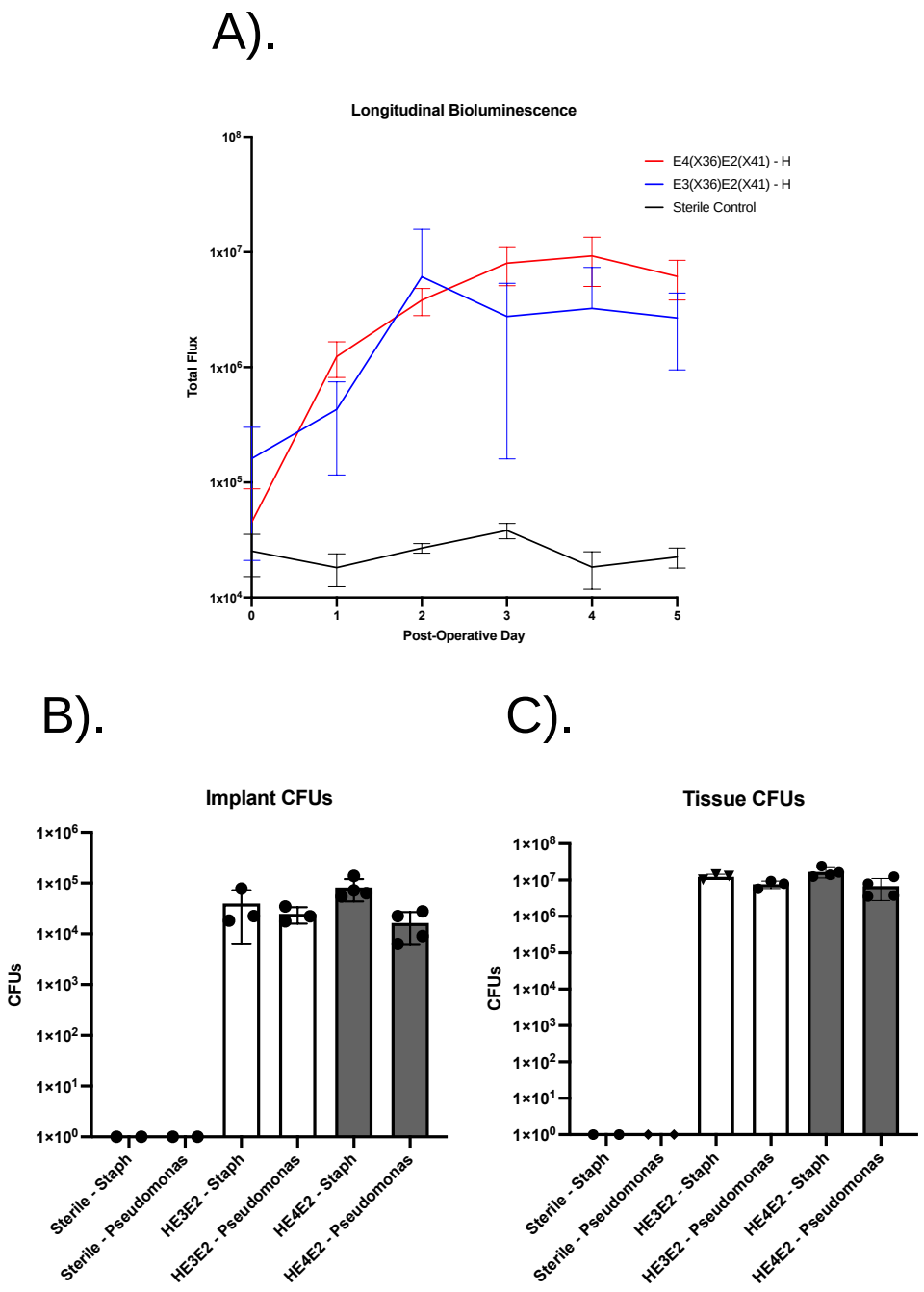


Figure 13. In vivo bioluminescent signal over time representing bacterial burden (A). Average implant colony forming units (CFUs) (B). Average tissue colony forming units (CFUs) (C).

The chosen inoculum for future experiments will be E3(Xen36)E2(Xen41) considering that the addition of non-antibiotic impregnated hydrogel increases CFUs and burden of infection. E3 MSSA was chosen in anticipation of the addition of a third pathogen, *E. coli* (Xen14).

