***In vitro* staphylococcal aggregate morphology and protection from antibiotics is dependent on distinct mechanisms arising from postsurgical joint components and fluid motion**

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**Abstract**

Considerable progress has been made toward elucidating the mechanism of *Staphylococcus aureus* aggregation in synovial fluid. In this study, aggregate morphology was assessed following incubation in several simulated postsurgical joint conditions. Using fluorescently labeled synovial fluid polymers, we show that aggregation occurs through two distinct mechanisms: direct bridging between *S. aureus* cells and host fibrinogen, and an entropy-driven depletion mechanism facilitated by hyaluronic acid and albumin. By screening surface adhesin deficient mutants (*clfA, clfB, fnbB,* and *fnbA*), we identified the primary genetic determinant of aggregation in synovial fluid to be Clumping factor A. To characterize this bridging interaction, we employed an atomic force microscopy- based approach to quantify the binding affinity of either wild type *S. aureus* or the adhesin mutant to immobilized fibrinogen. Surprisingly, we found there to be cell-to-cell variability in the binding strength of the bacteria to immobilized fibrinogen. Super high resolution microscopy imaging revealed that fibrinogen binding to the cell wall is heterogeneously distributed at both the single cell and population level. Finally, we assessed the antibiotic tolerance of various aggregate morphologies arising from newly deciphered mechanisms of polymer-mediated synovial fluid-induced aggregation. The formation of macroscopic aggregates under shear, were highly tolerant of gentamicin, while smaller aggregates, formed under static conditions were susceptible. We hypothesize that aggregate formation in the joint cavity, in combination with shear, is mediated by both polymer-mediated aggregation mechanisms, with depletion forces enhancing the stability of essential bridging interactions.

**Importance**

The formation of a bacterial biofilm in the postsurgical joint environment significantly complicates the resolution of an infection. To form a resilient biofilm, incoming bacteria must first survive the initial invasion of the joint space. We previously found that synovial fluid induces the formation of *Staphylococcus aureus* aggregates, which may provide rapid protection during the early stages of infection. The state of the host joint environment, including the presence of fluid flow and fluctuating abundance of synovial fluid polymers, determines the rate and size of aggregate formation. By expanding on our knowledge of the mechanism and pathogenic implications of synovial fluid-induced aggregation, we hope to contribute insights for the development of novel methods of prevention and therapeutic intervention.

**Introduction**

Despite rigorous efforts by orthopedic surgeons to maintain operating room sterility, 1-2% of primary joint replacement surgeries still result in infection1,2. The most common pathogen associated with the development of chronic prosthetic joint infections (PJIs) is the bacterium *Staphylococcus aureus. S. aureus* predominance is due to a variety of toxic virulence factors, including the ability to form bacterial biofilms3,4,5. Bacteria residing in a mature biofilm are more difficult to culture in a diagnostic test and display considerable recalcitrance to antibiotic treatment6,7. Until recently, it was assumed that to form a resilient biofilm in the postoperative joint space, infecting cells had to first survive against applied antimicrobial agents and host immune system defenses whilst entering in a susceptible planktonic state. This preconception has been questioned by numerous groups reporting that synovial fluid, the viscous fluid which occupies the joint cavity, induces the rapid formation of bacterial aggregates8,9,10,11. Under applied shear in a flow cell system, synovial fluid induced aggregates are capable of attaching and forming biofilms on various orthopedic materials12. Further, these aggregates have been observed in the synovial fluid aspirates of chronically-infected patients, suggesting that they can facilitate long-term bacterial survival13.

Considerable progress has been made towards elucidating the mechanism of synovial fluid induced staphylococcal aggregation. Previous work has determined that the fluctuating composition and mixing of synovial fluid, mimicking postsurgical joint conditions, determines both the size and rapidity of aggregate formation *in vitro*14,15. These factors are heavily dependent on the elapsed time following surgery and the degree of patient ambulation14. For example, only minute quantities of synovial fluid are present immediately following surgical site closure16. However, as the joint space gradually refills, the concentration of various polymers including hyaluronic acid, fibrinogen, and albumin will increase17,18. The complexity of synovial fluid composition and the dynamic effects of fluid flow warrant investigation into the specific mechanics of bacterial aggregation.

To develop novel, targeted therapeutics it is essential that we first understand the mechanisms by which *S. aureus* invades the post-operative joint space. Prior work completed by ourselves and others has shown that *S. aureus* interacting with host factors, namely fibrinogen, is involved in synovial fluid-induced aggregate formation14,10. This process is mediated by a “bridging mechanism” in which fibrinogen forms a bridge between two or more cells. We have also found that the aggregation process is influenced by bacterial cell concentration, synovial fluid viscosity and mixing, suggesting that collision probability is an important factor14. Under static conditions, fibrinogen first mediates the formation of distinct, branched aggregates. The stimulation of fluid flow then initiates a secondary phase in which individual bacterial cells and smaller aggregates coalesce into large, free-floating aggregates in a fibrinogen-dependent manner. In addition to host factors, it is known that *S. aureus’* “microbial surface components recognizing adhesive matrix molecules” (MSCRAMMs), including clumping factors and fibronectin binding proteins, are important in the aggregation process. While it is known that these factors facilitate direct bacterial interaction with synovial fluid polymers, it is not yet clear which specific adhesins are required for synovial fluid-induced aggregation. Moreover, the relative differences in the attractive forces of these proteins with their corresponding synovial fluid polymer target is undefined.

 In addition to direct interactions between host and bacterial factors, we also found that “depletion aggregation,” an entropy-driven process occurring when high concentrations of non-adsorbing polymer push the bacteria together, is facilitated by an interaction between hyaluronic acid and protein19,20,21. In contrast with a bridging mechanism, aggregation through polymer depletion yields a tightly packed, globular aggregate morphology.

