**Reduction in *Pseudomonas aeruginosa* and *Staphylococcus aureus* Biofilms from Implant Materials in a Diffusion Dominated Environment**

James P. Moley, BS a,\* Mary S. McGrath, BS a,\* Jeffrey F. Granger, MD b, Paul Stoodley, PhDa,b, †, Devendra H. Dusane, PhD a

a *Department of Microbial Infection and Immunity, The Ohio State University, and*

b *Department of Orthopaedics; The Ohio State University, Columbus, Ohio 43210, USA*

**Short title:** Diffusion Mediated Biofilm Eradication

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**Correspondence:**

†**Paul Stoodley**, PhD

Director, Campus Microscopy and Imaging Facility (CMIF),

Professor, Departments of Microbial Infection and Immunity and Orthopedics,

The Ohio State University, 716 Biomedical Research Tower,

460 West 12th Avenue, Columbus OH 43210.

Tel: +1 614 292 7826

E-mail: Paul.Stoodley@osumc.edu

**Abstract**

Antibiotic-loaded calcium sulfate beads (CS-B) are used to treat biofilm related periprosthetic joint infections (PJI). A previous study has shown that such beads are effective in reducing lawns biofilms grown on agar plates; however, the ability of CS-B to eradicate biofilms grown on solid orthopaedic material surfaces has not been investigated. We grew biofilms of bioluminescent strains of *Pseudomonas aeruginosa* Xen41 and a USA300 MRSA *Staphylococcus aureus* SAP231 on an ultra-high molecular weight polyethylene (PE), hydroxyapatite (HA), and 316L stainless steel (SS) coupons for three days under static growth conditions, with daily nutrient exchange. The coupons were rinsed with sterile phosphate buffered saline (PBS) to remove planktonic bacteria and placed in a petri dish, surrounded by four either antibiotic vancomycin and tobramycin loaded (CS-BV+T) or unloaded beads (CS-BU). A thin layer of agar was overlaid to simulate a periprosthetic infection where an implant abuts soft tissue, then incubated for 72 hours. The amount of biofilm was measured by bioluminescence imaging (BLI) for activity and viable cell count (CFUs). Coupons exposed to CS-BV+T showed a significant reduction in the amount of biofilm within 24 hours, regardless of the bacterial strain or material type. Whereas, coupons exposed to control CS-BU had no effect on bacteria over 72 hours. **Statement of Clinical Significance:** Antibiotic-loaded calcium sulfate beads (CS-B) were effective in significantly reducing mature biofilms of *P. aeruginosa* and *S. aureus* from orthopaedic relevant surfaces in our novel *in vitro* periprosthetic-soft tissue model.

**Keywords:** Biofilm; antibiotic-loaded calcium sulfate beads; *Pseudomonas*; *Staphylococcus*; implant

**Introduction**

 Orthopaedic periprosthetic joint infections (PJI) result in significant hospitalization burden, morbidity, and are directly related to increasing costs related to treatment and patient care [1]. As the number of worldwide PJI cases increases, biofilm formation is being studied as a key virulence factor related to its antibiotic tolerant properties [2]. Two of the most commonly implicated pathogens that result in biofilm formation and PJI are *Pseudomonas aeruginosa* and *Staphylococcus aureus* [3]. One clinical method of eradication of these biofilms that has been tested involves the application of antibiotic-loaded, synthetic calcium sulfate beads (CS-B). When placed adjacent to growing biofilms, CS-B loaded with tobramycin, vancomycin, and both antibiotics have been shown to prevent bacterial colonization and significantly reduce lawn biofilms grown on agar plates and on biofilms grown on polystyrene Petri plates [4, 5]. Here we extend our *in vitro* characterization studies to evaluate the ability of CS-BV+T to kill mature biofilms grown on orthopaedic surfaces in a novel periprosthetic-soft tissue model. The rationale is that in a PJI, diffusion will be limited in areas such as adjacent tissue, or when the joint is immobilized, limiting the spread of antibiotics released from antibiotic-loaded cement Orthopaedic materials made up of ultra-high molecular weight polyethylene (PE), hydroxyapatite (HA), and 316L stainless steel (SS) have all been implicated in PJI cases and shown to harbor bacteria and established biofilms [6, 7]. We test the relative ability of *P. aeruginosa* and *S. aureus* to form biofilms on different surfaces to address the question of whether antibiotic-loaded calcium sulfate beads can reduce biofilms on representative orthopaedic surfaces in a diffusion dominated environment.

