Quantifying The Natural History Of In Vivo Biofilm Formation And Bacterial Persistence In A Murine Model Of Implant-associated Staphylococcus Aureus Infection

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Introduction: Staphylococcus is responsible for ~80% of chronic infections in bone (osteomyelitis), where it persists as biofilm consisting of bacteria embedded in a complex glyocalyx structure [1]. Biofilm formation is a multi-step process that begins with the initial adherence of bacterial cells to the implant surface, followed by rounds of cell division and secretion of matrix components, and finally maturation of the biofilm into a source of new single cells that can emigrate to cause other infection sites [1,2]. Despite its clinical importance, the in vivo formation and maturation of biofilm during the establishment of implant-associated osteomyelitis has not been elucidated, largely due to the absence of quantitative methods. Hence, we developed a scanning electron microscopy (SEM) based assay system to quantify in vivo biofilm formation in a murine model of implant-associated osteomyelitis, and used it to investigate the natural history of Staphylococcus aureus (S. aureus) biofilm formation and maturation.

Methods: All animal studies were performed on IACUC approved protocols. Two methicillin-sensitive S. aureus (MSSA) strains (UAMS-1 and SH1000) and one methicillin-resistant S. aureus (MRSA) strain (USA300LAC) were used in this study. A flat stainless steel wire (cross-section 0.2 mm x 0.5 mm; MicroDyne Technologies, Plainville, CT) was cut to 4mm length and bent into an L-shaped implant whose long side was 3mm, short side was 1mm (Figure 1A). After sterilization, the wires were incubated in an overnight tryptic soy broth culture with or without bacteria for 30 minutes then air dried for 2 minutes. The inoculating dose of bacteria was determined to be: 1.56 ± 0.38 × 10^5 CFU of SH1000, 1.88 ± 0.55 × 10^5 CFU of UAMS-1 and 2.11 ± 1.16 × 10^5 CFU of USA300LAC, per implant. Six-week old C57Bl/6 mice were anesthetized, and prepared for implantation. After a 5mm incision was made to expose the medial cortex of proximal tibia, a hole was drilled in medial cortex with a 23G needle (0.65 mm in diameter) without penetrating the lateral cortex; then the lateral cortex was drilled through the medial hole with a 30G needle (0.3 mm diameter). The implant was inserted from the medial side and press-fit with its long end and its L tightly pressed against the medial side of the tibia. Mice were sacrificed on days 1, 2, 3, 4, 7, 14, 28 and right tibiae were harvested. Tibiae with implants were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde and then decalcified. To remove an implant and not damage the biofilm, bone and soft tissue anterior to the implant was entirely removed with a scalpel, then the implant was lifted straight out (normal to the tibia) so tissues strongly attaching to the posterior surface of the implant were retained. SEM images were taken with all wires in the same orientation (Fig 1B). A 1 mm² region of interest (ROI) was defined by the pin surface that had been located between medial and lateral cortex. To measure area of the biofilm, pictures of the ROI were cropped and converted pixel-by-pixel to either black (stainless surface) or white (biofilm) using imageJ (NIH, Bethesda, MD) software (Fig. 1B). Percent Biofilm Area was defined using this formula: %biofilm area = tissue area (white) / total area (black plus white). To define biofilm growth in more detail, the ROI was divided into 3 sub-regions: the medial, intramedullary, and lateral (Fig.
Figure 1

A) Scheme of the implant; B) SEM image obtained at 1,000X showing the top surface of the implant and the 1mm² ROI (black rectangle), which was cropped (middle image) and then converted into a binary image (lower image) that used to quantify the %biofilm area. White bar indicates 200µm. C) The three sub-regions in ROI.

Results: Representative SEM images of the *S. aureus* and biofilm are presented in Figure 2 to illustrate the natural history of
biofilm formation in our model, which was similar for all strains tested. The initial event of adhesion appears to occur ex vivo, and the *S. aureus* persist as small clusters without biofilm matrix around them for the first 24hrs following implantation. Biofilm formation was detected at 48hrs, which was the peak of *S. aureus* proliferation, as evidenced by the large clusters of bacteria. At day 4, the *S. aureus* was surrounded by a honeycomb-like matrix, which appeared to grow and completely cover the bacteria at day 7. Evidence of biofilm maturation and bacterial emigration was present at day 14 in the form of empty lacunae and excavated *S. aureus* that were observed on the biofilm surface. No remarkable differences in the appearance of the *S. aureus* or biofilm could be detected between the day 14 and day 28 SEM images.
Figure 2 SEM pictures of low magnification (5,000 X: left column; bar = 10nm) and high magnification (15,000X: right column; bar = 1nm). Quantification of the ROI demonstrated that for all strains, the %biofilm area significantly increased over time until the peak at day 14, uniformly covering about 40% of the wire (Figure 3). ROI sub-region analysis showed that biofilm formation initiates in the medial side of the wire, and grows laterally until the peak at 14 days when the implant is uniformly covered by biofilm. Again there was no difference
between 14 and 28 days. Representative SEM pictures (25X) of wires harvested at 1, 3, 7, 14, 28 days after surgery. For each time point, the primary photograph (top) and the converted binary image (bottom) of the ROI is presented. B) The %biofilm area of each time point is presented as the mean ± SD N=5 wires per group (*: P<0.05 vs. No bacterial)

Discussion: Although biofilm is broadly acknowledged to be the primary challenge in preventing and eradicating S. aureus
Infections, particularly in bone, our ability to study biofilm formation in vivo has been limited due to the absence of quantitative models. Here we demonstrate an approach in which the natural history of S. aureus biofilm on an implant can be qualitatively and quantitatively analyzed in a murine model of osteomyelitis. Consistent with the current dogma of infection that includes 4 distinct phases [1,2], we demonstrate evidence of: 1) bacterial adhesion; 2) robust S. aureus proliferation from 2 to 4 days after implantation 3) decreased proliferation and increase biofilm-matrix formation with encapsulation of the bacteria at day 7; and 4) biofilm degradation and S. aureus emigration at day 14. Furthermore, we developed a ROI analysis method in which the %biofilm area on the implant can be quantified. In this approach, significant biofilm commenced around day 7 and peaked by 14 days in all three strains tested. The sub-region analysis provided evidence that although the bacteria uniformly coat the wire prior to implantation, biofilm formation initiates on the medial side where the implant is first introduced into the tibia. Together with other information obtained from this study, we interpret this finding to be that chronic osteomyelitis initiates as an acute soft-tissue infection and travels along the foreign body as biofilm until it infects the bone. Further research with this model is warranted to formally test this, as well as novel interventions that can prevent and/or eradicate S. aureus infection and biofilm formation.

**Significance:** We demonstrate a novel approach to evaluate and quantify the natural history of S. aureus biofilm formation in vivo on a stainless implant in a model of chronic osteomyelitis. This model can be used to evaluate the effects of anti-S. aureus therapies to prevent and/or eradicate biofilm formation.

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**References:**

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