In the present work, we employed a combination of atomic force microscopy, confocal imaging and *S. aureus* adhesin knockout mutations to identify the relative contribution of the host and bacterial factors required for aggregate formation. By narrowing down the mediators of the adhesin-mediated interactions and assessing the effects of purely biophysical forces, we determined the relative contribution of “polymer bridging” and “polymer depletion” aggregation mechanisms. To evaluate the bridging mechanism, we screened *clfA, clfB, fnbA,* and *fnbB* *S. aureus* knockout mutants for their ability to aggregate in synovial fluid and interact with fibrinogen. Using a synthetic synovial fluid containing fluorescently labelled adsorbing and non-adsorbing polymers, we also determined the spatial arrangement of each polymer relative to the bacterial cells, allowing further differentiation between the two mechanisms.

We also investigated how these various aggregation mechanisms influence antibiotic susceptibility. It has previously been shown that fulminant bacterial aggregates formed through either polymer bridging or depletion have the capacity to display antibiotic recalcitrance20,10. Less is known regarding the aggregate size and time dependency on the acquisition of tolerance. We hypothesize that smaller aggregates, which form during shorter time scales or under quiescent conditions may also be tolerant and clinically relevant immediately following surgical site closure.

Through the following work we show that synovial fluid-induced aggregation can occur through distinct mechanisms which, *in vivo,* is likely dependent on the state of the joint environment. We report that the primary bridging mechanism requires direct interaction between host fibrinogen and bacterial Clumping factor A. Further, this specific interaction is essential for conferring protection from antibiotic challenge, corroborating previous studies10. While bridging aggregation with fibrinogen was required to confer bacterial protection, we observed the formation of smaller aggregates formed through an alternative polymer depletion mechanism. This suggests that biophysical interactions alone are sufficient for aggregation to occur. While the aggregates formed through polymer depletion were susceptible to gentamicin, our data suggests that non-adsorbing synovial fluid polymers could be strengthening the aggregate phenotype in an additive manner. We speculate that aggregate formation in the joint cavity is dually mediated by both mechanisms acting together to enhance bacterial survival thus increasing the chances of chronic infection development.

**Results**

***Staphylococcus aureus* clumping factor A is required for macroscopic aggregate formation under shear but not for branching under static incubation**

Key surface adhesins binding to fibrinogen is an essential prerequisite for aggregate formation under shear14,10. To further understand the molecular forces governing aggregation through fibrinogen binding, the interactive forces of single *S. aureus* wild type and quadruple adhesin mutant (*ΔclfA ΔfnbAB clfB*::Tn) cells were studied by atomic force microscopy (AFM). AFM directly measures bond strength through a dynamic process of pushing and pulling on ligand-receptors of living bacteria. Interactions between wild type cells and fibrinogen or synovial fluid exhibited similar medians of ~ 60% binding frequency (**Fig. 1a**). Similarly, the strength of these interactions was comparable with medians of ~ 1.5 nN (**Fig. 1b**). While adhesive forces were also observed between quadruple mutant strain cells and fibrinogen or synovial fluid, they were significantly less frequent (~ 14 %) and weaker (~ 170 pN) compared to the wild type (**Fig. 1a and Fig. 1b**). Control studies with AFM cantilevers coated with hyaluronic acid and BSA presented even lower frequency of binding (~ 5%) and weaker forces (~ 100 pN). Consistent trends were observed between the adhesin mutant and whole synovial fluid. Force histograms of these are shown in **Supplemental Figure 1**.

To narrow down the individual surface protein(s) required for potential specific bridging interactions with fibrinogen, we screened double isogenic adhesin mutants of our wild type *S. aureus* strain (**Fig. 2a**). Macroscopic imaging of fibronectin-binding protein mutant, AH4392 (*ΔfnbAB*), (**Fig. 2c**) and clumping factor double mutant, AH4065 (*ΔclfA, clfB::Tn*) (**Fig. 2d**), revealed that clumping factors A and B are required for aggregate formation under shear, while fibronectin- binding proteins A and B are dispensable. We subsequently imaged single clumping factor mutants AH4037 (*ΔclfA*) (**Fig. 2f**) and AH2905 (*clfB::Tn*) (**Fig. 2e**) following 1-hour of dynamic incubation. While the clumping factor B mutant could form free-floating macroscopic aggregates in 10% bovine synovial fluid (BSF), the clumping factor A mutant was unable to replicate this phenotype. Moreover, complementation of clumping factor A back into the double clumping factor mutant strain (*∆clfA, clfB*::Tn, pJM01(*clfA*)) (**Fig. 2g**) recovered the dynamically formed synovial fluid aggregate phenotype, confirming that clumping factor A is essential. Finally, the experiment was repeated using exponentially growing cells from a 2-hour day culture to verify that differing expression levels of any adhesins during stationary phase cells were not influencing our observations (**Supplemental Figure 3**). Regardless of growth phase, the previously observed phenotypes remained consistent.

Because the initial period following surgical site closure has little fluid flow, we next screened the ability of the isogenic clumping factor mutants to aggregate in the absence of shear (**Fig. 3a**). Unlike the dynamic, macroscopic aggregates, there was no aggregation deficiency resulting from mutations in clumping factor A (**Fig. 3c**) or B (**Fig. 3d**). Interestingly, complementation of clumping factor A yielded slightly larger aggregates (**Fig. 3e**), however, the difference in average particle size was not statistically significant (P>0.05) (**Fig. 3f**). To justify our use of 2D images to quantify the effective diameter of the aggregates we also imaged the 3-dimensional structure of the aggregates in a subset of experiments. (**Fig. 3g-i**). As expected, the static aggregates settled to a single plane, and therefore, 2-dimensional quantification was deemed sufficient.

