Supporting Information

# Micrococcal-Nuclease-Triggered On-Demand Release of Vancomycin from Intramedullary Implant Coating Eradicates *Staphylococcus aureus* infection in Mouse Femoral Canals

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**Content**

1. **Methods**
2. **Supplementary Figures**

## 1. Methods

**Materials.** All chemicals were purchased from Sigma-Aldrich unless specified. For water contact angle and XPS characterizations, Ti6Al4V plates (0.5-mm thick, TMS Titanium, Poway, CA) were cut into 1×1 $cm^2$ square pieces and subjected to surface modifications. For *in vivo* studies, Ti6Al4V wires (0.5 mm in diameter, Goodfellow Corporation, Coraopolis, PA) were cut into 1-cm long pins for surface modifications. The methacrylated oligonucleotide (Oligo, Acid end-mC-mG-T-T-mC-mG-Acrydite end) was synthesized by IDTDNA (Coralville, Iowa) and used as received. *Staphylococcus aureus* (*S. aureus*, ATCC 25923) was purchased from ATCC and bioluminescent *S. aureus* strain Xen29 was purchased from Perkin Elmer.

**Enzymatic Oligo Digestion Monitoring.** The digestion of the methacrylated oligo by micrococcal nuclease (MN, 0.1-1.0 U/μL) was monitored by aqueous gel permeation chromatography (GPC) on a Varian ProStar HPLC system with two PL Aquagel−OH columns (Agilent Technologies) and a UV detector. The eluent was aqueous PBS (10 mM, pH 7.4) and the flow rate was 1.0 mL/min. Detection of the oligo probe and its fragments upon digestion was enabled by UV absorbance at 260 nm. Extinction coefficient of 2′-O-methyl–modified nucleotides was assumed to be the same as that of RNA. All measurements were performed in triplicates.

**Synthesis of PEGDMA-Oligo and PEGDMA-Oligo-Vanco hydrogels.** To synthesize the PEGDMA-Oligo hydrogel with varying crosslinking densities, a hydrogel precursor solution containing PEGDMA (7.5, 10 or 15 wt%), the methacrylated oligo (75 μM) and VA-086 (initiator; 1 wt%) in deionized (DI) water was freshly prepared. For the hydrogel disc preparation, 50 μL of the precursor solution was photo-crosslinked in an 8-mm in diameter Teflon mold under 365-nm irradiation for 10 min. All hydrogels were washed in DI water for 72 h with frequent fresh water

changes to remove excess radical initiators or any untethered methacrylate prior to further experiments.

Vancomycin was covalently attached to PEGDMA-Oligo hydrogel via EDC/NHS coupling. First the PEGDMA-Oligo hydrogel was equilibrated in 900-µL PBS (10 mM, pH 5.0) for 60 min. Then, EDC and NHS (7.5 mM, 50 µL each) were added to the equilibrated hydrogel and allowed to react for 30 min in RT. The activated hydrogel was then transferred into a vancomycin solution (0.5 mL, 150 µM in 10 mM PBS, pH 8.0) for amidation at RT for 4 h. All covalently modified PEGDMA-Oligo-Vanco hydrogels were washed in DI water for 72 h with frequent fresh water changes to remove unreacted/excess EDC, NHS and/or vancomycin prior to further use.

The oligonucleotide coupling efficiency within the hydrogel was determined by a leaching experiment. Briefly, the PEGDMA-Oligo-Vanco hydrogel (50 µL, 8 mm diameter) was immersed in DI water (0.5 mL) and the absorbance of the supernatant collected every hour for six hours and once after 24 and 48 hours was recorded at 260 nm. The accumulative vancomycin collected from the supernatant after 48 h was considered not covalently coupled to the hydrogel. By this experiment, we determined a 90% coupling efficiency of vancomycin on the 15 w/v% PEGDMA hydrogel, which is considered adequate and applied to all subsequent experiments.

***In Vitro* Release Kinetics of Vancomycin from Modified Hydrogels.** The PEGDMA-Oligo-Vanco hydrogels (50 µL, 8 mm diameter) were immersed in 0.5 mL of sterile PBS (10 mM, pH 7.4) containing 0.1 U/µL of MN and incubated at 37 ºC. At predetermined time points (1, 1.5, 2, 3, 4, 6, 12, 16, 24 h), the amount of vancomycin released from the hydrogel matrix was calculated from the absorbance measured with the UV spectrophotometer at 280 nm. All the measurements were performed in triplicates.

