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Bacterial Interactions with Immobilized Liquid Layers

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Bacterial interactions with surfaces are at the heart of many infection-related problems in healthcare. In this work, the interactions of clinically relevant bacteria with immobilized liquid (IL) layers on oil-infused polymers are investigated. Although oil-infused polymers reduce bacterial adhesion in all cases, complex interactions of the bacteria and liquid layer under orbital flow conditions are uncovered. The number of adherent *Escherichia coli* cells over multiple removal cycles increases in flow compared to static growth conditions, likely due to a disruption of the liquid layer continuity. Surprisingly, however, biofilm formation appears to remain low regardless of growth conditions. No incorporation of the bacteria into the layer is observed. Bacterial type is also found to affect the number of adherent cells, with more *E. coli* remaining attached under dynamic orbital flow than *Staphylococcus aureus*, *Pseudomonas aeruginosa* under identical conditions. Tests with mutant *E. coli* lacking flagella confirm that flagella play an important role in adhesion to these surfaces. The results presented here shed new light on the interaction of bacteria with IL layers, highlighting the fundamental differences between oil-infused and traditional solid interfaces, as well as providing important information for their eventual translation into materials that reduce bacterial adhesion in medical applications.

1. Introduction

Infections contracted in clinical settings are often a consequence of bacterial adhesion and biofilm formation on implants, extracorporeal devices, and surgical tools. Treatment and prevention of such fouling requires the administration of drugs that contribute to the ever-increasing problem of antibiotic resistance,[1] and can also increase risk of patient mortality.[2] Slippery liquid-infused porous surfaces, inspired by the carnivorous *Nepenthes* pitcher plant, were recently introduced as a new approach to prevent bacterial biofouling in both industrial and medical applications without the need for administering antibiotics.[3–5] This approach represents a paradigm shift in materials science, where instead of a solid surface, bacteria are presented with an inherently dynamic and self-healing immobilized liquid (IL) layer that is held in place by a chemically matched substrate.[3,6,7]

Epstein et al. were the first to demonstrate that the presence of an IL layer of fluorinated oil on expanded polytetrafluoroethylene (ePTFE) significantly reduced biofilm coverage of *Staphylococcus aureus* and *Escherichia coli* (by 97.2% and 96%, respectively) after 48 h of growth under static conditions, compared to ePTFE without an IL layer.[4] Similarly, *Pseudomonas aeruginosa* biofilm coverage was reduced by 99.6% after 7 d of growth under gentle flow (10 mL min^{-1}).[4] Other works have reported similar results for bacterial biofilm formation on IL layers on oil-infused polymers: *S. aureus* under static conditions[8] and *P. aeruginosa* under flow.[9] Recently, Li et al. studied various strains of *P. aeruginosa* grown on porous liquid-infused surfaces under flow in a continuous reactor,

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finding that most resulted in less than 1% biofilm coverage.[10] However, they also discovered that the strain *P. aeruginosa* with the highest drug resistance also showed the highest surface coverage, 12% after 7 d of incubation, pointing to potential differences among different bacterial types.

Bacterial adhesion and biofilm formation on solid surfaces is a complex phenomenon.[11,12] Generally, bacterial adhesion can be classified as either reversible or irreversible, as judged by the ability to remove surface-associated bacteria with a gentle rinse.[11] Reversible attachment can be mediated by chemiosmotic forces and cellular surface proteins, while irreversible attachment is achieved through production of extracellular polysaccharides and ligand-specific protein interactions.[13] For flagellated species such as *E. coli* and *P. aeruginosa*, flagella can play an additional role in both reversible and irreversible attachment on solid surfaces.[14–16] In nearly all cases, however, bacteria that interact with a solid will either irreversibly attach to a surface and begin producing extracellular polymeric substances (EPS) to eventually form a biofilm, or they will leave the surface and return to the planktonic state.[17] The shear stress created by flow conditions has been shown to enhance bacterial cell attachment to surfaces for a number of different species in a variety of ways, including the activation of catch-bonds in *E. coli*[18] and increasing the surface residence time of *P. aeruginosa*.[19]

Little detail is currently known about how bacteria interact with IL layers on oil-infused substrates, especially under complex flow conditions. However, this information is necessary to enable the rational design and optimization of materials that take advantage of the unique properties of IL layers in medical applications. In this work, we investigate in detail bacterial interactions with IL layers, comparing adhesion under both static and complex flow (dynamic) conditions, differences across three clinically relevant species, and, for *E. coli*, the effect of flagella. We focus specifically on IL layers created using silicone oil-infused polydimethylsiloxane (PDMS), as this is an easily fabricated material made from nontoxic, widely available components already used in medicine. Furthermore, various types of this material have already proven useful as fouling-release coatings in marine settings.[20,21] However, in this work, infused PDMS was used specifically to serve as an anchor for an immobilized liquid overlayer.