**Hyaluronic acid and bovine serum albumin localize outside of the aggregates while fibrinogen binds to the bacterial cell wall proteins**

Due to the fluctuations in the relative concentrations of hyaluronic acid, albumin and fibrinogen following surgery, we next assessed the spatial distribution of each polymer in relation to cells following 1-hour of either static or dynamic incubation. To visualize the localization of hyaluronic acid and fibrinogen, we composed a synthetic synovial fluid containing each polymer with differentiating fluorescent labels based on the previously described composition by Knott et al10. Following dynamic incubation, the synthetic synovial fluid stimulated the formation of a free-floating aggregate from the wild type bacteria but not the adhesin mutant (**Fig. 4a**).

Similarly, on microscopic examination, the fibrinogen closely co-localized with the wild type bacteria, and not the adhesin mutant (**Fig. 4b**). Conflicting with our observations using fibrinogen alone14, the quadruple adhesin mutant still aggregated on the microscopic scale with the other two polymers indicating a potential role for hyaluronic acid either alone or in combination with albumin. Under static conditions, both the adhesin mutant and the wild type formed the branched aggregate phenotype and closely co-localized with the fibrinogen (**Fig. 4c**), corroborating our findings that in the absence of shear the mutant is still able to interact with the proteins, likely through other surface adhesins. However, with fibrinogen alone, the mutant did not aggregate, even under static conditions14. Interestingly, using super high-resolution imaging of the interaction between fibrinogen and the bacterial surface of the wild type strain, we observed a heterogenous distribution of the polymer around the cells. While some cells were completely enveloped, others displayed little to no co-localization of fibrinogen with the cell wall (**Fig. 4d**). This resulted in a bimodal distribution of the percent coverage when quantified by image analysis (**Fig. 4e**).

**Bovine serum albumin (BSA) alone is not capable of stimulating aggregate formation at low or high concentrations**

Due to the relative abundance of albumin in the joint environment and the pathogenic attributes associated with bacterial interaction, we next evaluated the effect of bovine serum albumin (BSA) on aggregate formation under shear. At low concentrations of BSA, there was no observed aggregate formation by wild type *S. aureus* (**Fig. 5b**), however, following incubation in high concentrations of BSA, there was a moderate degree of immediate aggregation (**Fig. 5a**) but no significant increase in size over time.

**High concentrations of hyaluronic acid under shear yield depletion aggregation following long term incubation on an orbital shaker**

Because depletion aggregation is characterized by a dependency for high concentrations of non-adsorbing polymer to be present, we considered the effect of both low (0.3 mg/mL) and high (3 mg/mL) abundances of high molecular weight hyaluronic acid. Similar to bovine serum albumin, there were no significant differences between the saline and low concentration hyaluronic acid-treated bacteria after 3-hours of dynamic incubation (**Fig. 5b**). In contrast, a high abundance of the polymer stimulated the formation of small bacterial clusters at the early time points (**Fig. 5a**). The morphology of the aggregates, in conjunction with the concentration-dependent effect, indicates that longer incubations with high molecular weight hyaluronic acid present are capable of stimulating depletion aggregation of *S. aureus.*

**A combination of hyaluronic acid and bovine serum albumin facilitate depletion aggregation under dynamic conditions in a concentration-dependent manner**

After 1-hour of dynamic incubation, the combination of the two polymers at high concentrations yielded the formation of microscopic aggregates approximately 3 um2 in diameter (**Fig. 5a**). At the 3-hr time point the tightly packed aggregates had grown to approximately 5 um2 (**Fig. 5c**). In contrast, the addition of low concentrations of polymers stimulated some aggregation but did not elicit an increase in size over time (**Fig. 5b**). To visualize the distribution of hyaluronic acid and bovine serum albumin relative to the bacteria, we conducted confocal microscopy with fluorescently labelled polymers and FM-464 stained wild type *S. aureus*. As previously observed, the hyaluronic acid was localized to the space between the aggregates (**Fig. 5d**). Interestingly, while the bacteria were capable of binding BSA directly, BSA was also co-localized with the fluorescent hyaluronic acid in the inter-proximal spaces (**Fig. 5e**).

**The macroscopic aggregate phenotype formed under shear confers protection from gentamicin challenge**

Recent studies have reported that synovial fluid aggregates formed under specific conditions (nutrient availability under shear) display considerable recalcitrance to antibiotic administration10. Therefore, we next tested the antibiotic tolerance of each aggregate phenotype generated by potential postsurgical conditions. Dynamically formed aggregates formed under shear in a combination of 10% bovine synovial fluid and 10% tryptic soy broth were more tolerant to gentamicin challenge relative to untreated, planktonic cells or statically formed aggregates (**Fig. 6a**), corroborating findings from Knott et al, 2021. The bacteria forming the branched aggregates under static conditions were slightly less susceptible than the planktonic cells, however, the protection was non-significant.

 Because we previously identified the importance of fibrinogen bridging for the formation of the macroscopic aggregate phenotype, we also conducted antibiotic tolerance assays with our single *clfA* mutant strain. While wild type, dynamically formed aggregates are more tolerant to antibiotic challenge compared to planktonic and statically aggregated bacteria, the protection was abolished in the absence of clumping factor A. Regardless of incubation treatment, there was an approximate 6-fold reduction in bacteria following gentamicin challenge (**Fig. 6b**). When the dynamic aggregate phenotype was restored in the clumping factor A complement strain, the protection was recovered, however, the log reduction was not significantly different than the planktonic cells or static aggregates (**Fig. 6c**). Taken together, these data indicate that without the introduction of fluid flow, the aggregates are easily cleared by gentamicin. Furthermore, the presence of clumping factor A was required under the applied conditions to form the macroscopic aggregate phenotype, suggesting interaction with fibrinogen is essential to confer protection.

