**The influence of patterned surface features on the accumulation of bovine synovial fluid induced aggregates of *Staphylococcus aureus***

Niraj Gupta1, ⴕ, Tripti Thapa Gupta1, \*, ⴕ, Khushi Patel2, and Paul Stoodley1,3,4

1Department of Microbial Infection and Immunity, The Ohio State University, Columbus, Ohio, USA

2College of Public Health, The Ohio State University, Columbus, OH 43210, USA

3Department of Orthopeadics, The Ohio State University, Columbus, Ohio, USA

4National Centre for Advanced Tribology at Southampton (nCATS) and National Biofilm Innovation Centre (NBIC), Mechanical Engineering, University of Southampton, Southampton, UK

\*[nirajgupta.bme@gmail.com](mailto:nirajgupta.bme@gmail.com)

ⴕ These authors contributed equally to this work

**ABSTRACT**

Periprosthetic joint infection (PJI) after joint replacement is a major clinical issue requiring multiple surgeries and antibiotic interventions. Recent *in vitro* research has shown that PJI staphylococcal strains rapidly form antibiotic resistant free-floating aggregates in the presence of bovine synovial fluid (BSF). Staphylococcal aggregates are also present in human PJI joint fluid. However, the influence of surface roughness and fluid shear on the attachment and retention of such aggregates to surfaces is not known. Our aim was to assess how surface roughness and fluid shear stress influenced the attachment and retention of *S. aureus* BSF mediated aggregates on smooth and rough patterned titanium in flow cells compared to non-aggregated cells. *S. aureus* attachment of aggregates was significantly greater than single cells but was independent of surface roughness, however on the patterned surfaces aggregates preferentially accumulated in the grooves. Fibrous components in the BSF were also colocalized with the grooves. After a 24 hr attachment and incubation period different shear stresses were applied. There was significant detachment from flat surface at 1 mL/min (τw = 0.03 Pa) but minimal detachment from the patterned surfaces, even at flow rates as high as 13.9 mL/min (τw = 0.42 Pa). The retention of bacterial aggregates and biofilm by rough surfaces exposed to shear might be an important consideration of the colonization location on orthopedic implants which can have a wide range of roughness and surface features as well as influencing efficacy of shear-based debridement methods such as pulse lavage.

**IMPORTANCE**

Periprosthetic joint infections occurring after joint replacement is a major clinical problem requiring repeated surgeries and antibiotic interventions. *Staphylococcus aureus* is the most prominent bacteria causing most implants related infections. *S. aureus* can form a biofilm which is defined as a group of bacteria attaching together with the formation of an envelope that is resistance to antibiotics. The attachment and retention of these bacteria on the implant surfaces is not clearly understood. Recent *in vitro* research investigations have shown that staphylococcal strains rapidly form aggregates in the presence of bovine synovial fluid (BSF) in the joints and allows bacteria time to attach to the implant surface leading to biofilm formation. Thus, in this study, we examined the attachment of aggregates on titanium surfaces with varying roughness and found robust bacterial attachment and retention along the ridges and grooves which co-localized with the deposition of fibrous components present in the BSF.

**KEYWORDS:** *S. aureus*, orthopedic infections, aggregates, synovial fluid, biofilm, titanium, shear-stress, roughness

**INTRODUCTION**

Orthopedic infection is a global problem after total joint arthroplasty (TJA) such as total knee arthroplasty (TKA) and total hip arthroplasty (THA). TJA is accompanied by some complications, the most challenging of which is periprosthetic joint infection (PJI) (1). PJI is one of the most challenging complications of total joint arthroplasty occurring in 2% of patients following joint replacement and most common cause of revision for failed knee arthroplasty (16.8 to 25.2%) (2-4). These infections require multiple surgeries undergoing implant removal and re-implantation and prolong antibiotic treatment (1, 5). A recent report shows that 26% of patients undergoing arthroplasty die due to PJI within 5 years (5). The use of antibiotics, body exhaust systems, and laminar airflow in the operating room have decreased the incidence of PJI, however the infections associated with PJI have not eliminated (6). Instead, the likelihood of developing a recurrent infection is significantly increased (6, 7). A major complication in diagnosing and treating PJIs is due to biofilm growing on the surfaces of the implant and periprosthetic tissue.