**Hydrogel Coating of Ti6Al4V Pins.** To enhance the adhesion and stability of the hydrogel coating, Ti6Al4V pin surfaces were first modified using dopamine methacrylate (DopaMA). Ti6Al4V pins were immersed into 0.5 mL of ethanol containing 1mg/mL of DopaMA for 12 h at RT. The pins were then rinsed with ethanol 5 times to remove excess of DopaMA and dried under vacuum for 4 h. The DopaMA-coated pins were immersed into freshly prepared hydrogel precursor solution containing 15 wt% PEGDMA, 75 μM of methacrylated oligo and 1 wt% of VA-086. It was then partially photo crosslinked under 365 nm irradiation for 2 min. Pins are then removed from the pre-gel condition and then irradiated for 5 min to complete the crosslinking. All hydrogels coated pins were washed in DI water for 72 h with frequent fresh water changes to remove excess radical initiators or any untethered methacrylate prior to further experiments.

***In Vitro* Antibacterial Activity of Ti6Al4V Pins Coated with PEGDMA-Oligo-Vanco Hydrogel or PEGDMA-Oligo Hydrogel with/without Physically Encapsulated Vancomycin**. The therapeutic efficacy of the PEGDMA-Oligo-Vanco hydrogel was evaluated by its ability to inhibit *in vitro* suspension culture of *S. aureus* (method I) or its ability to inhibit *S. aureus growth* on agar plate when delivered in the form of Ti6Al4V coating (method II). PEGDMA-Oligo hydrogel without covalent vancomycin tethering or that with physically encapsulated but subsequently washed away vancomycin served as negative controls. Method I. PEGDMA-Oligo-Vanco, PEGDMA-Oligo or PEGDMA-Oligo/Vanco (after washing away physically encapsulated vancomycin) hydrogels (50 μL, 8 mm diameter) were immersed in 0.5 mL of LB media containing 130 CFU of *S. aureus*. Cultures were incubated for 48 h at 37 °C with shaking. Total CFU counts were determined at 24 and 48 h by sample turbidity ($OD_{600}$), measured using UV-Vis spectroscopy at 600 nm and calculated based on standard curves. Method II. 20,000 CFU of *S. aureus* were spread evenly on LB agar plates and incubated for 10 min at 37 °C with shaking. Ti6Al4V pins

coated with PEGDMA-Oligo-Vanco, PEGDMA-Oligo or PEGDMA-Oligo/Vanco (after washing away physically encapsulated vancomycin) were then placed on the plates and the culture was continued for 24 h at 37 °C with shaking. The clear zones surrounding the pins were photo-documented as an indication of local antibiotic activities as a function of pin coatings.

**Water Contact Angle Measurement.** For the water contact angle measurements, Ti6Al4V plates (10 mm × 10 mm) were used. The static water contact angles of Ti6Al4V substrates before and after the surface coating by DopaMA was recorded on a CAM200 goniometer (KSV Instruments). A droplet (3 μL) of Milli-Q water was placed on the substrate and the contact angles (left and right) of the droplet were recorded after 30 s. The left and right contact angles of each droplet, and three substrates of each sample group were averaged and reported as averages ± standard deviation.

**X-ray Photoelectron Spectroscopy (XPS)**. Surface compositional analyses of Ti6Al4V and Ti6Al4V-DopaMA substrates were carried out on a Thermo Scientific K-Alpha XPS equipped with an Al $K_\alpha$ radiation source under the pass energy of 200 or 50 eV (for survey or high-resolution scan) and the spot size of 400 μm. Survey scan spectra were obtained from five consecutive scans of a randomly chosen area of interest.

***In vivo* Studies.** All animal procedures were carried out per procedures approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee (IACUC). CL57BL/6 male mice ages 6-10 weeks and weights 22-28 g were anesthetized with 2-3% isoflurane-oxygen throughout surgery and bilateral intramedullary pins were inserted following bacteria inoculation. Briefly, a 2 mm skin incision was made over the knee exposing the joint. A medial incision was made to dislocate the patellar tendon, and the patella and patellar tendon were moved laterally to reveal the intercondylar notch of the femur. A 25-gauge needle was introduced onto the patellar grove and used to reamed through the femur. 4 μL of sterile LB media or Xen29

*S. aureus* solution ($10^4$ CFU/mL) was injected into the femoral canal (0 or 40 CFU Xen29 per femur) using a micro pipettor equipped with a 25-gauge needle tip. A sterile unmodified Ti6Al4V rod (0.5 mm in diameter and 10 mm in length) or those coated with PEGDMA-Oligo-Vanco or PEGDMA-Oligo hydrogels via a dopamine intermediate coating was fit into the previously reamed femur. The patella and patellar tendon were placed back into position and the skin was closed with 5-0 nylon sutures. Subcutaneous injections of buprenorphine were given immediately pre-operation and every 12 h post-operation for 48 h. No unexpected or unusually high safety hazards were encountered throughout the study.