**Bacteria residing in depletion aggregates formed by high concentrations of hyaluronic acid and bovine serum albumin are minimally protected from antibiotic challenge**

 Following 3-hours of dynamic incubation in high concentrations of hyaluronic acid and bovine serum albumin, we observed the formation of microscopic bacterial aggregates. After, 3-hours of incubation on an orbital shaker in either hyaluronic acid, bovine serum albumin, or a combination of both polymers, wild-type *S. aureus* was challenged with gentamicin. While both mono-treatments of either hyaluronic acid or bovine serum albumin, as well as the combination treatment of both, conferred some protection (<2-fold), any enhancement in bacterial survival was non-significant compared to the planktonic control (**Figure 7**).

**Discussion**

The ability of *Staphylococcus aureus* to rapidly aggregate in synovial fluid potentially enhances the virulence of the pathogen in the postsurgical joint environment. Due to these probable pathogenic attributes, a considerable amount of effort has been placed on characterizing the mechanics and pathogenic implications of synovial fluid-induced aggregation. Current work in the field has focused on defining the formation and characteristics of large, free-floating aggregates formed under specific conditions. Our group posits that these conditions neglect a multitude of potential effectors of aggregation, such as the presence and absence of fluid flow, as well as the rapid fluctuations in polymer concentration and viscosity. By manipulating such factors, we show that aggregation can occur through distinct mechanisms, yielding unique aggregate morphologies. Through the present work, we delve deeper into the specific mechanisms driving synovial fluid induced aggregation*,* aiming to simulate postsurgical joint factors, and assess the antibiotic tolerance of arising phenotypes.

Fibrinogen has been widely documented to be important for synovial fluid aggregate formation8,10. In the absence of fibrinogen, the large macroscopic aggregates no longer form under shear10,14. Building on this, our group found that under static conditions, representative of a sedentary joint, fibrinogen was unnecessary for the formation of smaller, branched aggregates14. Through atomic force microscopy measurements, we revealed that deleting key fibrinogen binding proteins weakens the adhesive forces between the bacteria and host polymer, but that this inhibition does not prevent static aggregation. This finding suggests that *S. aureus* may be interacting with fibrinogen through alternative microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), and that these weaker, secreted virulence factors may be sufficient for maintaining the small aggregate morphology in the absence of shear forces22,23. It also suggests that other polymers within the synovial fluid may be playing an additive or stabilizing role in aggregation.

Interestingly, super high-resolution microscopy allowed us to observe the heterogeneous binding of fibrinogen to the cell wall (**Fig. 4f**). While preliminary, this observation complements our AFM data which depicts large variability in single cell force measurements within a single population. We speculate that this patchiness may explain the branching structures we observe in the initial stages of aggregate formation under static conditions15,14. Heterogeneous expression may be expected to form strong localized bridging bonds in the presence of fibrinogen, but potentially weak attraction or even electrostatic repulsion between cells where adjacent surfaces with no adhesins are in proximity.

Although strong binding to fibrinogen may not be required for the formation of small, static aggregates, it is evidently a primary requirement of the macroscopic aggregate phenotype under shear. Despite a consensus in the field appreciating the essentiality of fibrinogen for aggregate formation, the specific bacterial genetic determinant(s) facilitating this interaction remained undetermined. By screening double and single clumping factor *S. aureus* mutants, we show that the formation of this phenotype is dependent on direct interaction between fibrinogen and clumping factor A. In the context of synovial fluid-induced aggregation in the postsurgical joint space, we speculate that this aggregate morphology will not arise until synovial fluid has refilled to a point where the fibrinogen concentration is abundant and joint flexion is restored.

In contrast to aggregate formation under shear, none of the mutant strains displayed aggregation deficiencies under static conditions. The clumping factor A complementation mutant yielded slightly larger aggregates compared to the other strains, although the increase in size was non-significant. We suspect this effect could be attributed to a higher copy number associated with the complementation plasmid. These data indicate that clumping factor A is the genetic determinant of aggregation when fluid flow (high shear) is present, but not in quiescent systems. Recent work suggests that the strength of fibrinogen binding by *clfA* is enhanced under high shear stress in order to facilitate attachment and decreased under low to allow for bacterial spread and colonization24. This force sensitivity may explain the requirement of *clfA* to form the macroscopic aggregate phenotype under shear. While the clinical relevance of each phenotype is dependent on multiple factors, the relative composition of the joint fluid and presence of fluid flow appear to be defining attributes. It is possible that at very high shear stresses, the shearing force will overcome the adhesion forces resulting in either the breaking up of aggregates or inhibiting their formation to begin with.

While the bridging mechanism mediated by *clfA* binding of fibrinogen is playing an important role,it is also plausible that other synovial fluid polymers may elicit alternative or additive effects. While neither hyaluronic acid nor bovine serum albumin could stimulate large aggregate formation alone, the combination of the two yields tightly packed globular aggregates. It bears mentioning that while *S. aureus* is capable of binding bovine serum albumin through the expression of surface proteins, such as *ebh*, the morphology and concentration dependence of this effect suggests that the driving mechanism is not solely bridging25,26. This is further supported by our findings that bovine serum albumin alone was unable to stimulate aggregate formation, even at high concentrations of bacteria and polymer. Hyaluronic acid, in contrast, is an abundant, non-adsorbing polymer in synovial fluid which has been shown to stimulate the formation of depletion aggregates at high polymer and bacterial concentrations20. This form of aggregation is entropically driven and dependent on random bacterial collisions in the environment. Therefore, the underlying mechanism is biophysical and thus will occur independent of bacterial surface adhesins. While the bacterial concentrations used in these assays were insufficient to stimulate depletion aggregation with hyaluronic acid alone, we suspect that the combination of both polymers together was able to recover this effect.