**Materials and Methods**

*Bacteria and Growth Conditions*

 Bioluminescent strains of *Pseudomonas aeruginosa* PAO1 derivative (Xen 41, PerkinElmer, USA) and *Staphylococcus aureus* (SAP231, a transformed USA 300 MRSA strain) [1] were used as previously described [2]. *P. aeruginosa* Xen 41 was grown in Luria Broth (LB; Sigma Aldrich, USA) and *S. aureus* SAP231 was grown in Brain Heart Infusion broth (BHI; Sigma Aldrich, USA) respectively at 37°C overnight on shaker conditions set at 200 RPM. Overnight cultures were used as an inoculum for biofilm formation on coupons.

*Biofilm Formation on Coupons*

 Biofilms were grown on PE, HA, and SS coupons (BioSurface Technologies, Bozeman, MT). PE and 316L SS are commonly used in the orthopaedic implants [10] and HA constitute around 65% of the mineral component of human bone [11]. The coupons had a diameter of 1.27 cm, surface area of 1.27 cm2, and roughness of 0.51 micrometers measured at 20°C by contact profilometry (MAHR PS-1) according to standard methods (ISO4288/ASMEB461). Briefly, sterile coupons were aseptically transferred to 24-well clear bottom microtiter plates (Corning Inc., USA). Overnight cultures of *P. aeruginosa* Xen41 or *S. aureus* SAP231 were added to 2 mL of growth media (BHI or LB broth respectively) to achieve the final cell concentration of approximately 1 × 105 CFU/mL. The aliquots were added to microtiter plate wells containing PE, HA, and SS coupons and incubated for 3 days at 37°C with 5% CO2 under static conditions for biofilm formation with daily media exchanges.

*Preparation of Calcium Sulfate Bead with Loaded (CS-BV+T) and Unloaded (CS-BU) Antibiotics*

 The antibiotic-loaded (CS-BV+T) and unloaded (CS-BU) beads of size 4.8mm were prepared as described previously [12]. The unloaded beads were prepared by combining 20g of calcium sulfate hemihydrate (CaSO4: 0.5 H2O; Sigma-Aldrich, USA) with 6ml of ultrapure sterile distilled water (Thermofisher Scientific, USA). The paste was transferred to a mold mat, and once set the resulting hemispherical beads were removed. The antibiotic-loaded beads were prepared to a formulation that has been described clinically [8]. 20g of CaSO4 powder was blended with 240 mg of tobramycin sulfate (Sigma-Aldrich) and 1000 mg of vancomycin hydrochloride (Sigma-Aldrich) for approximately 10 seconds. When well mixed, 6ml of ultrapure water was added to form the paste which was transferred to the mold mat and allowed to set for approximately 12 hours before removal.

 CaSO4 cement beads have a number of advantages over acrylic based beads with respect to the local release of antibiotics [5]. Since the beads are fully absorbable, there is longer sustained release than acrylic which shows an initial burst of release but then much of the antibiotic remains locked within the bead limited by the slow diffusion time. Second, with acrylic, once the local concentration of antibiotic at the surface has dropped to below the minimum inhibitory concentration (MIC) level, the material becomes an unprotected foreign body, susceptible to bacterial colonization and biofilm formation [13]. Lastly, the temperature due to the exothermic reaction during setting of CaSO4 is much lower than that of acrylic making it compatible with a wider range of heat labile antibiotics.

*Bead Exposure*

 Following a 3-day incubation, all coupons were rinsed twice with sterile phosphate buffered solution (PBS; Dulbeco’s, Gibco, USA), placed in the center of a 100 × 15 mm petri dish (Fisher Scientific, USA), and surrounded by four beads in direct contact and equidistant apart as shown in Fig. 1 and Fig. 2. The coupons with *P. aeruginosa* and *S. aureus* biofilms grown were exposed to antibiotic-loaded (CS-BV+T) and unloaded (CS-BU) beads. They were overlaid with a thin layer of molten agar and incubated for 72 hours at 37°C and 5% CO2. Experiments were performed in triplicate.

*Bioluminescence Imaging (BLI)*

 Prior to biofilm removal for cell count enumeration, BLI was performed using an *in vivo* imaging system (IVIS 100, Xenogen) on one representative coupon from each treatment and control group as previously described [2]. IVIS provides a non-destructive method of assessing the relative amount of actively growing biofilm [3]. The images were captured in grayscale intensity where white was the most metabolically active and black represented no activity. Lack of signal could be attributed to biofilm removal, inactivation or killing or a combination thereof. The relative amount of biofilm on the coupon was quantified on IVIS images by measuring the bioluminescent intensity using a FIJI image analysis software [4] measuring a circular region of interest (ROI) over the coupon. A heat-map color scale was also generated for visual clarity where, red represented the highest metabolic activity and blue/ black indicted no activity.