Using iterative sonication–vortexing removal cycles to compare numbers of adherent cells, we show that while significantly fewer *E. coli* remain attached to the surface when grown under static conditions, this difference initially disappears in cultures grown under dynamic conditions, with surfaces showing similar number of adherent cells as solid controls. Interestingly, no equivalent increase in biofilm formation is observed. Visualization of the surfaces suggests that the increase in adherent cells is due to a disruption of the liquid layer. However, further removal cycles show that these cells are poorly attached and more easily removed from these surfaces than from controls. We further show that *E. coli* appears unique in its ability to adhere to IL layers under dynamic conditions, as tests on *S. aureus* and *P. aeruginosa* show decreased numbers of adherent cells under similar dynamic conditions. Further work with flagella-deficient *E. coli* mutants suggests that these differences may be partly due to flagella. The results provide important information about the factors affecting the interaction of bacteria with IL layers, and will help inform their future development and application as antiadhesion surfaces in healthcare settings.

2. Results and Discussion

2.1. Dynamic versus Static Growth Conditions

Figure 1 shows comparisons between PDMS and PDMS infused with silicone oil after exposure to a clinical isolate of *E. coli* for 48 h under static or dynamic conditions. Dynamic conditions were created by placing the samples on an orbital shaker at 100 rpm. In Figure 1A, representative images of the surfaces with crystal violet (CV)-stained biofilms are shown. Although both noninfused surfaces show biofilm, the dyed biomass on the sample grown under static conditions is darker and more pronounced than that of the sample grown under dynamic conditions. This is likely due to the fact that more bacteria can settle on the surface in the absence of any flow to remove them.

The infused PDMS surfaces exposed to both static and dynamic conditions appeared cleaner than their respective noninfused controls. However, there does appear to be a small amount of biofilm present on the infused surface exposed to dynamic conditions, while the infused surface exposed to static conditions appears completely clean, as previously reported.[4,8,9] The biofilm coverage results are quantified in Figure 1B, which confirms the somewhat greater amount of biofilm coverage for noninfused samples exposed to static conditions compared to noninfused samples exposed to dynamic conditions (96.4 (±4.7)% versus 51.0 (±24.2)%, respectively, $P = 0.046$). However, this difference is slight compared to the drastic difference in surface coverage between infused and noninfused samples: coverage drops to 0.3 (±0.1)% for infused samples under static conditions and to 4.5 (±2.6)% for infused samples under dynamic conditions ($P < 0.05$).

Figure 1C shows the colony-forming unit (CFU) counts for all of the samples after dipping to remove planktonic bacteria, followed by one vortex (30 s) and sonication (3 min) cycle to remove adherent bacteria. The results are normalized such that the average of the noninfused PDMS samples is equal to 1, to facilitate a more accurate comparison between the noninfused and infused samples. Although the infused samples exposed to static conditions show a decrease of approximately two orders of magnitude under this treatment, the samples grown under dynamic conditions show no decrease in adherent bacteria with infusion. While the presence of a small amount of biofilm coverage on the infused dynamic samples may explain the presence of a small number of adherent bacteria, it is surprising to see the number of adherent bacteria on these samples is similar to noninfused control samples, where the percent biofilm coverage is ten times larger. These results suggest that either (1) the *E. coli* are adhering to the IL layer under dynamic conditions, but not producing EPS as they are on the controls, (2) the EPS that is produced is removed while the producing bacteria remain behind, or (3) the bacteria and EPS are becoming mixed into the oil layer.

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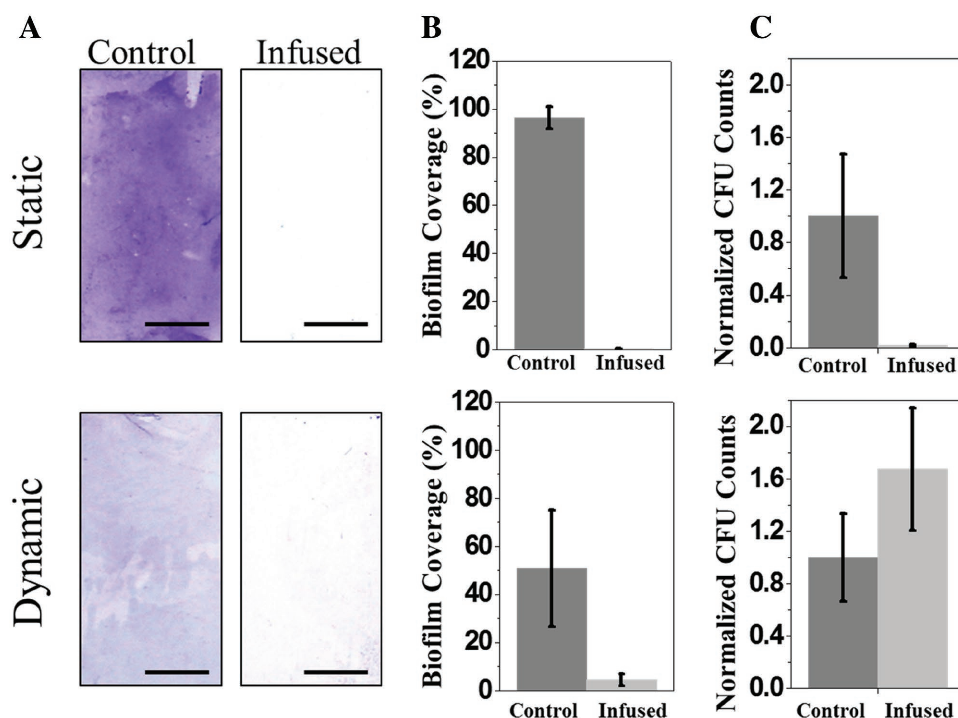

Figure 1. *E. coli* biofilms grown under static (upper row) and continuous orbital shaking (lower row) conditions: A) Representative images of the surfaces with the biofilm stained purple. Scale bars are 0.5 cm. B) Percent biofilm coverage from image analysis. C) Colony-forming unit counts.