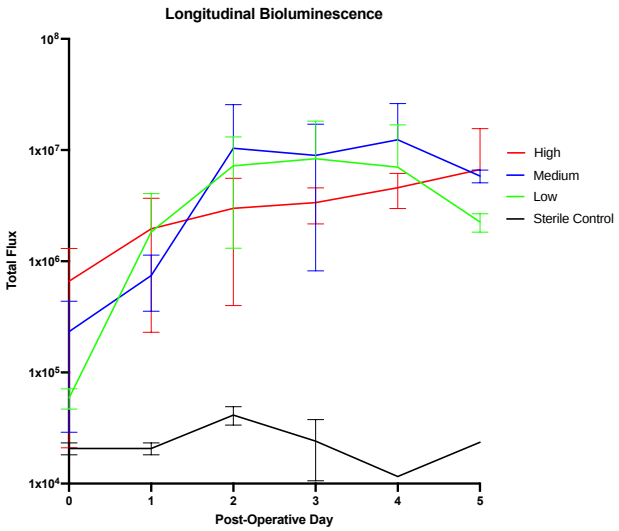
In Experiment 3, we added a third pathogen, *E. coli* (Xen14) to the experiment. The following inoculums/experimental groups were tested:

- Sterile Control – 2

- High - E3(X36)+E2(X41)+E3(X14) – 4
- Medium - E3(X36)+E2(X41)+E2(X14) – 4
- Low - E2(X36)+E2(X41)+E2(X14) – 4

Interestingly, in the low inoculum group one mouse expired on POD3 and POD5. In addition, one mouse in the medium inoculum group expired on POD5. No mice expired in the high inoculum group.

A).



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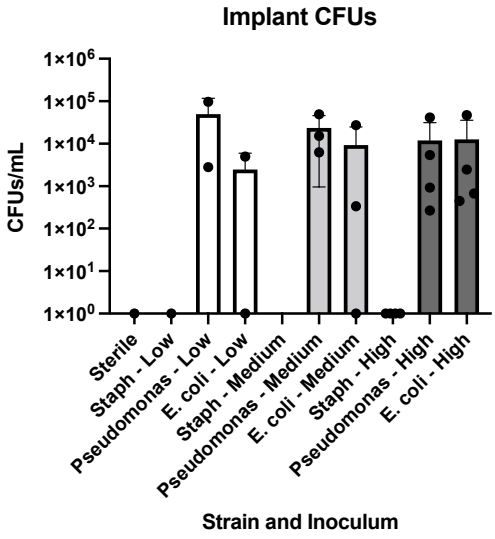


C).

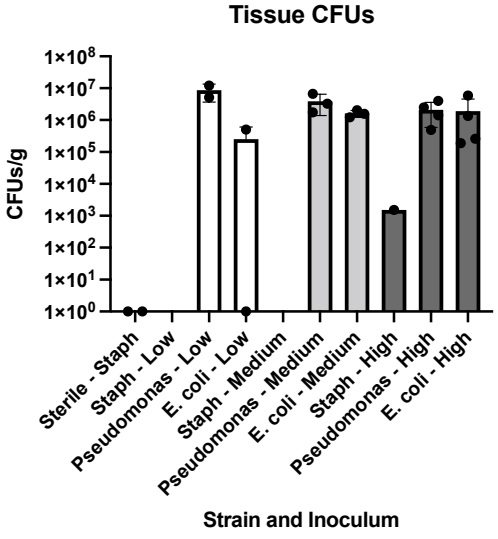


Figure 14. *In-vivo* bioluminescent signal over time representing bacterial burden (A). Average implant colony forming units (CFUs) (B). Average tissue colony forming units (CFUs) (C).

It is evident according to implant and tissue CFUs that lower inoculums of Xen36 *S. aureus* and Xen14 *E. coli* allow for more robust growth of Xen41 *P. aeruginosa*, leading to higher mouse mortality in the low and medium inoculum groups. Furthermore, the addition of a second gram negative pathogen, *E. coli*, allows for the both gram negative *P. aeruginosa* and *E. coli* to outcompete *S. aureus* with the exception of the high inoculum group. We will choose a final inoculum of E5(Xen36)E2(Xen41)E3(Xen14) allowing for a balance of E5 gram negative and E5 gram positive species.

In-Vivo Hydrogel Polymicrobial Efficacy:

Experiment 1:

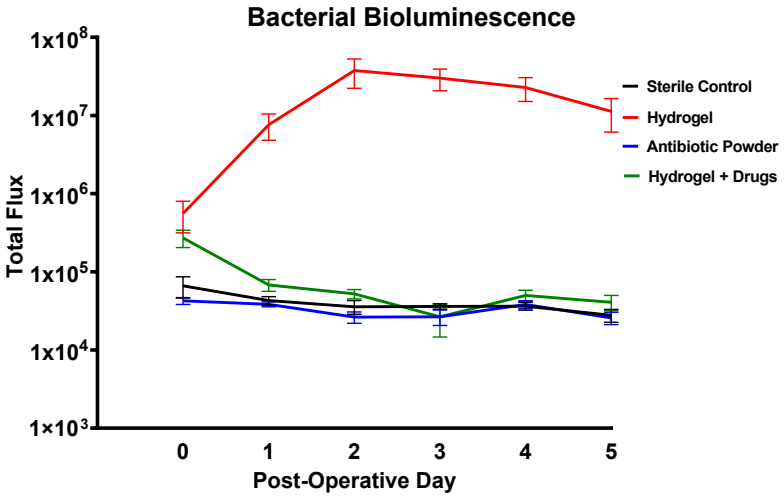
The goal of our first experiment was to identify the antimicrobial efficacy of our drug loaded hydrogel against our established E5(Xen36)E2(Xen41)E3(Xen14) polymicrobial animal model. Our experimental groups were as follows:

- Sterile Control – 2
- Infected Hydrogel – 4
- Intrawound antibiotic powder (vancomycin + tobramycin) – 4
- Hydrogel + Drugs (vancomycin + tobramycin + tranexamic acid + lidocaine) – 4

We intentionally loaded our hydrogel with all four agents to determine if multi-drug impregnation would interfere with antimicrobial efficacy. Please see drug concentrations below:

- Vancomycin: 4mg
- Tobramycin: 4.572mg
- Tranexamic acid: 5.56mg
- Lidocaine: 1.384mg

A).



B). C).

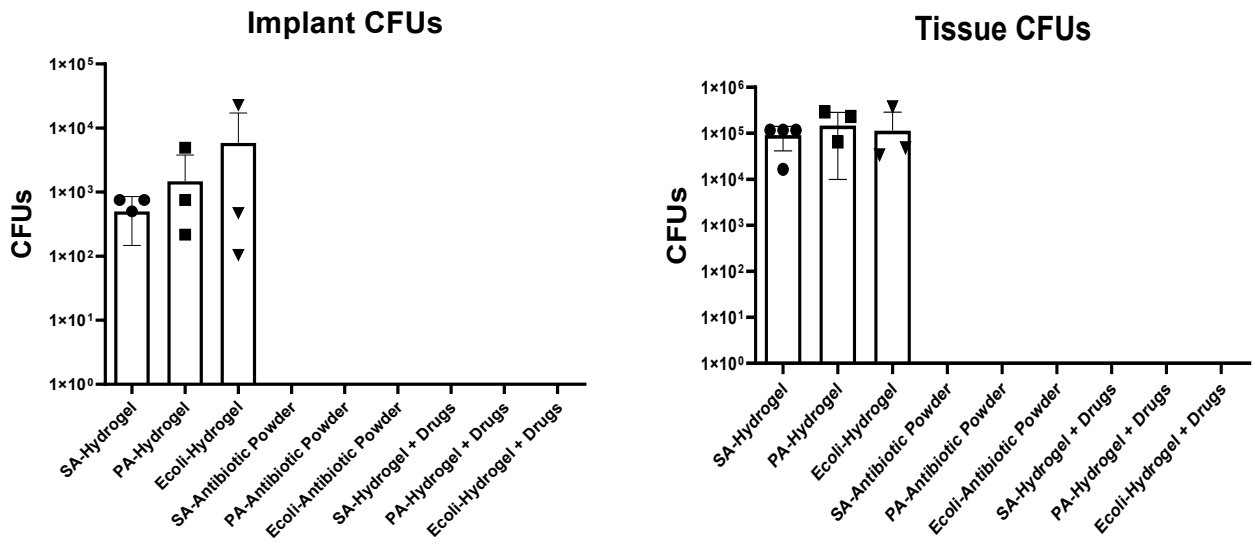


Figure 15. *In-vivo* bioluminescent signal over time representing bacterial burden (A). Average implant colony forming units (CFUs) (B). Average tissue colony forming units (CFUs) (C).

In Figure 15A, the antibiotic powder group lead to the immediate killing of bacteria in the wound as the bioluminescent signal in overlapping with sterile on POD0. In the drug loaded hydrogel group, one can observe a gradual killing of bacteria until the group meets sterile control on POD3. In Figures 14B and 14C, both antibiotic powder and drug loaded hydrogel eliminated polymicrobial CFUs completely by POD5, which is promising for our hydrogel. Our next polymicrobial efficacy experiment aims to increase our power and will also focus on renal toxicity, serum antibiotic levels, and antimicrobial susceptibility testing.