**Micro Computed Tomography (μCT).** Mice were scanned 1 day (to ensure proper pin placement and normal femoral anatomy) and 21 days post-operation on a Scanco vivaCT 75 system (Scanco Medical, Switzerland) at an effective voxel size of 20.5×20.5×20.5 $\mu m^3$. The proximal and distal femoral growth plates were located to establish the center slice of the femur and 100 consecutive slices were analyzed on both sides for a total analysis length of ~4mm. A global threshold of 260 (minimum bone densities of 549.7 mg HA/$cm^3$ and above) was applied to calculate bone volume fraction (BVF) and bone mineral density (BMD) using Scanco Medical's analysis software. When analyzing cortical thickness, a threshold of 50 (minimum bone densities of 63.2 mg HA/$cm^3$ and above) was utilized to include the entire contoured cortical bone space despite any porosity or lesions within the cortical bone.

***In Vivo* Bioluminescence Imaging.** The bioluminescence imaging of *S. aureus* within the infected mouse femoral canals was carried out using IVIS-100 imaging system (Perkin-Elmer) on day 2, day 7, day 14 and day 21 post-op. Mice were anesthetized with 5% isoflurane-oxygen and placed on the imaging platform. The bioluminescence image was recorded with a 5 min overall exposure

time with open emission filter. Background subtraction was performed with uninfected femoral ROI.

**Explant Bacterial Counts.** At 21 days post infection, mice were euthanized, and the IM pins were retrieved. Each retrieved pin was placed in 0.5 mL of LB media in an eppendorf tube and vortexed for 5 min to dislodge all surface-bound bacteria from the pin. The CFU counts were determined by serial dilution on LB agar plates.

**Femoral Histology and Organ Pathology.** At 21 days, immediately after IM pin removal, femoral explants were fixed in periodate-lysine-paraformaldehyde (PLP) for 48 h at 4 ºC followed by decalcification in 18% aqueous ethylenediaminetetraacetic acid (EDTA, pH 8.0) for 2 weeks with bi-weekly solution changes.[1] The decalcified femurs were subjected to serial dehydration, paraffin embedding, and longitudinal sectioning (6 µm in thickness) before staining for hematoxylin and eosin (H&E) for cellularity, osteogenic marker alkaline phosphatase (AP, fast blue) and osteoclast lineage marker tartrate-resistant acid phosphatase (TRAP, fast red) for bone remodeling, and by gram stain kit (Abcam) for gram positive *S. aureus* (blue). Heart, lung, kidney, liver, pancreas, spleen, and ribs were collected at 21 days post-operation and fixed and stained by H&E for comparison with organs retrieved from healthy age-matched controls.

**Statistics and Study Design.** All statistical analysis was performed using Prism 7.0 (GraphPad Software Inc.) Shaprio-Wilk normality testing was used to evaluate data distribution. Pair-wise comparisons passing normality test were analyzed with Student's *t*-test while the Mann-Whitney rank-sum test was used for pair-wise comparison of non-parametric data. Multiple group comparisons passing normality test were analyzed using one-way analysis of variance (ANOVA) with Tukey's post hoc while non-parametric multiple group comparisons were analyzed using the Kruskal-Wallis test with Dunn's post hoc testing. Multi-variant comparisons were carried out

using two-way ANOVA with Tukey's post hoc test. *P*-values of <0.05 were considered significant. All data was presented as mean ± standard deviation (S.D.).

**Reference cited:** 1. Kutikov, A. B.; Skelly, J. D.; Ayers, D. C.; Song, J., Templated repair of long bone defects in rats with bioactive spiral-wrapped electrospun amphiphilic polymer/hydroxyapatite scaffolds. *ACS Appl Mater Interfaces* 2015, *7* (8), 4890-901.

## 2. Supplementary Figures


**Figure S1**. Image of LB agar plate containing PEGDMA-Oligo-Vanco and PEGDMA-Oligo coated Ti6Al4V pins after 24 h *S. aureus* culture.