To further investigate the lack of difference between CFUs measured on infused versus noninfused samples exposed to dynamic conditions, we visualized the samples using confocal microscopy and a triple-reporter system. In this system, the PDMS fluoresced green, the silicone oil overlayer orange, and the *E. coli* red. The results are shown in **Figure 2**. In these images, the red bacteria appear elongated due to the weak flow of the lubricant and aqueous phase during imaging that typically takes several tens of seconds (z-stack). These flows originate from noncomplete equilibration of the samples before imaging. The apparent motion of the bacteria is thus not due to their intrinsic swimming, but rather due to their motion with the flow of the surrounding media, and thus all bacteria seem to move uniformly in the images. The samples that were exposed to static growth conditions showed a layer of silicone oil covering the PDMS substrate, regardless of where on the sample the image was taken (Figure 2A and Figure S1 (Supporting Information)). However, the layer was not necessarily uniform in thickness, ranging from ≈15 to 35 µm. The samples kept under dynamic conditions showed even more variable thicknesses, however, with localized dewetting causing the oil to pool (Figure 2A and Figure S1 (Supporting Information)). This pooling was only observed when the samples were placed under aqueous medium. In air, the oil layers self-healed as expected. Nevertheless, such pooling is in agreement with the increase in biofilm coverage observed for infused samples exposed to dynamic conditions (vs static conditions) shown in Figures 1 and 2, as bacteria may be able to adhere more strongly to the areas where the IL layer is depleted.

Overall, the visualization results suggest that while static conditions yield a more consistent oil overlayer than dynamic conditions, the system is still sensitive to various conditions and may not be perfectly uniform. The difference in the overlayer observed for the samples kept under dynamic conditions may have been due in part to the presence of turbulent flow where the fluid came in contact with the edges of the square samples, which would have been more pronounced near the outer edges of the dish due to the higher velocity of fluid flow at those points. Previous work visualizing the stability of immobilized fluorinated liquid layers under laminar flow in water showed that they remain intact under these conditions, and did not appear to be completely stripped away even at shear stresses of up to 2.32 kg m^{-1} s^{-2}.[22] However, the fact that the layers examined in that work were not exposed to turbulent flow conditions and were only investigated in contact with pure water, not complex medium, may explain the differences. The use of silicone oil in the work presented here, as opposed to the more inert fluorinated oils used in other studies, may have also contributed. Recent results by other researchers using silicone oil layers has suggested that a disruption of the layer at edges and corners can result in increased bacterial presence in these areas.[23]

Visualization of bacterial attachment on samples exposed to dynamic conditions showed individual bacterial cells to be present only at the surface of the oil layer; no bacteria were observed to be incorporated into the oil (Figure 2B, side view and Figure S2 (Supporting Information)). However, more bacteria were present on the dynamic samples compared to the static samples (Figure 2B, top view), in agreement with the increase in CV absorbance observed in Figure 1B. These results support the hypothesis that *E. coli* are adhering more strongly to the infused surface under dynamic conditions, rather than becoming incorporated into the oil layer. However, whether

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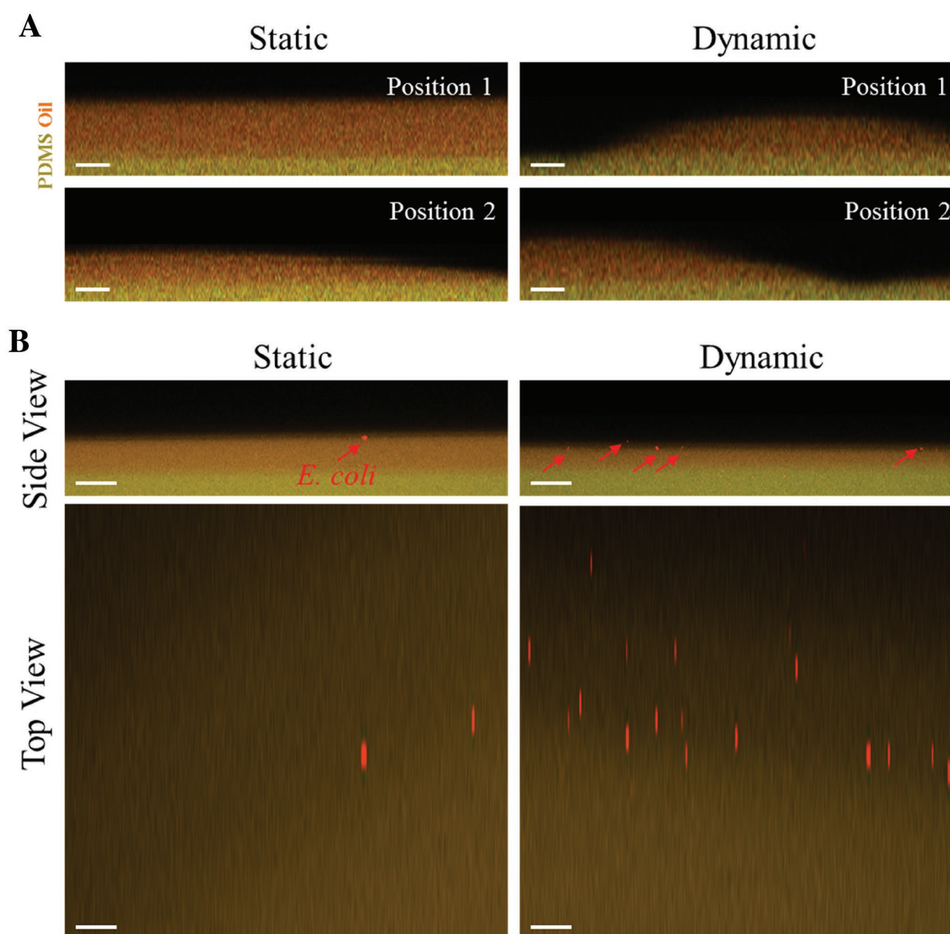