Experiments 2 & 3:

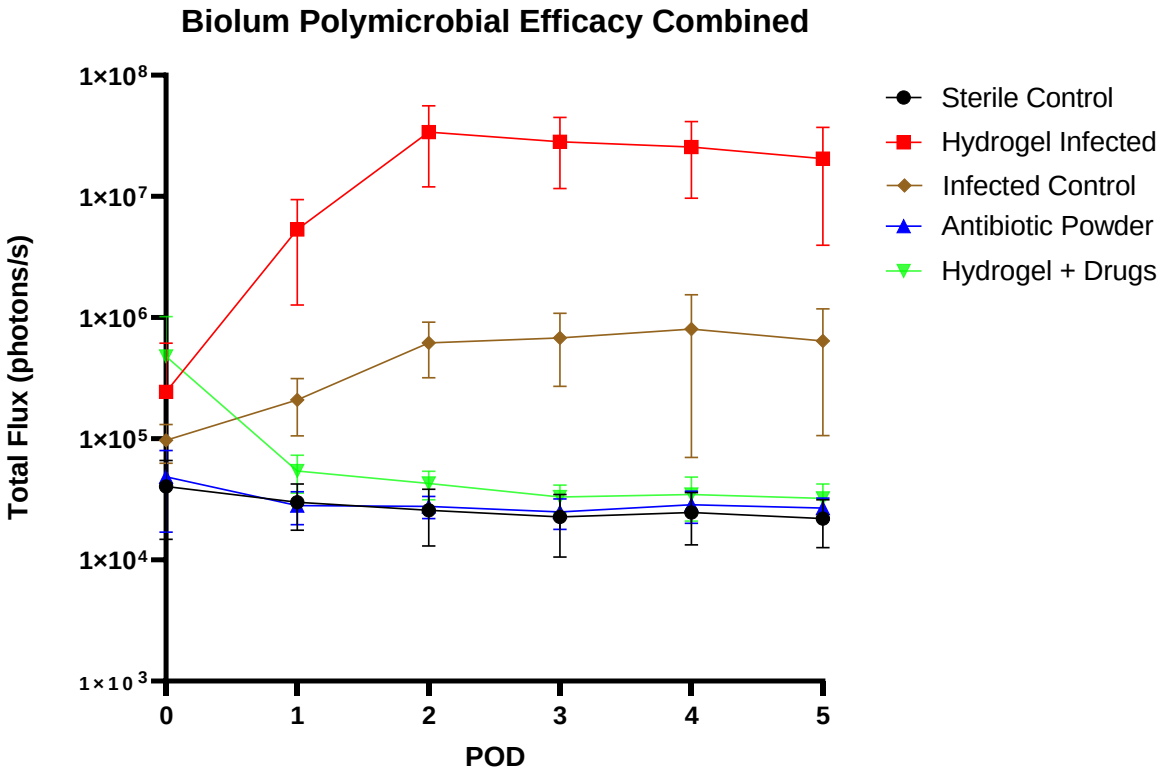
After completing experiment one, we performed two additional experiments to increase power and verify the efficacy of our treatment arms. After compiling the data from our three experiments, our experimental breakdown per group is below (N=61).

- Sterile Control – 6
- Infected Control – 10
- Infected Hydrogel – 15
- Intrawound antibiotic powder (vancomycin + tobramycin) – 15
- Hydrogel + Drugs (vancomycin + tobramycin + tranexamic acid + lidocaine) – 15

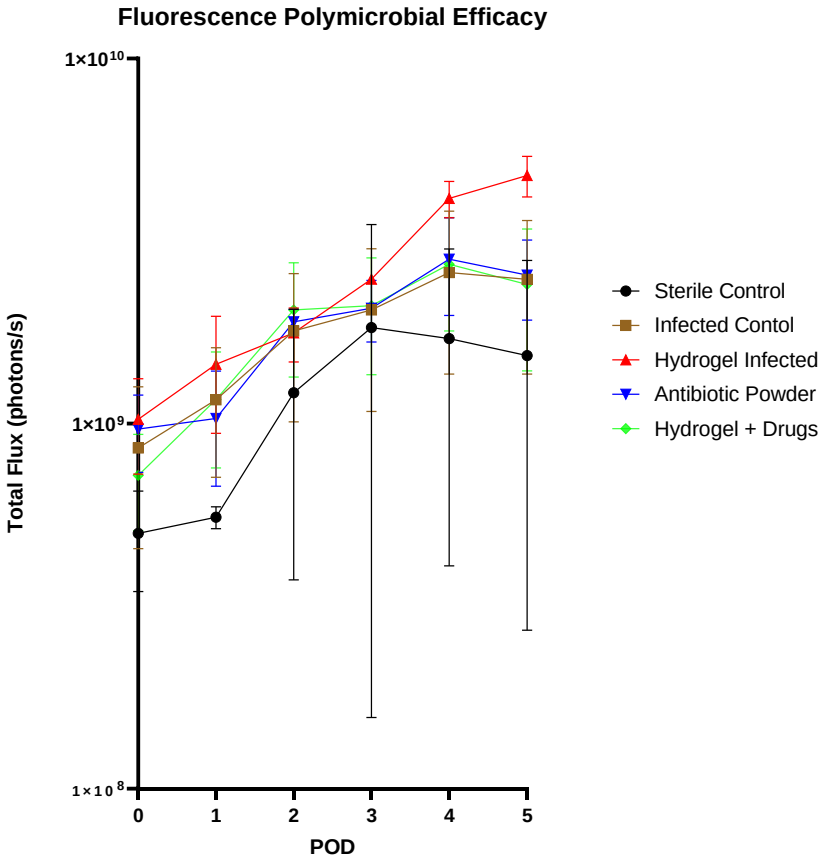
Experimental endpoints include daily in-vivo bioluminescence and fluorescence imaging, implant and tissue CFUs, kidney histology, microCT imaging, and antibiotic susceptibility testing (if applicable). We continued to use the following quantities of the drugs below for our hydrogel and antibiotic powder groups.