*S. aureus* is the prominent bacteria responsible for causing PJI with biofilms and aggregates observed on the surface of implanted joint devices and the surrounding tissue (8). It is also the most frequent pathogen associated with metal surfaces, and acute and chronic osteomyelitis (9).

During PJI, *S. aureus* biofilms and aggregates have been observed on the surface of implant prosthesis and the surrounding tissue (8). The attachment of these bacteria on the surfaces depends on various factors such as flow conditions, surface chemistry, surface hydrophobicity, surface roughness, and structures in the surface of the cell such as pili, fimbriae, and flagella (10-12). Most often bacterial attachment is initiated in surface irregularities that serve as microenvironments where bacteria are sheltered from unfavorable environmental factors to promote their survival (13). Rougher surfaces are believed to promote bacterial attachment due to increased surface area and depressions that provide more favorable and additional sites for colonization (14). The effects of surface roughness have been studied over a wide range of physical scales (15, 16) for the attachment of planktonic bacteria. Research studies are more concentrated towards observing bacterial attachment on irregular surface topographies, however the bacterial attachment behavior on patterned microscale and nanoscale topographies is comparatively less (17). Rowan *et al*. fabricated flat surfaces with evenly distributed square corrals, approximately 10 µm across were able to trap *E. coli* cells (18). In other study (19), Hou *et al.* used varied patterns of PDMS to study the effects of surface microtopography on bacterial attachment and biofilm formation. It was demonstrated that *E. coli* attached and formed biofilms in the valleys between protruding square features regardless of the dimension of the features (2-100 µm tested) and spacing between adjacent features (5-20 µm tested). All the above-mentioned studies were carried out with the planktonic bacteria. In contrast, our study looked over the attachment of bovine synovial fluid (BSF)-mediated bacterial aggregates on the patterned surface which might be important in the initial course of bacterial attachment. Host factors responsible for bacterial attachment include serum or tissue proteins such as fibronectin, fibrinogen, albumin, and laminin (14, 20). These proteins have been shown to rapidly coat a biomaterials when it is implanted (21, 22). Some of these plasma protein components adsorbed on blood-exposed devices did promote *S. aureus* attachment which is an essential step in the establishment of bacterial colonization and infection (23). Some *in vitro* and few *in vivo* studies have shown that fibrinogen, fibronectin, vitronectin, thrombospondin, and von Willebrand factor promote *S. aureus* attachment when immobilized on artificial surfaces (23-29).

We previously looked at the attachment of free-floating aggregates to different surfaces and found that there was a decrease in surface attachment in BSF induced aggregation after 5 and 15 minutes of seeding cells (30, 31). In addition, in another study, we investigated the role of preformed aggregates in synovial fluid on biofilm development in comparison with biofilm formed from single cells. Both of our above-mentioned studies demonstrated reduced number of BSF induced aggregates attachment compared to the single cells attachment on flat titanium (Ti) surfaces. However, in the present study, we hypothesize that the attachment of those aggregates might be different on a patterned surfaces as they will have a possibility to get physically captured in ridges and grooves of rough surfaces. Moreover, in our previous study, the bacterial attachment/growth was done in a flat Ti. Medical implants can have both flat surfaces as well as rougher, porous surfaces to encourage osteointegration. To create a range of surfaces that may be representative for various implants, we modified flat Ti coupons with roughness values that resembled surface roughness of real medical implants (32). In this regard, patterned surfaces were designed by engraving parallel lines with 50 and 100 µm depths and 500 µm peak-to-peak distance. The flow cell with patterned and flat Ti surfaces were run for 24 hours with and without BSF in Ringer’s solution (RS) under 0.0042 Pa shear stress. Bacterial attachment was then assayed qualitatively by confocal and SEM microscopy and quantitatively by CFU assay. In addition, the adsorption of proteins and hyaluronic acid which might have facilitated the bacterial attachment were visualized by confocal microscopy. Furthermore, various wall shear stresses by varying flow rates in the flow cell were applied to observe the differences in robustness of attachment on the flat and patterned surfaces.

**MATERIALS AND METHODS**

**Bacterial strain and culture conditions**

Green fluorescent protein (GFP) expressing methicillin-resistant *Staphylococcus aureus* (MRSA) strain AH1726 (LAC (AH1263) + pCM29 (PsarA\_sGFP, camR) (33) was used in this study. This strain of S. aureus (AH1726) contains GFP expressing plasmid in the same background strain (AH1263) and both have been previously shown to form biofilms (34, 35). Bacterial stocks of *S. aureus* (AH1726) were maintained at -80°C in 20% glycerol and streaked onto tryptic soy agar (TSA) plates that were incubated overnight at 37°C in the presence of 5% CO2. A single bacterial colony from the streaked plate was used to inoculate 25 mL of tryptic soy broth (TSB) for each experiment. Culture tubes were incubated at 37°C for 17-18 hours at 200 rpm.