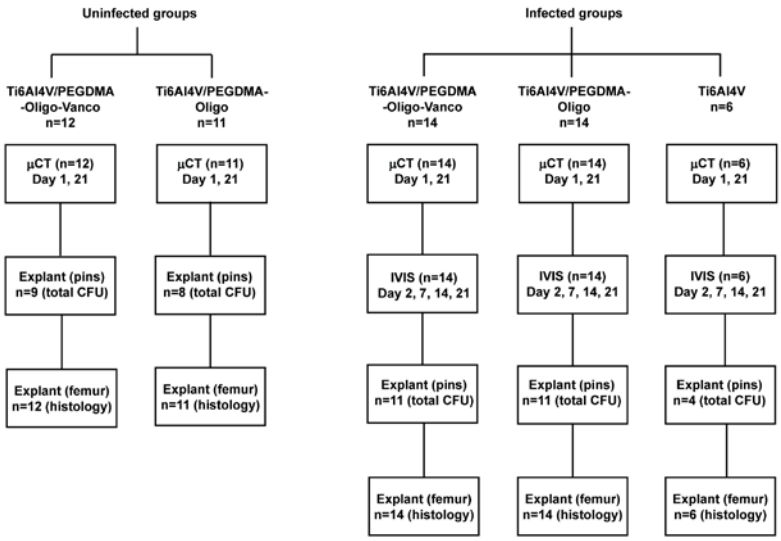


**Figure S2**. *In vivo* study design.

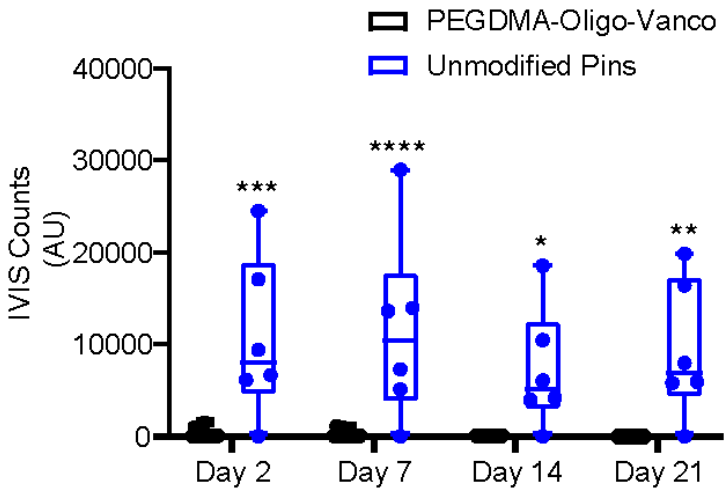


**Figure S3**. Longitudinal bioluminescence intensities of mouse femoral canals injected with 40 CFU Xen-29 *S. aureus* and inserted with IM pins with (n = 14) or without (n = 6) PEGDMA-Oligo-Vanco coating at 2, 7, 14, and 21 days. Error bars represent standard deviations. * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ (two-way ANOVA).

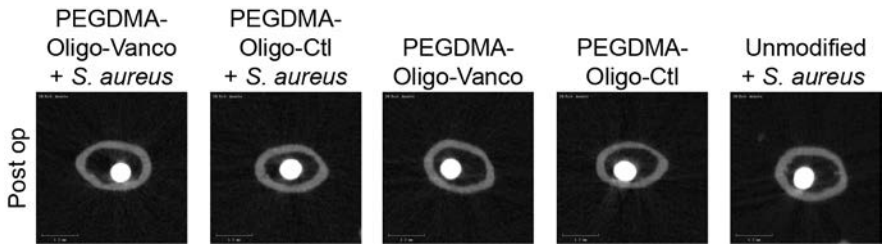


**Figure S4.** Post-op µCT axial images of the center slices of the mouse femurs confirming proper positioning of the inserted IM pins.