- Vancomycin: 4mg
- Tobramycin: 4.572mg
- Tranexamic acid: 5.56mg
- Lidocaine: 1.384mg

A).


Biolum Polymicrobial Efficacy Combined
Total Flux (photons/s)
1×10⁸
1×10⁷
1×10⁶
1×10⁵
1×10⁴
1×10³
0
1
2
3
4
5
POD
Sterile Control
Hydrogel Infected
Infected Control
Antibiotic Powder
Hydrogel + Drugs

B).


Fluorescence Polymicrobial Efficacy
Total Flux (photons/s)
1×10¹⁰
1×10⁹
1×10⁸
0
1
2
3
4
5
POD
Sterile Control
Infected Contol
Hydrogel Infected
Antibiotic Powder
Hydrogel + Drugs

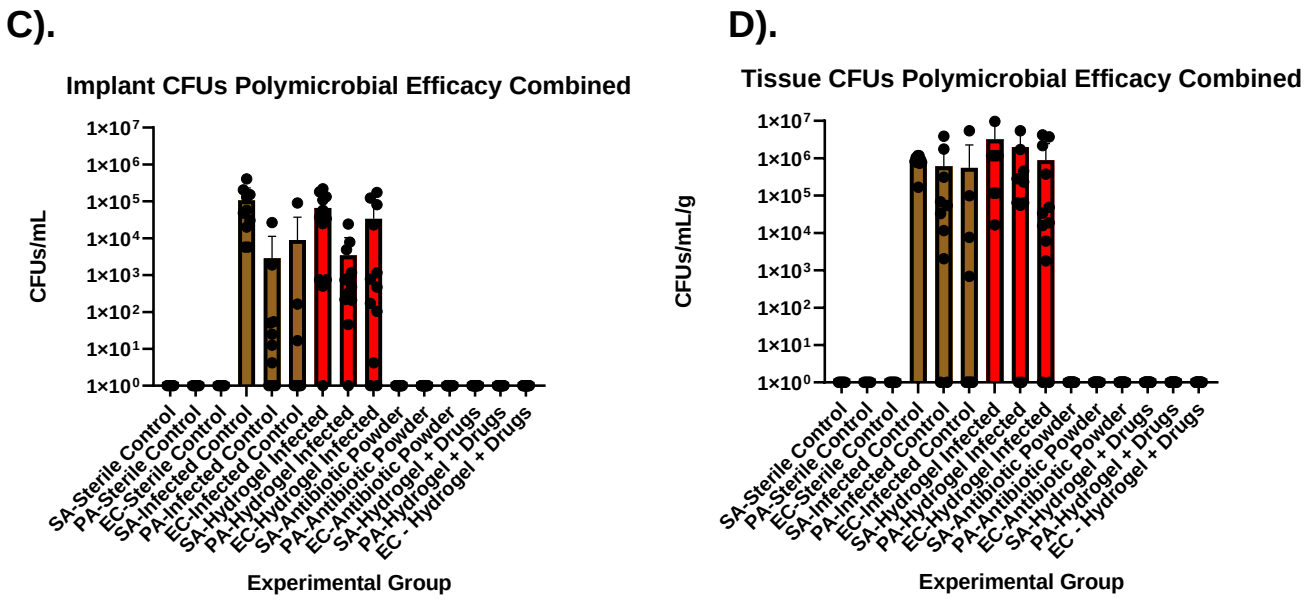


Figure 16. *In-vivo* bioluminescent signal over time representing bacterial burden (A). *In-vivo* fluorescent signal over time representing granulocyte recruitment (i.e. neutrophils) (B). Average implant colony forming units (CFUs) (C). Average tissue colony forming units (CFUs) (D).