**Bacterial aggregate formation**

One mL of the overnight culture of *S. aureus* (108 CFU/mL) was centrifuged at 21,000 xg for 1 minute. The supernatant was removed, and the pellet was washed in PBS and then resuspended in 10% synovial fluid (100 µl) in 900 µL RS. This is referred to as +BSF later. 10% synovial fluid was chosen as the aggregation process was found to be similar in 10% - 20% synovial fluid (36). Higher concentration of synovial fluid possesses higher viscosity and challenging to experiment with, and as a result, 10% synovial fluid has been used in this study. For single cells suspension preparation, 1 mL of the overnight culture was centrifuged at 21,000 xg for 1 minute and after removing supernatant, the pellet was washed and resuspended in 1 mL RS. This is referred to as -BSF later. The aggregates and single cell suspensions were then injected into two parallel channels of a flow cell (Fig. 1) using 1 mL syringes. We chose Ringer solution as it is isotonic with physiological concentrations of salts and therefore representative of the osmotic conditions that bacteria might experience in the body.

**Biofilm growth assay**

A chemostat model comprised of a flow cell with chambers dimension of 13mm width X 0.34mm depth X 39mm length (FC 270-AL, BioSurface Technologies, Bozeman, MT, USA) and a peristaltic pump (IPC ISM932A, Cole-Parmer, Vernon Hills, IL, USA) were employed for biofilm growth assay. Ti coupons (10 mm diameter and 2 mm thickness, BioSurface Technologies) were placed in the flow cell with the ridges and grooves positioned parallel to the direction of fluid flow. The chemostat model was assembled as shown in Fig. 1(A) and the channels were purged with respective fluids assuring the absence of air bubbles. It was then transferred to a chamber maintained at 37°C. Approximately after one hour of warm up period, the flow channels were inoculated with 1 mL of aggregates and single cells with starting cell concentration of 108 CFU/mL. The system was left under static conditions to allow attachment for 1.5 hours. The channel inoculated with aggregates and single cells were continuously supplied with 10% BSF in RS and RS respectively. In this study, 108 CFU/mL bacterial inoculum concentration was used for the biofilm growth assay, however, previously two different concentrations of 108 CFU/mL and 103 CFU/mL were used to investigate biofilm formation between aggregates and single cells (37). The flow rate was maintained at 0.139 mL/min creating a wall shear stress of 1.69 X 10-4 Pa and run for 24 hours at 37°C for biofilm formation with Reynolds number (Re) of 0.49 suggesting laminar flow. Furthermore, for the attachment and detachment study to investigate the robustness of bacterial attachment, higher flow rates of 13.9 mL/min and 5 mL/min representing shear stress values of 1.69 X 10-2 Pa and 6.09 X 10-3 Pa and Re of 49.32 and 17.74 respectively were applied for both patterned and flat surfaces.

**Bacterial enumeration**

The coupons recovered from the biofilm growth assay were gently dip rinsed once in PBS and transferred to conical tubes each containing 5mL of PBS. The tubes were sonicated for 5 minutes followed by 3 minutes vortex to dislodge biofilm attached to the surfaces. The bacterial suspensions were then plated for bacterial enumeration with the micro dilution method. This method and similar methods have shown to be more effective in quantifying CFUs present in aggregates/biofilm on a coupon (37-39). The bacterial count obtained in CFU/mL was converted to CFU/cm2 and reported in Fig. 3. It should be noted that all coupons were assumed to be flat during the calculation of surface area of the coupons since grooves of depths 50µm and 100µm do not have significant effect (approximate increase of 50% of total surface area) on the CFU/cm2 values reported in logarithmic scale.