Figure 2. Confocal images of the liquid overlayer. A) Greater differences in thickness appear within a sample after 48 h incubation with bacteria under dynamic conditions compared to static conditions. Scale bars are 5 µm. B) Top and side views of an infused surface after exposure to *E. coli* for 48 h under either dynamic or static conditions. More bacteria (red) appear on the dynamic sample versus the static sample, however the bacteria are always localized on the surface of the oil layer rather than incorporated into it. Scale bars are 20 µm.

or not they are producing EPS that is then separated from the surface, or whether their EPS production is inhibited, are questions that should be the subject of future investigations, and will likely require in-depth analysis of the activation of relevant gene pathways, such as Cpx.[24] Nevertheless, it is known that the restriction of flagellar motion by a solid surface can serve as a signal for some bacteria to form a biofilm.[25,26] One explanation, therefore, may be that the presence of a liquid layer, even a partial one, continues to allow flagellar motion to a limited degree and does not trigger the full cascade of biofilm-forming signals within the *E. coli* cells.

2.2. Bacterial Adhesion

To give a more complete picture of the attachment of *E. coli* to the infused PDMS surfaces relative to noninfused controls, we tested their relative adhesion strength through multiple vortexing and sonication cycles followed by CFU counts. The combination of sonication and vortexing was designed to remove the majority of bacteria in one cycle,[27] implying that the remaining bacteria would be firmly attached to the surface. Confocal microscopy of the surfaces after the initial gentle rinse to remove planktonic organisms and again after the first removal cycle showed that the IL layer was completely removed by this vortex–sonication process, at least to the level visible by microscopy (Figure S3, Supporting Information).

The absolute CFU counts for both infused and noninfused PDMS samples under dynamic and static conditions are shown in **Figure 3**A, while the results normalized with respect to noninfused controls are given in Figure 3B. The normalized results were found to fit well to a modified stretched exponential function. The stretched exponential function was originally developed to describe relaxation processes in complex systems such as the adsorption and desorption of polymers on a surface,[28] but has also recently been used to describe the desorption of bacteria from a surface under flow.[19] This equation relates material or cells being adsorbed or desorbed from a surface to time. In our case, the modified function is given by

$$\mathrm{CFU}_{\mathrm{norm}} = (1-b) * e^{-n^{x}} \tag{1}$$

where b represents the change in CFU counts compared to the noninfused control upon the first removal cycle (with a more

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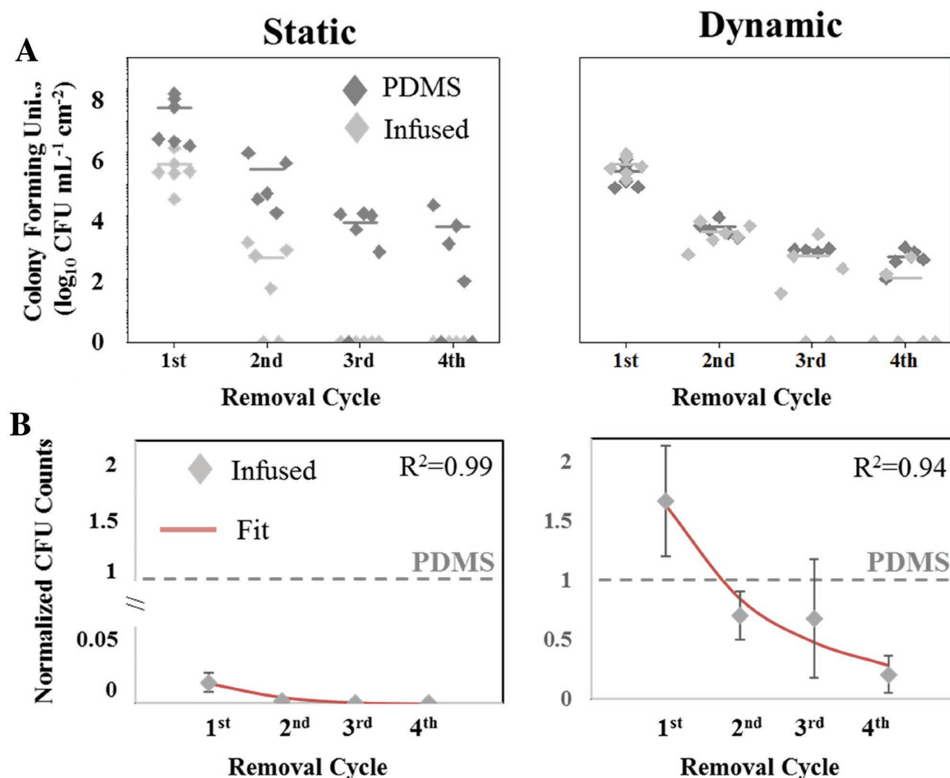