*Colony Forming Units (CFU)*

 Following exposure to the treatment arms at 24 and 72 hour time points, coupons from each experimental arm were rinsed with PBS. The biofilm was removed by placing the coupons into 10 mL of PBS in a 50 mL Falcon tube (Corning) and sonicating at a frequency of 35 kHz for 15 min in a sonicator water-bath (Fisher Scientific FS7652H). Sonication was repeated three times with a 10 seconds interval for vortexing in between. A 10-fold serial dilution was prepared and the diluted samples were plated onto a respective agar for enumeration of *P. aeruginosa* and *S. aureus* CFUs after 24 hours of incubation at 37°C, 5% CO2. The colonies were expressed on the basis of calculated coupon area as CFU/cm2.

*Statistical Analysis*

Statistical comparisons between the geometric means of CFU/cm2 from control and experimental arms were performed using Excel software (Microsoft) using an unpaired 2-tailed Student’s *t*-test assuming equal variances. Differences were considered significant for a *p*<0.05.

**Results**

*Bioluminescence Imaging (BLI)*

To make a rapid assessment of the efficacy of biofilm eradication that could be compared to the viable cell counts, BLI using IVIS was taken and the images were compared with coupons of each treatment (Fig. 3A and 3B). Coupons exposed to unloaded beads, CS-BU showed a rapid growth and spreading of bacteria from the biofilm present on the coupon materials within the first 24 hours that completely covered the agar surface at 72 hours. Interestingly, the bacteria on the SS coupons in particular showed the highest bioluminescent signal around the edges of the coupon, suggesting that this zone had the most adherent bacterial biofilm activity. The PE coupon surface was more bioluminescent than the HA and SS surfaces suggesting higher adherent biofilms on PE. For coupons exposed to CS-BV+T, there was a significant reduction in intensity values over time with both bacterial strains (Fig. 4A and 4B). This suggested the efficient killing of biofilms by antibiotic-loaded CS-B in a diffusion dominant environment.

*Viable Cell Count*

 After 3 days, biofilms of both strains had grown to approximately 109 CFU/cm2 on PE, HA, and SS (Fig. 5A-B). While there were no significant difference in CFU counts among the three materials (*p*>0.05), PE surfaces harbored the most bacteria followed by HA and SS, a similar pattern to that noted in bioluminescent imaging. The difference in growth between *P. aeruginosa* and *S. aureus* was not significant (*p*>0.05). After 24 hours of incubation, all coupons in direct contact with CS-BV+T showed non-detectable levels (<7 × 101 CFU/cm2) of bacteria (*p*<0.05), with an approximate seven log reduction. There were no statistically significant differences among surface materials and bacterial strains. All coupons exposed to CS-BU showed nearly one log reduction in CFU count over the first 24 hours with no differences among strains and surface types. This reduction might be due to depletion in nutrients within the surrounding agar medium. At 72 hours, coupons exposed to CS-BV+T showed a similar (seven log) reduction compared to the starting biofilm (*p*<0.05), but in this case a few cells were cultured which were between 9 × 101 to 5.5 × 102 CFU/cm2. Coupons exposed to unloaded beads (CS-BU) at 72 hours showed an approximate one log reduction as compared to the initial 3 day biofilm (0 hours exposure) with no statistical significance (*p*>0.05) among bacterial strains or surface types. Bioluminescence intensity color images and image analysis combined together with CFU counts highlighted a gradual reduction in biofilms for coupons exposed to CS-BV+T as compared with coupons exposed to CS-BU.

**Discussion**

Bacterial colonization of orthopaedic implant surfaces can lead to biofilm formation and the development of PJI [5]. Given the significant risk of infection and devastating impact of PJI, it is clear that effective means of eradicating bacteria and preventing biofilm formation on these surfaces is critical. Here, we show that antibiotic-loaded calcium sulfate beads placed adjacent to a biofilm colonized surface were capable of eradicating biofilms of *P. aeruginosa* and *S. aureus* grown on three different materials relevant to PJI surfaces.

 Growth of bacteria on the coupons was patchy in nature, indicating potential for additional analysis of surface texture, hydrophobicity and biofilm adherence which might explain the heterogeneity. The CFU counts show a dramatic reduction in viable cells within the first 24 hours of antibiotic-loaded bead exposure which indicates the potential biofilm killing power of V+T exposure. While there were no significant differences among surface material type, the bioluminescence and cell counts showed that PE surfaces harbored the most bacteria while SS surfaces harbored the least, however, there was only 1- log difference demonstrating that even though the materials were different, they were all capable of harboring significant amount of adherent biofilms. Similar rates of adherence and subsequent removal with V+T was seen in a previous study which examined the role of CS-BV+T both alone and in combination with pulse lavage exposure on 316L SS coupons [16]. The current study expands on that work by using three orthopaedic materials and growing biofilms on agar in a diffusion dominated environment. The IVIS and FIJI analysis of the biofilms in the current study supports the cell count data and similarly reveals the utility of antibiotic-loaded calcium sulfate in treating mature biofilms growing on orthopaedic surfaces in quiescent locations in the joint space.