**Coupon modification and characterization**

Medical implants are comprised of different roughness. To be within the roughness values of these implants (32), grade 5 Ti coupons (10 mm diameter and 2 mm thickness) were machined to make parallel grooves and ridges with pk-pk distance of 500µm and ridge to groove depth of 50 µm (50:500) and 100 µm (100:500). As an example, Fig. 1(B) shows a normal photograph which shows the pattern of a modified clean coupon with ridge-to-ridge distance of 500µm. In addition, Fig. S1 and S2 in supplementary materials show the side views of these coupons to illustrate the flatness of the tops and bottoms of the grooves. For flat surfaces, the coupons were manually sanded using an aluminum oxide sanding sheet (436A38, Grainger P600, USA) for 4-5 minutes. The roughness of these coupons was measured using Zeta 20 Optical Profilometer (Zeta, CA, USA) and Ra value was calculated. An Ra value is calculated over the entire measured area and is given by the arithmetic average of deviations from the mean. Ra is used for measuring the roughness of machined surfaces which dictates general variations in overall profile height characteristics (40). Furthermore, the water contact angle values for these surfaces were measured using sessile drop method where 75µL of distilled water was dropped on each surface and side view photographs (Fig. S1) were taken. These photographs were used to measure the contact angles in ImageJ (41). The Ra and contact angle values in Table 1 represent the mean ± SD of three measurements.

**TABLE 1** Average surface roughness (Ra) on flat and patterned titanium surfaces

|  |  |  |
| --- | --- | --- |
| Coupons (depth:pk-pk) | Ra (µm) | Contact angle (θ°) |
| 100:500 | 26 ± 0.47 | 90.09 ± 0.93 |
| 50:500 | 22 ± 0.26 | 91.14 ± 1.65 |
| Flat | 0.195 ± 0.03 | 69 ± 3.67 |

**Confocal Laser Scanning Microscopy assessment of *S. aureus* attaching in presence (+BSF) and absence (-BSF) of synovial fluid on Ti surfaces**

The biofilm formed on coupons were imaged under 10X objective lens using a confocal laser scanning microscope (CLSM) (FluoView FV10i, Olympus, PA, USA)). To identify the adsorbed proteins and hyaluronic acid on the Ti surfaces, fibrinogen 0.3 mg/mL (Fisher, F35200 Alexa fluor 647 conjugate), hyaluronic acid 0.2 mg/mL (Sigma, F1177, Fluorescein hyaluronic acid), and SYPRO Ruby Biofilm Matrix Stain (F10318, Fisher Scientific, USA) were used. Fluorescent fibrinogen (blue) and hyaluronic acid (green) were used to assess where these synovial fluid components might attach. SYPRO was used as a general protein stain.

**Qualitative Scanning Electron Microscopy (SEM) assessment of bacteria attached in presence (+BSF) and absence (-BSF) of synovial fluid on Ti surfaces**

For SEM images, coupons with bacteria were fixed according to a procedure described previously (42, 43) with some modifications. The following chemicals used for SEM were purchased from Thermo Fisher Scientific (Norwalk, IL, USA). Coupons were placed in a 24-well plate and soaked in a prefixing agent containing 2.5% Glutaraldehyde in 0.2M cacodylate buffer (pH 7.4) for 24 hours at room temperature. The coupons were then rinsed with cacodylate buffer three times. After the final rinse, the coupons were dehydrated in increasing concentrations of ethanol (70%, 90%, and 100%) three times each for 5 minutes. Finally, the coupons were soaked in 100% Hexamethyldisilazane (HMDS) twice for 10 minutes each, coated with gold-palladium and then viewed under a SEM (Quanta 200, FEI, Hillsboro, OR, USA) at an accelerating voltage of 10 kV. The acquired images were then false-colored using photo editing software (Adobe Photoshop® ver. 22.4.2) to better visualize the bacterial accumulation in ridges and grooves of the patterned coupons.

**Application of shear stress to assess the robustness of biofilm attachment**

The flow cell with mature biofilm from the biofilm growth assay was transferred to a microscope (Leica DM2700 equipped with K3M camera, Leica Microsystems Inc., IL, USA) along with the fluidic connections. Various flow rates from 1 mL/min to 13.9 mL/min were applied. A particular flow rate was allowed to run for 1 minute and videos were recorded at 4 frames/sec (Video S1 and S2 in supplementary material). Images of coupon surface at multiple fields of view were captured before and after this experiment. To determine the biofilm attachment before flow and the residual attachment after flow, ImageJ (41) was employed to calculate the total surface area coverage by biofilms. This experiment was conducted three times for both flat and patterned surfaces.