In-vivo longitudinal bioluminescence demonstrates that the unloaded hydrogel group has the highest infection burden followed by the infected control group. The drug loaded hydrogel group demonstrates a gradual decrease in bioluminescent signal over three days until it reaches the line of sterile control, indicating the gradual elution of antibiotics over this time. The antibiotic powder group leads to the immediate killing of bacteria (Figure 16A). There is no statistical difference in granulocyte recruitment between our treatment groups, which indicates its safe use of hydrogel in our mouse model (Figure 16B). of the thirty mice who received either antibiotic powder or drug loaded hydrogel, all mice were culture negative at the end of the experiment (Figure 16C & D). This rendered it impossible to perform antibiotic susceptibility testing or sequencing. When comparing the infected control group to our unloaded hydrogel group, the presence of hydrogel alone seemed to increase the bacterial burden of gram-negative species harvested from implants and tissue (Figure 16C & D). This may suggest the need to cover for gram-negative species when choosing antibiotics to load in the hydrogel in the field. Kidney histology and microCT images are pending processing and analysis and will be included in the next DOD quarterly report. In addition, we plan to run a final small animal *in-vivo* experiment using our transgenic Lys-EGFP strain and utilize thromboelastographic (TEG) studies to measure the efficacy of drug loaded hydrogels to prevent fibrinolysis.

Ex-Vivo Hydrogel Thromboelastography (TEG):

Experiments 1 and 2:

We endeavored to determine the ability of tranexamic acid (TXA) loaded hydrogel to prevent fibrinolysis using TEG studies. We performed two experiments and focused on the maximum amplitude (MA), G, Lysis in 30 minutes (LY30). The maximum amplitude is representative of clot strength and normal values range between 44-64. G is another indicator of clot strength and normal values range between 3.6-8.5. LY30 is indicative of the percent of the clot that lyses in 30 minutes and normal values range from 0-8%. Our first experiment was to determine our ability to successfully induce clotting, induce fibrinolysis, and utilize dissolved TXA to prevent a fibrinolysis reaction. 200uM of dissolved TXA was used. Groups are listed below and there were 3 replicates for each group:

1. Normal blood – Thrombin (IIa) (clotting control)
2. Normal Blood – tPA (fibrinolysis control)
3. TXA Blood – Thrombin (clotting control)
4. TXA Blood – tPA

Average	MA	G	LY30
TXA + tPA	56.3333333	6.66666667	7.66666667
TXA + IIa	56.8333333	6.76666667	4.36666667
Blood + tPA	21.6	1.43333333	72.2666667
Blood + IIa	56.6	6.7	3.13333333

Figure 17. MA, G, and LY30 values for control groups in experiment 1.

In control group 1, we were able to successfully induce a strong clot with MA, G, and LY30 values of 56.6, 6.7, and 3.13%, respectively. In control group 2, we were able to induce fibrinolysis with MA, G, LY30 values outside of normal limits at 21.6, 1.43, and 72.26%, respectively. For control group 3, the addition of TXA did not disrupt our ability to induce clothing indicated by MA, G, and LY30 values of 56.83, 6.76, and 4.36%, respectively. Lastly, the addition of TXA was able to prevent tPA fibrinolysis with MA, G, and LY30 values of 56.33, 6.67, and 7.67%, respectively (Figure 17).

For experiment 2, the same 300uL TXA loaded hydrogel from *in-vivo* was generated (5.56mg dissolved TXA). In addition, 4 drug loaded hydrogel was also generated to show that multi-drug impregnated hydrogel would not disrupt its ability to prevent fibrinolysis, making it a procoagulant. We used a biopsy punch to make 56.5uL uniform hydrogel cylinders that could be incubated with 2mLs of mouse blood overnight to allow for TXA elution (Figure 18).