Figure 3. CFU counts of bacteria removed from PDMS and infused PDMS via vortex–sonication cycles (removal cycles) after 48 h of growth under either static or dynamic conditions. A) Absolute CFU counts. B) CFU counts from infused PDMS normalized to noninfused PDMS and fit with a modified stretched exponential function. The dashed line gives the values for noninfused PDMS at each removal cycle.

positive value indicating a larger initial decrease), n the cycle number, and x is a shape parameter which represents the severity of the drop in CFU counts in subsequent cycles (with a larger value indicating easier subsequent removal). Fitting with this function was intended to further parameterize the normalized removal curves and give a more quantitative basis for comparison. All resulting values are given in Table S1 (Supporting Information).

For samples grown under static conditions (Figure 3, static), the results reveal a decrease of approximately two orders of magnitude upon the first vortex–sonication cycle ($b = 0.96$, corresponding to a 98% decrease in removed CFUs), and a drop to zero CFUs upon the third removal cycle. These results support the hypothesis that when grown under static conditions, the few bacteria that remain on a liquid-infused surface after removal from the culture medium are poorly attached and can be relatively easily stripped off compared to noninfused controls.[4,8,9]

In contrast, bacteria attached to the infused surfaces under dynamic conditions (Figure 3, dynamic) appeared to show greater persistence with multiple cycles of vortexing and sonication. Unlike samples grown under static conditions, there was no decrease in CFUs removed from these samples compared to their noninfused controls upon the first removal cycle ($b = -3.47$, $P = 0.203$). However, there was a significant difference at the fourth and final removal stage ($x = 0.73$, $P = 0.017$), suggesting that *E. coli* that were adherent on the infused surfaces were still ultimately more easily stripped off compared to those attached to noninfused control surfaces.

Softening of the PDMS substrate by silicone oil swelling may be one possible explanation for the easier removal of bacteria incubated even under dynamic conditions. However, a recent study of *E. coli* attachment on PDMS substrates of varying stiffness showed that *E. coli* appeared to adhere more strongly to softer surfaces rather than harder surfaces.[29] Therefore, it is more likely that the reduced adhesion strength observed for *E. coli* is due to the presence of the infusing silicone oil and its transport to the interface rather than the softening of the PDMS matrix as a result of swelling.

2.3. Species-Specific Assays

As previously mentioned, different bacterial strains use different methods to attach and anchor themselves to surfaces. To test for differences in bacterial adhesion to liquid-infused surfaces, samples were exposed to the gram-positive coccal bacterium *S. aureus* and the gram-negative rod *P. aeruginosa* under dynamic conditions. One of the strengths of liquid-infused surfaces is the fact that this primarily physical approach to resisting bacterial attachment has been previously shown to be fairly consistent across a range of species.[4] However, most reports of bacterial attachment to liquid interfaces have tended to focus on a single species.[8–10] Furthermore, of these, only *P. aeruginosa* has been thoroughly tested under continuous flow conditions.[4,9,10]

The biofilm coverage results and normalized removal curves for *S. aureus* and *P. aeruginosa* grown under dynamic