**Statistical analysis**

All experiments in this study were repeated three times. The threshold for significance was set at *p* < 0.05. Statistical significance was determined by unpaired two tailed t-test, assuming equal variance using prism (Graphpad v8 software) or, one-way ANOVA followed by Tukey’s multiple comparison tests. All error bars in the charts indicate standard error of the means.

**RESULTS**

**Optical profilometry**

The topography of Ti surfaces was examined by measuring the surface roughness (Ra). The Ra value is listed in Table 1. The results indicate that there is 113-fold increase in the Ra values of coupons with 50:500 and 133-fold increase with 100:500 coupons when compared with that of flat Ti. This demonstrates that the patterned Ti surfaces are significantly rougher than the flat Ti.

**S*. aureus* attachment in presence (+BSF) and absence (-BSF) of synovial fluid on flat and patterned Ti surfaces with number of surviving cells based on CFU’s assay**

Confocal microscopy was used to investigate the bacterial attachment in presence (+BSF) and absence (-BSF) of bovine synovial fluid (Fig. 2). With a concentration of 108 CFU/mL, bacterial attachment was observed both in presence and absence of BSF. The bacteria grown only in Ringer’s solution (without BSF) shows substantial biomass distribution on the surfaces, whereas the bacteria aggregated in several particles and appeared to be randomly distributed in presence of BSF as visualized by the qualitative confocal images. In +BSF and -BSF, the attached bacteria were found both on the top and bottom surfaces of the patterned surfaces demonstrating the potential to attach at different depth of the surface. Moreover, the attachment of bacteria appears to be more abundant towards the pattern of the Ti surfaces in both +BSF and -BSF conditions signifying the bacteria alignment with different surface pattern. With CFU’s assay, the number of bacteria attached on the Ti surfaces were found to be significantly greater in +BSF than in -BSF in 50:500 (*p*=0.0016), 100:500 (*p*=0.0063), and flat coupons (*p*=0.0021) respectively. No significant differences was found between different roughness surface (*p*>0.05). The patterned and flat Ti surfaces had the same bacterial load with the coupons of different roughness in both +BSF in terms of CFU (Fig. 3).

**Scanning Electron Microscopy (SEM) images of the *S. aureus* attached in presence (+BSF) and absence (-BSF) of synovial fluid on flat and patterned Ti surfaces**

Fig. 4 shows the SEM images of bacteria attached in presence and absence of BSF. Many attached *S. aureus* aggregates of various sizes could be observed on different surfaces. Those aggregates were detected more commonly on the pattern surfaces of the Ti, whereas less aggregates were distributed on the flat surfaces. The single cells without BSF exposure appeared to be scattered uniformly over the surfaces. With SEM images, the bacterial cell distribution looks comparable in both patterned and flat surfaces.

**Confocal and SEM images demonstrating the adsorption of fibrinogen, hyaluronic acid, and other proteins on the patterned Ti surfaces**

The adsorption of fibrinogen, hyaluronic acid, and other proteins might have led to the differences in bacterial attachment in +BSF and -BSF as revealed by the confocal images (Fig. 5A and B). This staining could have been due to the attachment of the fluorescent polymers (fibrinogen and hyaluronic acid) with like molecules and other components in the synovial fluid that had previously attached to the surface. Fluorescent hyaluronic acid was predominantly staining both 50:100 and 100:500 Ti surfaces, whereas there was less signal from fluorescent fibrinogen. Similarly, proteins were stained by SYPRO ruby biofilm matrix stain on both 50:100 and 100:500 coupons. None of the fluorescently labelled fibrinogen or hyaluronic acid were stained on the flat surfaces (Fig. 5C). On the negative control surfaces which were only exposed to RS, no proteins were found to be stained by SYPRO staining and fluorescent hyaluronic acid and fibrinogen were not stained as well (Fig. 5D, E, and F). It should also be noted that the Fig. 5 shows the evidence of proteins on top of the patterns, however, there were proteins absorbed on the bottom of the patterns as well. In fluorescent HA and composite images in Fig. 5, some fibres can be seen extending towards the bottom. Similarly, in Fig. 2, although the panels represent only fluorescent images, aggregates attachments in both grooves and top rims can be observed.

In addition to the confocal microscopy images the SEM images (Fig. 6) show the deposition of the fibrous components on the patterned Ti surfaces. Those materials could be the BSF components such as fibrinogen and hyaluronic acid or the adsorption of fluorescent fibrinogen and hyaluronic acid added to the surfaces. These components were distributed more on the patterned surfaces compared to the flat. However, those fibrous components were not visualized on the surfaces without synovial fluid (-BSF).