Electrostatic interactions between bovine serum albumin and hyaluronic acid have been widely documented in the literature27. While these interactions are enhanced under acidic conditions, they are present at a neutral pH. As such, the effect that we observed with the combination treatment of both polymers together could be explained by an increase in the relative abundance of total polymer or possibly the formation of hyaluronic acid-albumin complexes. The formation of such complexes could presumably increase the overall size of the non-adsorbing polymer in the system and potentially recover the requirements of the depletion mechanism at our lower bacterial concentrations. Complementing our theory, confocal microscopic mapping of fluorescently labelled hyaluronic acid and bovine serum albumin indicate that the majority of both polymers localize to the space in between the aggregates, as opposed to fibrinogen, which localizes to the cell wall in a bridging fashion.

Of note, our assays were conducted under relatively high concentrations of bacteria which may limit our ability to extrapolate to the clinic. However, the true bacterial titers within an infected joint at various time points are poorly described. It is possible that growth within the joint space could yield the concentrations required for polymer depletion to attain relevance. Furthermore, localized inflammation and secreted bacterial virulence factors, such as hyaluronidases, may result in cleavage of the hyaluronic acid. This cleavage may alter the pH of the system, inherently strengthening the electrostatic interactions between hyaluronic acid and albumin polymers28,27. We suspect that in the context of a periprosthetic joint infection, hallmarked by an acidic microenvironment, this fluctuation may skew the utility of this mechanism. While these factors were not considered in the present study, they may affect the efficacy of a depletion mechanism and will be investigated in the future.

 It has previously been shown that the formation of large aggregates under shear confers recalcitrance to antibiotic challenge10. We have shown here alternative mechanisms by which aggregation can occur in synovial fluid under various conditions. Corroborating previous studies, we report that the formation of the macroscopic aggregate phenotype with fluid flow is a prerequisite for conferring antibiotic tolerance. Expanding on this finding, we show that the genetic determinant for the antibiotic tolerant phenotype is expression of *clfA*. Conversely, small, static synovial fluid aggregates, as well as those stimulated by the combination of hyaluronic acid and albumin, displayed comparable susceptibility to planktonic cells. It is probable that the antibiotic tolerance of polymer depletion aggregates observed in other studies, is attributed to the far higher bacterial inoculums which were deemed irrelevant in the present work20. We conclude that the rapid bridging mechanism through fibrinogen binding under fluid shear is required for the conferring of antibiotic tolerance to *S. aureus* aggregates under our simulated conditions.

**Conclusion**

The development of synovial fluid aggregates is a multi-factorial phenomenon which is heavily influenced by a rapidly changing microenvironment following a surgical procedure (**Fig. 8a and Table 2**). By manipulating potential postsurgical joint conditions, we report that aggregate formation is largely mediated by a polymer bridging mechanism through direct interaction of fibrinogen and clumping factor A. The temporal development of these macroscopic, gentamicin- tolerant bacterial aggregates (**Fig. 8b**) is dependent on fluid flow (**Fig. 8c**). In contrast, the formation of microscopic aggregates under static conditions does not require the presence of clumping factor A and confers little antibiotic protection (**Fig. 8c**). Independent of the bridging mechanism, we report high concentrations of other synovial fluid polymers (hyaluronic acid and albumin) can stimulate the formation of depletion aggregates, although this phenotype lacks recalcitrance to applied gentamicin.

**Methods**

**Bacterial Strains and Growth Conditions**

Bacterial strains were received in agar slants from the Horswill Laboratory (University of Colorado School of Medicine, Aurora, CO). Strains were streaked out on tryptic soy agar (TSA) (BD Biosciences, Heidelberg, Germany) to obtain isolated colonies. A single colony was used to inoculate 5mL of tryptic soy broth (TSB) (BD Biosciences, Heidelberg, Germany). Cultures were grown overnight in an orbital shaker (220 RPM) at 37-degrees (Innova 44; New Brunswick Scientific). To obtain day cultures, overnight cultures were diluted 1:000 into fresh TSB prior to placement in an orbital shaker (220 RPM) set to 37-degrees for 2-hours. Methicillin-resistant *S. aureus* LAC strain, AH1263, was used in all of the following assays14. Additionally, mutant strains AH4392, AH4065, AH4037, AH5614, AH2905 and AH4413 were utilized in macroscopic and microscopic imaging bovine synovial fluid assays29 (**Table 1**).

**Atomic Force Microscopy**

Overnight cultures of wild type strain, AH1263, and quadruple adhesin mutant, AH4413 (*ΔclfA ΔfnbAB clfB*::Tn), were grown to exponential phase (OD600 nm > 0.3) at 37°C in fresh TSB. Cells were harvested and washed in PBS and were used within 2 hours after harvesting to ensure cell viability30. Force measurements were acquired with a Bioscope Resolve AFM (Bruker) as described in Casillas et al 201931. An attached inverted microscope (Axiovert 200M; Zeiss) was used to position the AFM cantilever over *S. aureus* cells31. A total of 102 different *S. aureus* cells from 21 independent cell cultures were probed with Si3N4 cantilevers with nominal tips radius of 20 nm and an median spring constant of 0.14 nN/nm as determined by thermal tuning method. These AFM tips were coated with fibrinogen (Fg) (100 µg/ml), or a 10% synthetic synovial fluid, by immersion in their respective solutions for 45 min, and then rinsed in PBS. Control studies with AFM tips coated with hyaluronic acid and BSA and uncoated were also conducted. Negative binding controls included experiments with *S. aureus* AH4413 (*ΔclfA ΔfnbAB clfB::Tn*) cells. All the force measurements were conducted in PBS, at a single retraction velocity of 5.4 um/s generating over 60,000 force curves. To ensure specificity, only the final rupture peak was included in the post-collection analysis presented herein. Strength of interactions were plotted as a box and whisker plot, and as a histogram of force frequency. The latter was plotted to see the distribution of force values and was reported as percentage of force curves observed in a force range divided by total number of curves with adhesion events multiplied by 100.