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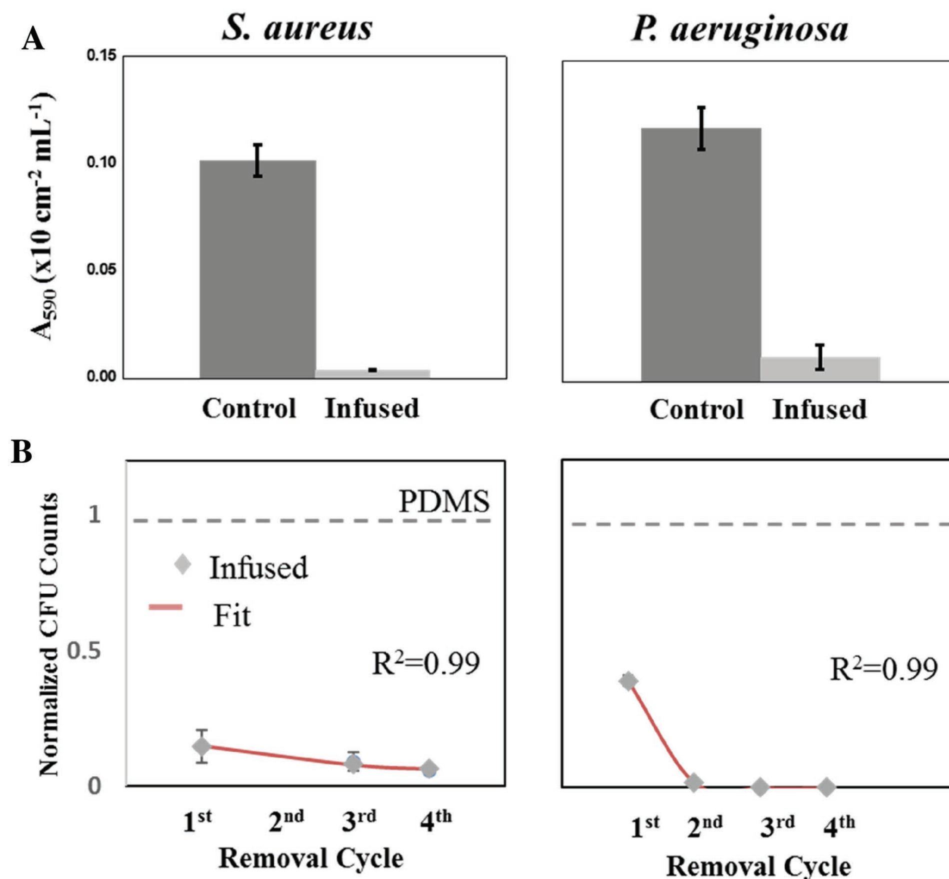

Figure 4. Comparison of *S. aureus* and *P. aeruginosa* attachment on PDMS and infused PDMS after 48 h under dynamic conditions. A) Biofilm stain absorbance. B) Counts normalized to the average noninfused PDMS values.

conditions are shown in **Figure 4**. Figure 4A shows the overall CV-stained biofilm coverage after only a dip in water to remove planktonic bacteria. *S. aureus* and *P. aeruginosa* are known to form thick, persistent biofilms, and the coverage results show a clearly thicker biofilm present on noninfused samples exposed to these two organisms compared to the *E. coli* biofilms in Figure 1. However, for both species, biofilm formation is significantly lower on the infused samples, in agreement with what has previously been shown both on infused polymers[8,9] and other types of IL interfaces.[4,10]

The normalized removal curves shown in Figure 4 exhibit drastically different results for both *S. aureus* and *P. aeruginosa* grown under dynamic conditions on infused samples compared to what was observed for *E. coli* subjected to the same treatment. For *S. aureus*, the first removal cycle was found to remove nearly all the adherent bacteria (b = 0.60), in a manner more similar to *E. coli* grown under static conditions. However, subsequent removal cycles revealed that the few bacteria that remained behind appeared to be even less likely to be removed than the *E. coli* (x = 0.44), with bacteria still being removed from the surface after the fourth cycle. Tests on *P. aeruginosa*, in contrast, revealed an intermediate decrease in CFUs removed during the first cycle (b = −0.06), but much less persistent attachment in the following cycles (x = 2.00), and a drop to zero CFU by the third cycle.

The removal curves for *S. aureus* and *P. aeruginosa* shown in **Figure 5**B reveal an interesting difference in relative adhesion to infused surface under dynamic conditions, especially in contrast to *E. coli*. The presence or absence of flagella may partially explain the observed results, as they were shown in previous experiments on textured superhydrophobic surfaces to play a definitive role in adhesion.[30] *E. coli* have multiple long flagella per cell; in contrast, *P. aeruginosa* have a single flagellum per cell, while *S. aureus* have none. The decreasing normalized CFU values for the first removal cycle reflect this (0.85, 0.39, and 0.15 for *E. coli*, *P. aeruginosa*, and *S. aureus*, respectively). These results may suggest that, to some extent, the relative strength of initial adhesion of a particular bacterial species to infused surfaces under dynamic conditions may be predicted by the number of flagella.

It is also clear from the subsequent removal cycles for all three species that other factors in addition to flagella are likely contributing to the interaction with the infused polymer surface and/or liquid overlayer. These may include cell shape and surface charge,[31] cell membrane composition,[32] and/or cell motility.[33] Now that the methods presented here have been shown effective at revealing the differences between different species, further tests should include a systematic investigation of these factors as well.