**Confocal microscopy and surface area coverage demonstrating the S*. aureus* attachment and detachment in presence (+BSF) of synovial fluid on patterned and flat Ti surfaces respectively at varying shear stress**

It is known that bacteria preferentially attach to rougher surfaces. Therefore, we hypothesize that the patterned surfaces will support aggregate attachment and retention on the surface. To test our hypothesis, varying shear stress was applied to the biofilm grown on Ti surfaces in a flow cell. Fig. 7 shows the attachment of aggregates on patterned and flat surfaces at pre- and post-flow. Even after application of 13.9 mL/min of flow rate, the biofilms on the patterned surface demonstrate robust attachment (Fig. 7B post-flow; see also video S1). On the other hand, the attached biofilm on the flat surface was removed at a flow rate as low as 5 mL/min (Fig. 7B post-flow; see also video S2).

The surface area covered by aggregates were calculated with Image J as shown in Fig. 8. There were significant differences between pre- and post-flow in both patterned surfaces (*p=*0.0042) and flat surfaces (*p*=0.0455). In terms of the biofilm attachment on these surfaces, there were no significant difference pre-flow (*p* < 0.05), however, after the experiment with varying flow rates, a significant difference (*p* = 0.0021) was found between the attached biofilm remaining on the two surfaces. This demonstrates a robust attachment of biofilm to the patterned surface despite the higher shear stress.

**DISCUSSION**

We previously studied the attachment of free-floating *S. aureus* aggregates to different surfaces and found a decrease in surface attachment in BSF induced aggregates after 5 and 15 minutes of seeding cells (30, 31) on flat Ti surfaces. However, many orthopedic surfaces are not flat but have larger scale textures and features such as edges, ridges and holes. In a study showing biofilms on actual implant materials, we found that biofilms accumulated on such features (32) We hypothesize that the attachment of those aggregates might be different to patterned surfaces as they have the possibility to get physically trapped between ridges and grooves of such surfaces as those could have the differences in roughness and might lead to difference in bacterial attachment, however in this study, the overall surface roughness values were taken into account. To test our hypothesis, patterned surfaces were designed by engraving parallel lines with varying depths and peak-to-peak distances on Ti coupons. The patterned coupons were positioned with the ridges and grooves parallel to the direction of fluid flow in the flow cell. It would also be interesting to see the effect of coupons placement with ridges and grooves at certain angle or perpendicular to the fluid flow. However, we anticipated pressure drop around the areas adjacent to the wall where the fluid is flowing from behind, or a complete skimmed flow where the aggregates trapped in the grooves would not see any shear force and get protection from detachment. On the other hand, with flow parallel to the patterns, we anticipated laminar flow and entire coupon could experience fluid flow. We plan to incorporate changing the orientation of the patterning to the flow stream in future work to better resemble clinical debridement in which flow can be from multiple directions relative to implant surfaces and patterning. We used Ti since this is a material relevant to orthopedic implants and conducive for machine milling. The confocal microscopy demonstrates the attachment of bacteria in the patterned surfaces with preferential attachment in the parallel grooves. The bacteria attaching in 50:500 and 100:500 surfaces in +BSF and -BSF shows the possibility of bacterial attachment in higher depth as well potentially in the implant surfaces where varying degree of depth or pocket is located.

The CFU’s assays shows higher attachment of bacteria to BSF exposed coupons of all textures compared to those exposed to RS. These results could be seen as contradicting with our previous work (30) where we had shown significantly higher number of aggregates in the -BSF group than in the +BSF. However, it should be noted that previously the experiments were carried out for up to 15 minutes only. In those experiments we quantified biomass by number of aggregates and surface coverage. However, since one aggregate was counted as one particle and even though BSF reduced the total number of aggregates, a single large particle may contain many cells, and in terms of total cells on the surface be equivalent to a surface covered with more numerous smaller aggregates. In the present study, we used sonication and vortexing (44) for the removal and disaggregation of the attached biomass which may also explain higher CFUs but lower numbers of attached aggregates in the +BSF when compared to -BSF. The staining with fluorescent fibrinogen, fluorescent hyaluronic acid, and a general protein stain by the confocal microscopy suggested that these components became trapped in the grooves as illustrated by their parallel staining. In contrast, absence of those components on the surface exposed to RS only suggests less attachment of bacteria because of absence of initial lodging sites.