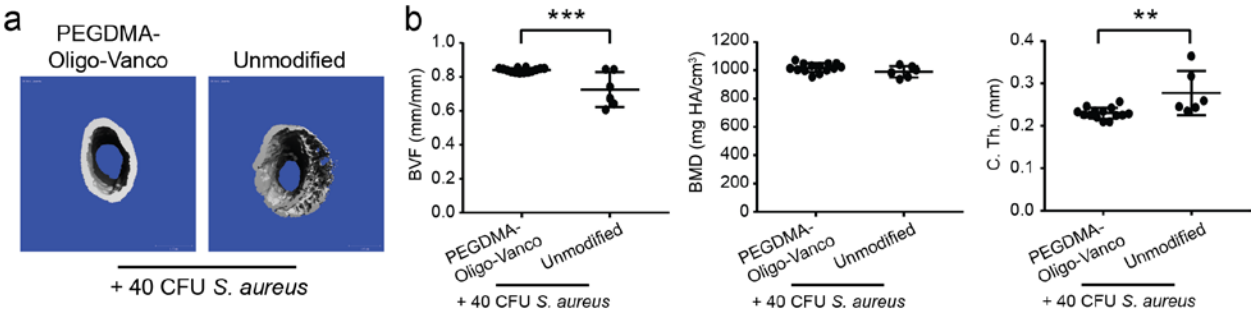


**Figure S5**. (a) 3D reconstructed µCT images of the distal femoral region with the IM rod contoured out; (b) quantification of bone volume fraction (BVF), bone mineral density (BMD), and cortical thickness (C. Th.) of femurs in the PEGDMA-Oligo-Vanco + *S. aureus* (n = 14) and unmodified Ti + *S. aureus* (n = 6) groups at 21 days post-op. Error bars represent standard deviations.*$p \leq 0.01$, **$p \leq 0.001$ (student's t-test).