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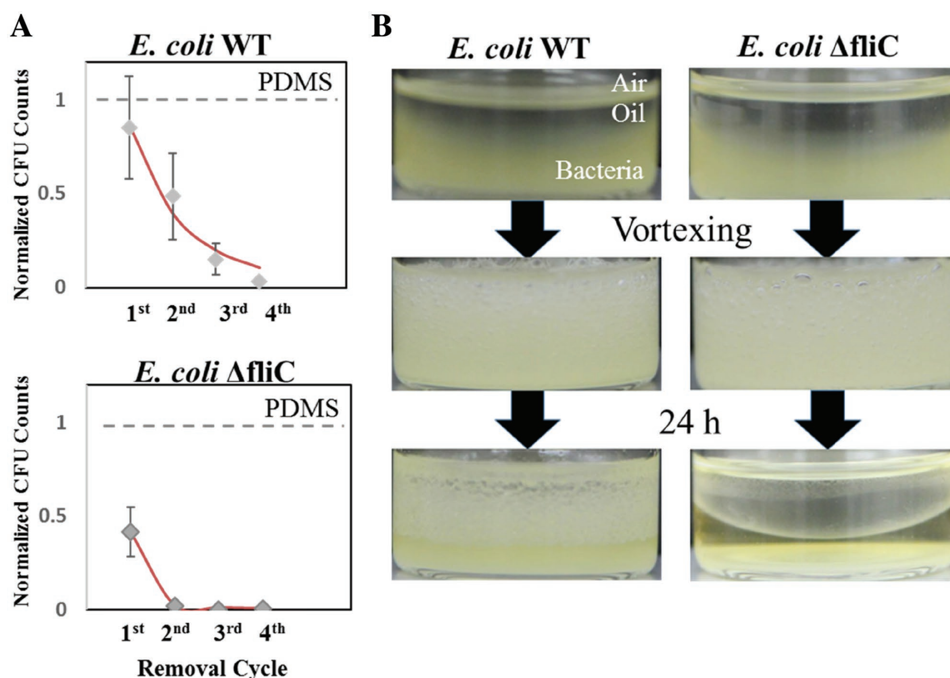

Figure 5. Adhesion of *E. coli* Wild Type (WT, with flagella) versus the ΔfliC mutant (without flagella) on noninfused and infused PDMS. A) CFU counts normalized to control PDMS values and B) a mixing assay showing the stability of an emulsion of the infusing liquid (silicone oil) with a bacterial solution.

2.4. Effect of Flagella

To further investigate the question of the relative importance of flagella in adhesion to the IL layers on our oil-infused samples, we tested *E. coli* with the flagellin subunit gene deleted (ΔfliC mutant),[15] as well as the wild type (WT) K12 strain from which it had been derived as a control. Previous work has revealed a critical role for flagella in the adhesion of *E. coli* to surfaces.[14,15]

The normalized and fitted CFU count results for *E. coli* with and without flagella are shown in Figure 4A. The results for the WT control show a pattern similar to that of the clinical isolate previously tested, with no significant decrease in removed CFUs upon the first removal cycle ($b = -1.37$, $P = 0.006$) and a similar degree of removal for the subsequent cycles ($x = 0.99$), becoming significantly different from the control upon the third removal cycle ($P = 0.022$). The degree of similarity observed in the removal curves between this WT K12 strain and the clinical isolate tested in Figure 3 further validate both the method and the fit as an approach to accurately quantify the relative degree of bacterial adhesion on infused PDMS surfaces.

The removal curve for the bacteria without flagella (*E. coli* ΔfliC) grown on IL layers under dynamic conditions shows a clear difference from the WT control (Figure 5A). Although the amount of bacteria removed from the surface in the first removal cycle was not as great as that of the samples grown under static conditions ($b = -0.12$ compared to $b = 0.96$, respectively), the degree of removal in subsequent cycles was much higher ($x = 3.78$ for *E. coli* ΔfliC compared to $x = 0.74$ for *E. coli* grown in static conditions). These results suggest that for the *E. coli* strain tested here, the flagella play an important role in adhesion to the oil-infused surfaces under dynamic conditions.

To further test the effects of both the presence and absence of flagella on interaction with the silicone oil present in our infused materials, we performed a mixing assay[15] in which a volume of silicone oil was vortexed with an equal volume of medium containing bacteria either with or without flagella (Figure 5B). This experiment was intended to confirm that the absence of flagella was indeed changing the way that the bacteria interacted with the oil itself. It was found that while both types of bacteria would initially produce an emulsion, the sample containing bacteria without flagella would separate after 24 h while the sample containing bacteria with flagella would remain as a stable emulsion. These results support what was observed in the removal curves – that *E. coli* without flagella are less likely to have stable interactions with the silicone oil.

One explanation for the observation that flagella contribute to adhesion to infused surfaces, but do not fully account for it may be the composition of the *E. coli* cellular membrane. The membrane is known to contain proteins with hydrophobic domains that could provide stabilization when in contact with the oil layer.[34] However, stabilization with the membrane alone does not appear to be as effective as stabilization with flagella, as flagella not only contain hydrophobic domains and can act as a surfactant, but also have the surface area and length to potentially penetrate down into the oil layer or spread out along the surface to improve contact. The fact that greater adhesion occurs most strikingly for samples grown under dynamic conditions may also tie in to the observation that movement of the medium appears to disrupt the oil overlayer. If the layer is deformable, there would be more opportunity for the flagella to interact with it and become stabilized. Further work should focus on the localization of the *E. coli* flagella with respect to the oil overlayer.