However, besides having similar number of CFU’s as the patterned surfaces, those components were not present on the flat Ti surfaces, suggesting that those components preferentially lodge to patterns. We next asked if the rough surfaces protected biofilm from shear induced detachment. We applied increasing shear stress to both coupons’ surfaces. In the patterned coupon, we observed minimal detachment of the biofilm at a flow rate as high as 13.9 mL/min (Fig. 7 B post-flow), however, in the flat coupon, a flow rate of < 5mL/min was enough to remove the majority of biofilm (Fig. 7 B post-flow). This demonstrates that the bacteria attached on the patterned surfaces were protected from high shear-stress. The surface area covered by the biofilms were significantly different in pre- and post-flow in both patterned and flat surfaces. Biofilm on the flat surface was reduced by approximately 99% reduction, but only approximately 25% on the rough surfaces suggesting that the topography of a surface played an important role in retaining biofilm despite physical attempts to remove them. This finding may provide some insights in orthopedic debridement where water jets are used to remove biofilm and bacteria from implants, bone, and soft tissue. It should also be noted that we were initially focused on the robustness of aggregates attachment due to the synovial fluid, conducting similar experiments in the absence of BSF were out of scope. However, it will be interesting to observe the effect on bacteria detachment in the absence of BSF as well. We anticipate the attached single cells getting washed away with increasing shear even from the patterned surface and plan to incorporate shear stress in the -BSF conditions in future experiments.

Furthermore, SEM images show fibrous components on the patterned surfaces but not on the flat and surfaces exposed to RS only. This observation leads to the conclusion that more bacterial attachment on the patterned surfaces is possible because of initial lodging of fibrous components. It is believed that an implanted device is immediately coated with host matrix proteins including fibrinogen, fibronectin, and collagen (23, 45, 46). *S. aureus* possesses numerous cell wall-bound surface proteins that contain bind domains for these matrix proteins, and such surface proteins are referred to as microbial surface components recognizing adhesive matrix components (MSCRAMMs)(46, 47). We envision using the surface analysis technique such as XPS which could characterize and quantify the molecules from BSF that may adhere to the surface in our future experiments. One study showed the contribution of fibrinogen in promoting *S aureus* attachment to hemodialysis tubing (23). The bacterial attachment on the tubing was significantly lower in clfA mutant compared with its wild type. On the other hand, the clfA overexpressing strain showed a greater than 2-fold increase in attachment to fibrinogen compared with its parent. Roughened Ti surfaces preferentially bind proteins such as fibronectin or vitronectin (48) and it has also been shown that rough Ti alloy adsorbed more fibronectin than the flat surface (49) suggesting the role played by surface energy in adsorption of proteinaceous layer (50). Because *S. aureus* have the binding sites for fibrinogen and other proteins, we believe that once those proteins and hyaluronic acid become adsorbed to rougher features on the surfaces, it facilitates more attachment of the bacteria by binding with them. In future, along with investigating the bacterial attachment, a potential method to avoid bacterial attachment would also be interesting to explore. On the other hand, when surface patterns such as channels are present, flow can cause recirculation zones around the patterns. It has been reported that sediment deposition in cavities will decrease for deeper patterns (higher aspect ratios) due to the presence of recirculation zones in these cavities (12, 51). A similar principle applies to the attachment of bacteria in this study. As different patterned surfaces have different surface roughness, there may be competing contributions from surface roughness effects and the recirculation zone induced removal of bacteria at a given flow rate. At relatively low flow rates, the relative contribution of roughness effect may outweigh removal effects due to recirculation flows. However, higher flow rates, the contribution from recirculation flows may outcompete roughness effects as previously described (12, 51). This can explain our finding of the same bacterial attachment and growth on the surfaces with different roughness since the biofilms were grown under very low shear stress of 0.042 pa.