**Macroscopic Imaging**

Overnight cultures of each strain were grown in TSB as described above and diluted to an OD600 of 0.2. 3mLs of the culture were pelleted by centrifugation (21,000 xg) (ThermoFisher, Sorvall Legend Micro 21R Microcentrifuge) and the supernatant aspirated. The bacteria were resuspended in phosphate buffered saline (PBS) or 10% bovine synovial fluid in a total volume of 3mLs. The total volume was transferred to a 35x10mm petri plate and placed on an orbital shaker (60 RPM) at room temperature for 1-hour. After 1-hour, the aggregates were imaged as previously described using a dual 12-megapixel camera secured 15 cm above the plate. Images were evaluated for the presence or absence of free-floating aggregates. The raw macroscopic images were then imported to FIJI for image analysis to quantify aggregate size (**Supplemental Figure 2**). Each image was converted to 8-bit and inverted. A threshold was then applied and altered to include aggregates which display defined edge morphology and eliminate artifact. Non-aggregating strains yielded the accumulation of microscopic aggregates and single cells forming a diffuse cloud-like pattern. Such artifacts including the edge of plate and diffuse cloud patterns were eliminated using the paint and fill features. Particles greater than 75 pixels (0.04 mm2) were analyzed for quantification of size.

**Confocal Microscopy and Image Analysis**

* **Isogenic Mutant Screen for Aggregation**

Overnight cultures of each strain were diluted to an OD600 of 0.2. 1mL of each diluted culture was pelleted and resuspended in PBS. The bacteria were incubated with nucleic acid, SYTO-9, for 30 minutes, washed twice, and resuspended in either PBS or 10% bovine synovial fluid (Lampire Biological Laboratories, Pipersville, PA). Imaging was conducted as previously described with 5 representative images collected for each strain at 60x magnification with an additional 2x zoom14. Images for quantification were captured in 2 dimensions in order to rapidly capture the dynamics of aggregate formation. Images were exported as TIFs to FIJI image analysis software for quantification of aggregate size as previously published14. Statistical significance was determined by one way ANOVA followed by Tukey’s multiple comparison post-test. Error bars indicate 2 biological replicates each consisting of 5 representative images. Representative Z stacks of the wild type strain were collected to verify that 2 dimensional images can be approximated as spheres. Stacks were imported and reassembled using Imaris 9.7 Software (RRID:SCR\_007370).

* **Fluorescent Synthetic Synovial Fluid**

Wild type strain, AH1263, and quadruple adhesin mutant, AH4413, were grown overnight as described above. Cells were stained with red plasma membrane stain, FM-464 for 30 minutes. Next, the bacteria were washed twice and resuspended in a synthetic synovial fluid containing commercially available fluorophore-conjugated polymers at concentrations comparable to 10% synovial fluid. A combination of PBS, Alexa Fluor 647-conjugated fibrinogen (0.175mg/mL) (Invitrogen, Waltham, MA), Fluorescein hyaluronic acid (0.3mg/mL) (Sigma-Aldrich, St. Louis, MO), and 1.9mg/mL bovine serum albumin (Fisher Bioreagents, Fisher Scientific) were added to the tube. The resuspensions were transferred to 35mm x 10mm, glass bottom confocal dishes with total volumes of 1mL or 3mLs (35 by 10 mm; MatTek Corp., Ashland, MA). Prior to confocal imaging, bacteria were incubated for 1-hour either statically (1mL total volume) or dynamically on an orbital shaker set to 60RPM (3mL total volume) at room temperature. Both strains were imaged macroscopically and using Olympus FluoView FV10i confocal laser scanning microscope. Confocal images were similarly captured at 60x with an addition 2x or 5x zoom.

* **Hyaluronic acid and Bovine Serum Albumin**

RFP-expressing *S. aureus,* AH3047, and GFP-expressing strain AH1726 were used to assess the effect of hyaluronic acid and bovine serum albumin on aggregate formation11. Bacteria were prepared as described above and resuspended in PBS supplemented with hyaluronic acid (0.3mg/mL or 3mg/mL) (1.5 to 1.8 MDa; Alfa Aesar, Haverhill, MA, USA), and bovine serum albumin (1.9mg/mL or 19mg/mL) (BSA) or a combination of the two polymers. Both strains were imaged macroscopically and using confocal microscopy as described above. Confocal images were similarly captured at 60x with an addition 2x or 5x zoom. 5 representative images were collected and exported to FIJI for particle quantification32.