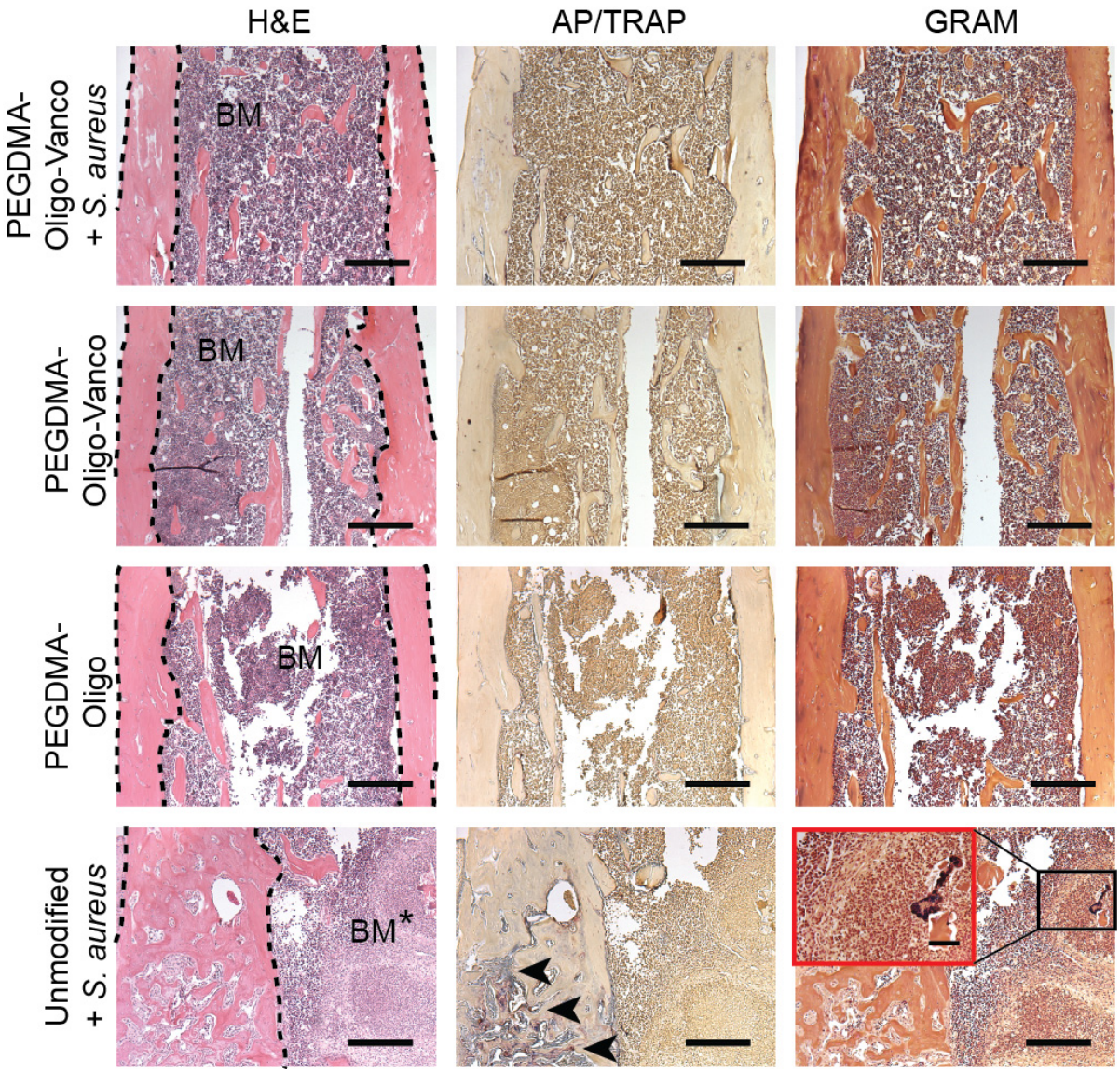


**Figure S6**. H&E, AP/TRAP, and Gram staining of explanted femurs treated with PEGDMA-Oligo-Vanco coated pins with and without inoculated *S. aureus*, PEGDMA-Oligo coated pin without *S. aureus*, and unmodified Ti with *S. aureus* at 21 days post-operation. Scale bars = 500 µm; Dashed lines outline the cortical bone; BM = bone marrow; BM* = infected bone marrow; Arrowheads indicate AP/TRAP activity; Black box outlines Gram positive stained bacteria (red box showing zoomed-in view at 50× magnification; Scale bar = 100 µm).

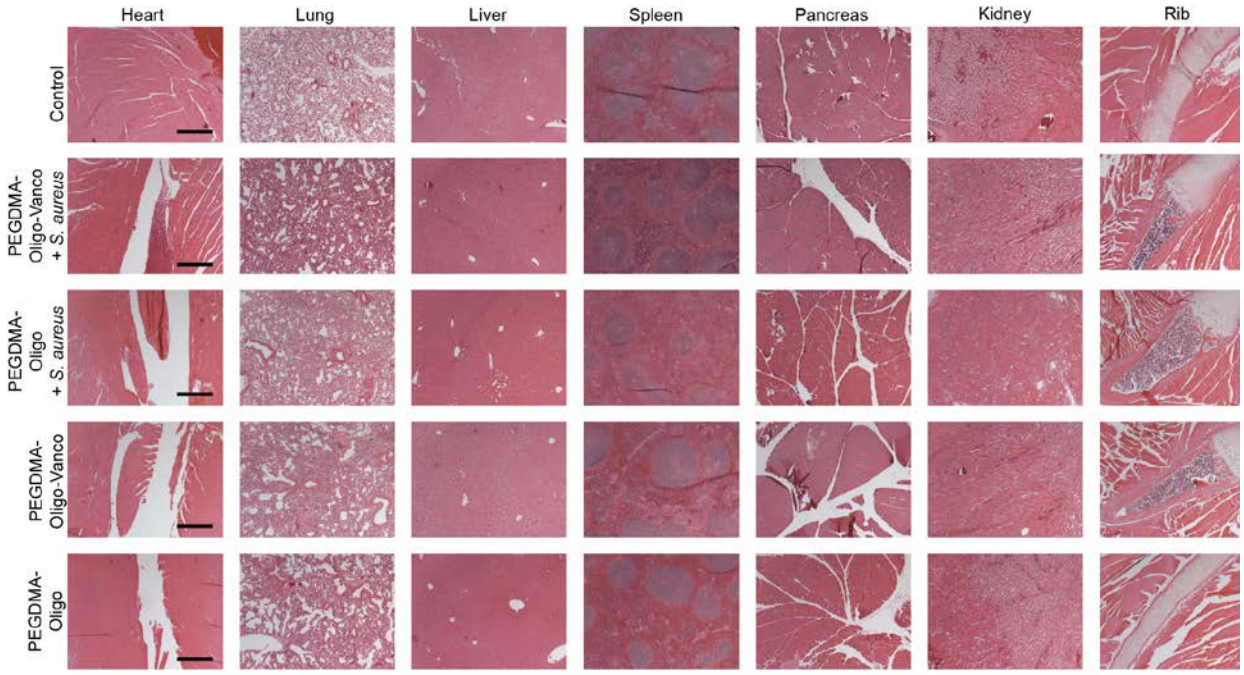


**Figure S7**. H&E stained sections of heart, lung, liver, spleen, pancreas, kidney and rib retrieved from the mice receiving various IM pin treatments in uninfected vs. infected femoral canals for 21 days. Organs retrieved from age-matched mice without any treatment serve as normal controls (top row). 50× magnification. Scale bars = 500 μm.