**CONCLUSION**

Our data provides evidence that the presence of bovine synovial fluid increases the attachment of *S. aureus* in the form of aggregates by total viable cells. In addition, the biofilm on a patterned surface was less easily removed by fluid shear stress compared to those on a flat surface. Since orthopedic implants have a wide range of surface features, textures and roughness’s and are subjected to a range of shear forces from the motion of joint fluid during flexion this may be provide clues as to where biofilm may accumulate. The deposition of fibrinogen, hyaluronic acid, and other proteins on to the patterned surfaces with ridges and grooves might have facilitated the bacterial attachment to these surfaces exposed to BSF. This study further adds knowledge of aggregates attachment on patterned surfaces that could aid the development of novel tools and techniques to treat PJI and other biofilm related infections on biomaterials. Specifically, it provides insight on designing the implant surfaces in a way that allows less or no attachment of BSF components so that the infection could be prevented by inhibiting the bacterial attachment.

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We declare no competing interests

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**FIG 1** (A) Flow system for biofilm assay and (B) patterned titanium coupon used for the experiment (left), and microscopic image showing the micropattern (right). The coupon had parallel grooves and ridges. Ridge-ridge distance (pk-pk) was 500 µm and ridge to groove depths were 50 µm (50:500) and 100 µm (100:500).

**FIG 2** Confocal images of bacteria imaged by GFP attached in the presence (+BSF) and absence of synovial fluid (-BSF) to patterned and flat Ti surfaces. The patterned patterns were 100 µm depth:500 µm pk-pk distance (100:500), 50 µm depth:500 µm pk-pk distance (50:500). Panel A and D are the images at lower magnification with focused ridges and unfocused grooves. Panel B and E are the zoomed -in ridges that are visible in A and D, while C and F are in focus zoomed- in grooves (dark areas in A and D). Panel H and J are zoomed- in images of G and I. Scale bars for A, D, G, and I are 100 µm, and B, C, E, and F are 50 µm.

**FIG 3** Synovial fluid increased the number of bacteria attaching to all surfaces. Number of bacterial cells recovered from flat and patterned Ti surfaces with textures of patterns of 50 µm depth: 500 µm pk-pk distance (50:500) and 100 µm depth:500 µm pk-pk distances (100:500). Statistical significance determined via unpaired two tailed t-test, assuming equal variance and a threshold of p < 0.05.

**FIG 4** False-colored SEM images of bacteria (blue) attached to different Ti surfaces (light red) in presence (+BSF) and absence (-BSF) of synovial fluid on flat and patterned Ti surfaces. The Ti coupons had three different textures: patterned patterns of 100 µm depth:500 µm pk-pk distance (100:500), 50 µm depth:500 µm pk-pk distance (50:500) and flat. The images were taken at 600X (1st and 3rd column) and 2400X (2nd and 4th column) magnification with scale bars of 100 µm (600X) and 20 µm (2400X) respectively. For patterned surfaces, the images were taken at the coupon grooves where machining patterns were visible. 2nd and 4th column images represent zoomed-in images for the 1st and 3rd column images.

**FIG 5** Confocal images of fluorescent fibrinogen and hyaluronic acid and general proteins attached to the Ti coupons after exposure to BSF (+BSF) or RS only as a negative control (-BSF). The Ti coupons had three different textures. Patterned patterns of 100 µm depth: 500 µm pk-pk distance (100:500), 50 µm depth:500 µm pk-pk distance (50:500) and flat. Scale bars are 100 µm.

**FIG 6** False-colored SEM images of fibrous components (green) attached to different Ti surfaces (blue) in presence (+BSF) and absence (-BSF) of synovial fluid on flat and patterned Ti surfaces. The Ti coupons had three different textures: patterned patterns of 100 µm depth:500 µm pk-pk distance (100:500), 50 µm depth:500 µm pk-pk distance (50:500) and flat. The images were taken at 600X magnification with scale bars of 100 µm.

**FIG 7** Fluorescent images of bacteria imaged by GFP attached in the presence (+BSF) of synovial fluid to patterned and flat Ti surfaces before and after flow. Scale bars are 100 µm. Post flow image for patterned surface was taken after a flow rate of 13.9 mL/min (wall shear stress of 1.69 X 10-2 Pa and Re = 49.32) was applied for 1 minute and for the flat surface, the post flow image was taken after a flow rate of 5 mL/min (wall shear stress of 6.09 X 10-3 Pa and Re = 17.74) was applied for 1 minute.

**FIG 8** Total surface area coverage measured by GFP signal in the presence of bovine synovial fluid to patterned and flat Ti surfaces before and after the application of increasing flow rates up to 13.9 mL/min. Error bars indicate the standard deviation, and the statistical significance was calculated via one-way ANOVA followed by Tukey’s multiple comparison tests.