**Super Resolution Microscopy**

To assess the direct interaction between fibrinogen and wild type *S. aureus,* the bacteria was super high-resolution images were taken with a Nikon Structured Illumination Microscope (SIM) (Tokyo, Japan) at the Campus Microscopy & Imaging Facility (CMIF) (Ohio State University). Wild type *S. aureus* was stained with SYTO-9 (Thermo Fisher Scientific, Waltham, MA) prior to a 1-hour incubation in Alexa Fluor 647-conjugated fibrinogen (0.175mg/mL) (Invitrogen, Waltham, MA). The images were collected with a 100X/1.49 oil SR HP Apo TIRF objective and a Hamamatsu Orca Flash 4.0 sCMOS camera. The green SYTO-9 channel was imaged with a 488nm laser with an emission Band Pass (BP) filter 500–545nm; and the red Alexa 647 channel was imaged using a 640nm laser with an emission BP filter 663–738nm. Analysis of the colocalization of fibrinogen to cell walls was calculated using a protractor overlay with 30° intervals to determine degrees of coverage. Individual cells with piecemeal colocalization had individual segments summed together to provide a final value. Cells actively dividing or outside of the focal plan were excluded from the data.

**Antibiotic Tolerance Assays**

* **Bovine Synovial fluid Aggregates**

Overnight cultures of AH1263 and AH4037 were prepared as previously described. Cultures were diluted 1:1000 into 20mL of TSB and grown to an OD600 of 0.2. 1mL aliquots of day culture were pelleted and the supernatant aspirated and discarded. The pellets were resuspended in either 1mL of PBS or 10% bovine synovial fluid in PBS. Aggregation was stimulated either statically on a benchtop, or dynamically on an orbital shaker (180 RPM) for 1-hour. TSB was added to each tube for a total concentration of 10% TSB in PBS and gently inverted to homogenize without disrupting the aggregates. 50 microliters of 10mg/mL Gentamicin (50x MIC) (GoldBio) were added to each culture and the tubes gently inverted again. Gentamicin was selected due to its’ frequent usage by orthopedic surgeons. Tubes containing bacteria, TSB, and gentamicin were placed in a 37-degree incubator for 1-hour. After a 1-hour antibiotic challenge, the bacteria were pelleted by centrifugation at maximum speed and the supernatant removed. Pellets were resuspended in 0.05% Trypsin-EDTA (Corning, New York, United States) and placed on a rocker for 30 minutes to break up aggregates. The bacteria were then pelleted, resuspended in PBS and washed twice prior to serial dilution for colony-forming unit (CFU) plating. Dilution plates were incubated overnight at 37 degrees.

* **Hyaluronic Acid-Bovine Serum Albumin**

Overnight cultures of wild-type *S. aureus,* AH1263, was prepared as described above. Cultures were diluted 1:1000 into 20mL of TSB and grown to an OD600 of 0.2. 1mL aliquots of day culture were pelleted and the supernatant aspirated and discarded. The pellets were resuspended in either 1mL of PBS, 3mg/mL hyaluronic acid in PBS, 19mg/mL of bovine serum in PBS, or a combination of both polymers. TSB was added to each tube for a total concentration of 10% and gently inverted to homogenize without disrupting the aggregates. 50 microliters of 10mg/mL Gentamicin (50x MIC) were added to each culture and the tubes gently inverted again. Tubes containing bacteria, TSB, and gentamicin were placed in a 37-degree incubator for 1-hour. After a 1-hour antibiotic challenge, bacteria were pelleted by centrifugation at maximum speed and the supernatant removed. Pellets were resuspended in 0.05% Trypsin-EDTA and placed on a rocker for 30 minutes to break up aggregates. The bacteria were then pelleted, resuspended in PBS and washed twice prior to serial dilution for colony-forming unit (CFU) plating. Dilution plates were incubated overnight at 37 degrees.

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**Competing Interest Statements**

The Authors declare no Competing Financial or Non-Financial Interests

**Data Availability Statement**

The data that support the findings of this study are available at Dryad repository: (https://datadryad.org/stash/share/YUKo5DQuPxfXEv3YqEWBPI\_GO48Nq9vMWWRjhwqs\_E.) doi: 10.5061/dryad.18931zd0x.

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**Tables**

**Table 1. Source of utilized strains from Horswill Laboratory.**

**Table 2. The effects of host and bacterial factors on the local postoperative joint environment and synovial fluid induced aggregation.**

**Figure Captions**

**Figure 1.** Box and whisker plots of *S*. *aureus* wild type and *∆clfA, ∆fnbAB, clfB*::Tn (quadruple adhesin mutant) binding (**1a**) frequency and (**1b**) force to fibrinogen and synovial fluid. Whisker ends 9th and 91st percentiles. Box ends 25th and 75th percentiles. Median is marked with a horizontal line. *p*-values calculated using Mann-Whitney where *p* < 0.0001 is indicated by \*\*\* between wild type and quadruple adhesin mutant (*∆clfA, ∆fnbAB,* clfB::Tn).

**Figure 2. Loss of *clfA* expression results in loss of dynamic synovial fluid aggregate phenotype.** After 1-hour of dynamic incubation in 10% bovine synovial fluid (BSF) on an orbital shaker (65 RPM), bacteria were imaged macroscopically. As previously reported, the wild type *S. aureus* formed large, free-floating aggregate (**2a**) while a quadruple adhesin mutant (*∆clfA, ∆fnbAB, clfB*::Tn) was unable to do so (**2b**). To further narrow required adhesins, we next screened double mutants of the same background strain. The double fibronectin-binding protein mutant (*∆fnbAB*) was still capable of forming the dynamic aggregate phenotype (**2c**), however, the double clumping factor mutant (*∆clfA clfB*::Tn) was phenotypically comparable to the quadruple mutant strain (**2d**). We next assessed the ability of single clumping factor mutants to aggregate. While clumping factor B was not required for the bacteria to aggregate in 10% BSF (**2e**), loss of clumping factor A (*∆clfA)* abolished the free-floating aggregate phenotype (**2f**). Lastly, complementation of clumping factor A back into the double clumping factor mutant strain (*∆clfA, clfB*::Tn, pJM01clfA) recovered the dynamically formed synovial fluid aggregate phenotype (**2g**).