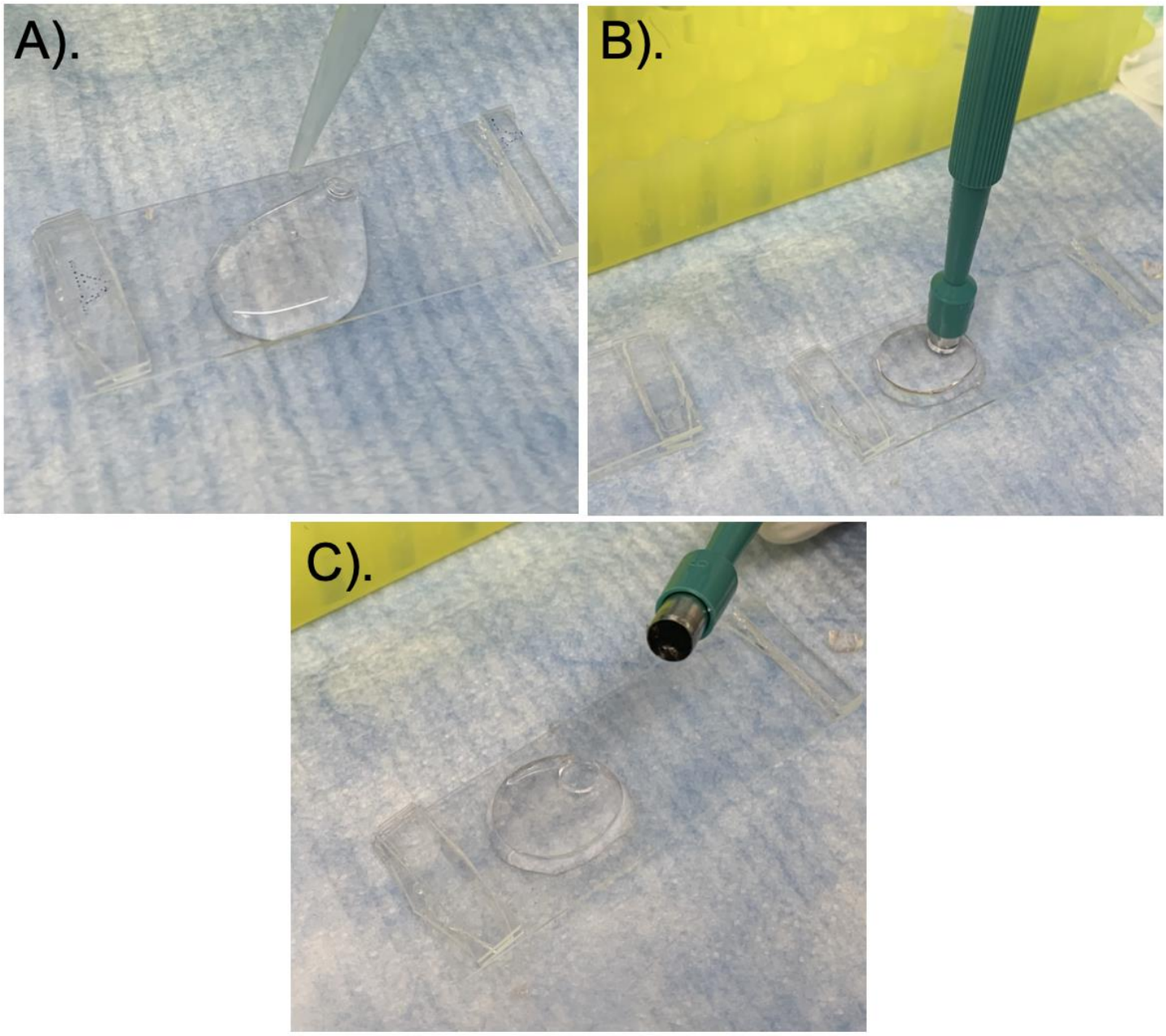


Figure 18. *Generation of cylindrical hydrogel for blood incubation using a biopsy punch. A). Pouring hydrogel with impregnated TXA. B). Solidified hydrogel is punched using biopsy tool. C). 56.5uL cylindrical hydrogel disk rests within biopsy punch.*

Assuming that 100% of TXA eluted into 2mLs of blood there would be 3.18mM of TXA in blood. This is well beyond the threshold of 200uM to inhibit fibrinolysis. Experimental groups are as follows:

1. Empty hydrogel + thrombin (clotting control)
2. Empty hydrogel + tPA (fibrinolysis control)
3. TXA hydrogel + tPA
4. 4-drug hydrogel + tPA

Average	MA	G	LY30
Hydrogel 4 Drug	49	5	4.4
Hydrogel TXA	56.133333	6.5	0.0666667
Hydrogel tPA Control	27.133333	1.9666667	84.6
Hydrogel IIa Control	48.633333	4.7333333	2.0333333

Figure 19. MA, G, and LY30 values for experimental groups in experiment 2.

For control group 1, the addition of hydrogel to blood incubated overnight did not disrupt our ability to generate a clot indicated by MA, G, and LY30 values of 49, 5, and 4.4, respectively. For control group 2, we successfully induced a fibrinolysis reaction indicated by MA, G, and LY30 values of 27.13, 1.97, and 84.6, respectively. For experimental group 3, TXA impregnated hydrogel was able to prevent fibrinolysis indicated by MA, G, and LY30 values of 56.13, 6.5, and 0.07, respectively. Lastly, 4 drug impregnated hydrogel did not disrupt TXA elution and fibrinolysis was inhibited indicated by MA, G, and LY30 values of 49, 5, and 4.4 respectively (figure 19).

These experiments demonstrated that TXA impregnated and multi-drug impregnated hydrogels can successfully prevent fibrinolysis in a TEG assay, which is an indicator of pro-coagulation efficacy.

We hope to have *in-vivo* small animal analgesic efficacy and safety studies (kidney histology) completed within the first half of 2023. In addition, synthesis of PEGDA has been scaled for large animal testing and >60g of PEGDA has been synthesized. Large animal modeling will begin Summer of 2023. All small animal work is complete.