 A previous study measuring the effectiveness of antibiotic-loaded CS-B at eradicating biofilm in a liquid environment showed a one log reduction after 24 hours and a five log reduction after 72 hours [5]. In our study, significant reduction (up to seven logs) was observed after 24 hours, followed by an increase in bacterial activity at subsequent time points thereafter. The differences may be due to varying methodology and bacterial strains. In the current study, biofilms were grown on coupon surface and antibiotic exposure was performed by placing beads immediately adjacent to the coupons in the agar. Thus the beads would be exposed to locally high concentrations of antibiotic as it slowly diffused from the bead. In fact the highest concentrations would occur in the vicinity of the bead at early time points. However, in the previous study [5], biofilms were exposed to antibiotics in a liquid environment. The eluted antibiotic would be diluted evenly throughout the medium, thus the initial concentration that the biofilm experienced might be lower as compared to the plate diffusion model where antibiotic concentration would increase as time progressed. Secondly, we used *S. aureus* SAP231 and *P. aeruginosa* Xen41, while in the previous study, methicillin-resistant *S. aureus* 16 (EMRSA-16) NCTC 13143 and *S. epidermidis* ATCC35984 were used.

 While the antibiotic beads suggested eradication after 24 hours with both bacterial strains, all experiments showed a subsequent small rise in bacterial activity at 48 and 72 hours, suggesting that not all bacteria were killed and further measures such as a greater bead packing density may be needed to completely eliminate biofilm. However, our system, like many *in vitro* models, did not have the presence of a host response that might be able to eradicate the remaining bacteria once the antibiotics had killed most of the biofilm bacteria. Although complete removal was not observed, the approximate seven log reduction seen across materials and both strains after 24 hours could have potentially disrupted the biofilm mechanism and structure, allowing antibiotics to have greater physical access to bacteria, as has been seen in dental biofilms [17]. Ongoing work to collect infected surgical explants from revision surgeries and study their interactions with antibiotic-loaded CS-B exposure will expand knowledge of their ability to eradicate biofilms *in vivo*.

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**Figure Legends**

Figure 1.Schematic outline of the method used in this study. “V+T” indicates beads containing both vancomycin and tobramycin. “U” indicates unloaded beads, used as controls. The experiment was performed in triplicate.

Figure 2.Four beads were placed around coupons of all three material types, ultrahigh molecular weight polyethylene (PE), hydroxyapatite (HA), and stainless steel (SS), respectively in this arrangement, in direct contact and at equidistance.

Figure 3(A). IVIS images showing emitted bacterial bioluminescence as an indicator of the amount of biofilm in unloaded (U) and experimental arms (V+T) against *Pseudomonas aeruginosa* Xen41. The heat-map scale is an indicator of relative intensity and activity where red being the highest, blue being low intensity, and black indicating no growth or activity. “U” represents the unloaded bead with no antibiotics while “V+T” represents the antibiotic-loaded beads.

Figure 3(B). IVIS images showing emitted bacterial bioluminescence as an indicator of the amount of biofilm of unloaded control (U) and experimental arms (V+T) against *Staphylococcus aureus* SAP231. The heat-map scale is an indicator of relative intensity and activity where red being the highest, blue being low intensity, and black indicating no growth or activity. “U” represents the unloaded bead with no antibiotics while “V+T” represent the antibiotic-loaded beads.

Figure 4(A).Coverage of *P. aeruginosa* biofilms on coupons measured as relative intensity units (RIUs) from the grayscale IVIS images. High values indicate a highly heterogeneous distribution of the biofilm on the surface of the coupon.

Figure 4(B).Coverage of *S. aureus* biofilms on coupons measured as relative intensity units (RIUs) from the grayscale IVIS images. High values indicate a highly heterogeneous distribution of the biofilm on the surface of the coupon.

Figure 5(A). CFU counts of biofilms of *P aeruginosa* Xen41 on ultrahigh molecular weight polyethylene (PE), hydroxyapatite (HA), and stainless steel (SS). \* indicates statistically significant reductions compared to 0 hour. CS-BU represents the control or unloaded bead-exposed set while CS-BV+T represents the antibiotic bead-exposed set.

Figure 5(B). CFU counts of biofilms of *S aureus* SAP231 on ultrahigh molecular weight polyethylene (PE), hydroxyapatite (HA), and stainless steel (SS). \* indicates statistically significant reductions compared to 0 hour. CS-BU represents the control or unloaded bead-exposed set while CS-BV+T represents the antibiotic bead-exposed set.