**Figure 3. Clumping factor A is not essential under static conditions.** Following 1-hour of static incubation in 10% BSF, wild type *S. aureus* (**3a**) a double clumping factor mutant a single clumping factor A mutant strain (**3b**), a single clumping factor B mutant (**3c**), a single clumping factor A mutant strain (**3d**) and a double clumping factor mutant with a clumping factor A complementation (**3e)** were imaged by confocal microscopy. FIJI image analysis software was used to quantify the average aggregate size (**3f**). Statistical significance was determined by one way ANOVA followed by Tukey’s multiple comparison post-test. Representative Z Stacks of the wild type *S. aureus* were collected following 1-hour of static incubation in 10% BSF and reassembled using Imaris Software (**3g-3i**).

**Figure 4. Fluorescent** synthetic **synovial fluid reveals localization of fibrinogen and hyaluronic acid polymers within aggregates.** FM-464 red plasma membrane stain was used to visualize both the adhesin mutant and wild type *Staphylococcus aureus* strains upon incubation in a synthetic synovial fluid consisting of fluorescein hyaluronic acid, Alexa Fluor conjugated fibrinogen, and bovine serum albumin (BSA). Following 1-hour of dynamic incubation on an orbital shaker both strains were imaged macroscopically (**4a**) and under confocal microscopy (**4b**) to assess free-floating aggregate formation and relative fibrinogen association, respectively. Both strains were also imaged in the synthetic synovial fluid following 1-hour of static incubation (**4c**) to evaluate the necessity of the various polymers and surface adhesins for development of the branching aggregate phenotype. Super high-resolution microscopy was used to assess direct interaction between fibrinogen and wild type *S. aureus* (**4d)**. The distribution of fibrinogen coverage within the representative image was quantified using image analysis (**4e**).

**Figure 5. Depletion aggregates form in physiological concentrations of hyaluronic acid and bovine serum albumin.** To test whether high polymer concentrations can drive depletion aggregation, independent of fibrinogen bridging, wild type strain AH1263 was incubated in hyaluronic acid, bovine serum albumin (BSA), or a combination of both on an orbital shaker (100 RPM). The average particle size was determined at 1 and 3-hr time points following incubation in high concentrations of polymer (**5a**) and low polymer (**5b**). Imaging with high concentrations of individual polymers stimulated clumping (**S2**) while a combination of high concentrations of both polymers yielded the formation of depletion aggregates (**5c**). Finally, we assessed the localization of the polymers relative to the aggregates. Imaging with fluorescently labeled hyaluronic acid and BSA was carried out at 60x with 2x zoom (**5d**) and 6x zoom (**5e**).

**Figure 6. The macroscopic aggregate phenotype formed under shear confers protection from gentamicin challenge.** Wild type *S. aureus,* AH1263 (**6a**) and clumping factor A mutant, AH4037 (**6b**) and clumping factor A complement strain, AH5614 (**6c**), were assessed for aggregate conferred tolerance to applied antibiotics. Following 1-hour of aggregate formation under static or dynamic conditions in 10% bovine synovial fluid, the bacteria were challenged with gentamicin (50x MIC) for 1-hour. A trypsin treatment was applied to break up the aggregates and bacterial viability was determined by dilution plating for colony-forming unit (CFU) counts.

**Figure 7. Depletion aggregate formation confers moderate protection from antibiotic challenge.** Wild type *S. aureus,* AH1263, was incubated for 3-hrs in PBS, 3mg/mL hyaluronic acid, 19mg/mL bovine serum albumin, or a combination of both polymers. Following incubation on an orbital shaker, the bacteria was challenged with 50x the minimum inhibitory concentration of gentamicin. A trypsin treatment was applied to break up the aggregates and bacterial viability was determined by dilution plating for colony-forming unit (CFU) counts.

**Figure 8. Working model of synovial fluid aggregate formation and subsequent protection from antibiotic challenge.** Host and bacterial factors which inhibit (**-**) or enhance (**+**) aggregate formation in the postoperative joint environment (**8a**). The formation of aggregates over time following exposure to synovial fluid in the presence (**8b**) or absence of antibiotics (**8c**). The red cells indicate dead bacteria whilst the yellow cells represent live bacteria.

**Supplemental Figure 1.** Histogram representation of the binding forces shown in Figure 1 between S. *aureus* wild type, and *∆clfA, ∆fnbAB, clfB*::Tn (quadruple adhesin mutant) and (**S1a**) fibrinogen and (**S1b**) synthetic synovial fluid.

**Supplemental Figure 2. Quantification of macroscopic, dynamically formed aggregates.** Following 1-hour of aggregate formation, raw images were collected and imported into FIJI image analysis software **(S2a**). The images were converted to 8-bit and inverted. A threshold was applied, and any artifact eliminated. The average aggregate size (**S2b**), total number of aggregates present (**S2c**), total aggregate area (**S2d**) and largest aggregate size (**S2e**) was determined for the wild type *S. aureus* and each mutant.

**Supplemental Figure 3. Exponentially growing cells maintain the same dynamic aggregate phenotype as stationary cells.** To account for differences in surface adhesin expression between growth phases, we repeated macroscopic imaging following dynamic incubation in 10% bovine synovial fluid (BSF) of cells harvested during exponential phase growth. The same phenotypes were observed for the wild type and each adhesin mutant strain regardless of growth phase.

**Supplemental Figure 4**. Aggregation of Wild Type *Staphylococcus aureus* in Hyaluronic Acid (HA) and Bovine Serum Albumin (BSA).