Results have been disseminated in a poster, entitled “Synthesis and Drug Release Kinetics of Robust Poly(ethylene glycol) Hydrogels for Field Dressings”, presented at UCLA’s Bioengineering Research Day. In addition, a further submission entitled “Testing a Rapidly Polymerizable Hydrogel Wound Dressing for Far Forward Care of War-Time Traumatic Blast Wounds in a Murine Model of Open Fracture” has been accepted for Podium presentation at the 2023 Musculoskeletal Infection Society (MSIS) Annual Meeting.

Large animal experiments

We have implemented a sub award with Dr. Joseph Wenke who moved to UTMB in Aug. 2022. His move, along with COVID delays led to delays in initiating large animal work. At present, we expect these experiments to proceed imminently now that all approvals are in place.

The IACUC has been approved, equipment has been purchased and received as part of the lab setup. The wound has been created in a cadaver sheep. The next step will be to schedule a couple of the control animals, then on to the experimental group.

All but a few small confirmatory things have been completed to start the study. The protocol has received both IACUC and ACURO approval. The primary outcome of the study will be the amount of bioluminescence from the bacteria within the wound. For this, we had to accomplish several things: block out all light from door, create a frame to hold camera over table the animal will be lying on in supine position, and set up camera, software, and computer to image wound. There were unforeseen logistical issues with acquiring a dedicated computer and challenges with getting the computer and camera to interface. This has been resolved.

We are currently in the process of performing the wound creation surgery, inoculating, and measuring bioluminescence of the bacteria within the wound. From this we are making adjustments and trouble shooting. There is still too much ambient light from computer so we are creating a frame and a cover over the limb and camera to reduce this. We expect that by the end of August that we will be able to refine the system enough to start the study.

IMPACT:

Nothing to Report. We cannot yet assess the impact of the science on the principle discipline.

What was the impact on other disciplines?

Nothing to Report. We cannot yet assess the impact of the science on the other disciplines.

What was the impact on technology transfer?

Nothing to Report. We have not yet initiated technology transfer.

What was the impact on society beyond science and technology?

Nothing to Report. We cannot yet assess the impact on society.

5. CHANGES/PROBLEMS:

In developing our small-animal polymicrobial model, the proposed E8 inoculum of 3 microbial agents are not feasible in our open fracture model as mice consistently perish during experiments. We have found that lower inoculums decrease lethality, but do not compromise intensity of soft tissue and bone infection.

While we were concerned about laboratory closures related to COVID, we began the funding period by taking a mathematical computational analysis approach to the PEG synthesis Subtask. We used computer programming to predict elusion criteria of several different hydrogels to see if we could predict optimal candidates for synthesis in case we could not initiate work in the lab due to work restrictions. Given that we were in fact able to begin in person lab work, these computational models have served as supplemental data rather than a change of approach. In fact, they have helped guide synthesis plans and are in part responsible for why we are ahead of schedule.

We continue to have concern about potential COVID related laboratory closure but we have implemented work SOPs that allow experiments to proceed with one person working at any given time. This has been in concordance with UCLA policy to minimize potential COVID exposures.

Changes that had a significant impact on expenditures

We have had no changes in expenditures at present.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals.

Nothing to report. As vertebrate animal use may be initiated earlier than Month 18 as proposed (Major Task 3, Subtask 1), we have completed all Institutional and DOD Animal Care and Use Documentation. We currently expect no delays in transitioning to animal work one Major Task 1 and 2 are complete.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Nicholas Bernthal
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0003-3338-5878
Nearest person month worked:	27
Contribution to Project:	Oversees and organizes laboratory meetings; guides conception and evaluation of PEGDA and hydrogels based on clinical needs; design and preparation of upcoming animal studies.
Funding Support:	

Name:	Andrea Kasko
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-2355-6258
Nearest person month worked:	27
Contribution to Project:	Oversees synthesis of PEGDA and hydrogels; evaluation of drug release
Funding Support:	

Name:	Elizabeth Pumford
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	0000-0001-8036-627X
Nearest person month worked:	22
Contribution to Project:	Synthesis of PEGDA and hydrogels; evaluation of drug release
Funding Support:	

Name:	Amaka Enueme
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	0000-0002-3337-0468
Nearest person month worked:	16
Contribution to Project:	Synthesis of PEGDA and hydrogels; evaluation of drug release
Funding Support:	

Name:	Zeinab Mamouei
Project Role:	Project Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0003-2898-1544
Nearest person month worked:	27
Contribution to Project:	In vivo hydrogel experiments using mouse models
Funding Support:	

Name:	Christopher Hamad
Project Role:	Postdoctoral Research Fellow

Researcher Identifier (e.g. ORCID ID):	0000-0002-8896-9857
Nearest person month worked:	17
Contribution to Project:	In vivo hydrogel experiments using mouse models
Funding Support	NIH T32AR059033

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES:

None