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3. Conclusion

The results presented here show that the interaction of bacteria with IL layers is a complex phenomenon involving the integrity of the oil layer itself and the morphology of the interacting cell. Importantly, in all cases, we show that bacterial attachment and biofilm formation on oil-infused samples were significantly reduced compared to noninfused controls, and that the removal of the attached cells was easier and required a smaller number of strip-off cycles. Within the set of oil-infused samples, we find that growing *E. coli* under dynamic orbital flow conditions initially results in the adhesion of a greater number of cells compared to growing *E. coli* under static conditions. Interestingly, this increase of attached cells does not correlate with an equal increase in biofilm formation. We further show that the number of adherent cells of two more clinically relevant species, *S. aureus* and *P. aeruginosa*, is much lower than that of *E. coli* grown under similar dynamic conditions, suggesting a species-dependence to the ability of IL layers to resist bacterial attachment. Finally, we demonstrate that these differences may be due in part to the presence of flagella, as flagella-deficient *E. coli* mutants adhered significantly less well under dynamic conditions than their WT counterparts.

Overall, our work shows that IL layers of silicone oil on infused PDMS are capable of reducing bacterial adhesion under dynamic conditions, even though the introduction of dynamic conditions affects the state of the IL layer. These results also support previous evidence that adhesion to IL layers can be species-, and even strain-dependent,[10] illuminating the need for further research into the interaction of IL layers and cell surface structures, such as adhesins, flagella, and fimbriae. An increased understanding of these interactions will allow for the modification of existing IL systems to better resist the adhesion of a broader range of microorganisms.

4. Experimental Section

Bacterial Stock Preparation: The WT bacterial strains cultured were *E. coli* strain J96, *E. coli* strain K12 W3110, *P. aeruginosa*, and *S. aureus*. The genetically altered cultures used were *E. coli* strain MC4100 mKate2::KanR and *E. coli* strain K12 W3110 ΔfliC. All stocks were generated from a stock agar plate, from which three to five colonies were transferred into growth media. All cultures were incubated overnight at 37 °C in the growth media, lysogeny broth (LB) - Miller (Becton, Dickinson and Company, Franklin Lakes, NJ), and kept shaking continuously in an orbital shaker. The mutant culture *E. coli* mKate2 was selected with 50 µg mL^{-1} of kanamycin sulfate (Life Technologies Corporation, Chicago, IL).

PDMS and Infused PDMS Preparation: Fluorescent PDMS stock was prepared by dissolving 850 µg of BODIPY FL alkyne laser dye (Exciton, Dayton, OH) into 10 mL of dichloromethane (Sigma-Aldrich, St. Louis, MO), then mixing with 100 g of Sylgard 184 silicone elastomer base (Dow Corning Corporation, Midland, MI). After mixing and degassing under vacuum overnight, the stock of fluorescent PDMS base was combined with its respective Sylgard curing agent in a 10:1 ratio and homogenized in a Thinky planetary centrifugal mixer (Thinky Corporation, Tokyo, Japan) at 2000 rpm for 1 min, then again at 2200 rpm for 1 min. Aliquots of 0.9 mL were then transferred into the square depressions (2.0 cm × 2.0 cm × 0.5 cm) of a mold master. The master was kept under vacuum for 2 h to extract air trapped in the PDMS, then at 70 °C overnight to facilitate PDMS curing. The cured PDMS squares were removed from the mold, cut in half, and sterilized in a glass dish via autoclave without a drying cycle. Infused PDMS samples were created by aseptically immersing the autoclaved PDMS coupons in 10 cSt silicone oil (Gelest, Morrisville, PA), previously saturated overnight with BODIPY PM597-8C9 laser dye (Lumiprobe, Hallandale Beach, FL) and filtered through a 0.22 µm nylon syringe filter (Sigma-Aldrich, St. Louis, MO).

Biofilm Growth: Difco tryptic soy broth (Becton, Dickinson and Company, Franklin Lakes, NJ), made with distilled, deionized water at 30 mg mL^{-1} and containing an additional 1.5% (w/v) NaCl (Sigma-Aldrich, St. Louis, MO), was used to induce *E. coli* and *S. aureus* biofilms. 25 mg mL^{-1} LB Miller Broth + 0.2% sodium citrate (w/v) (Sigma-Aldrich, St. Louis, MO) made with distilled, deionized water was used to induce *P. aeruginosa* biofilms. Noninfused and infused PDMS samples were placed into 6-well Falcon plates (Fisher Scientific, Pittsburgh, PA), and each well was filled with 7 mL of biofilm inducing media and 70 µL of bacterial culture (1:100 inoculum to media). Kanamycin was added at 50 µg mL^{-1} for selection of *E. coli* mKate2 prior to inoculating media. Triplicates were kept for both infused samples and their noninfused controls. Immediately following inoculation, all samples were incubated at 37 °C for 48 h to allow for proper biofilm formation. To simulate dynamic conditions during this period, one set of samples was kept shaking continuously at 100 rpm via placement on an orbital shaker inside the incubator. This speed value was chosen as it was the highest that could be achieved without spilling the culture medium out of the containers.

Crystal Violet Staining and Analysis: Biofilm coverage was analyzed by staining samples via a 10 min incubation in a 0.1% (w/v) solution of CV in deionized, distilled water. This was followed by gentle rinsing in deionized, distilled water to remove excess stain, air-drying, and photography with a Canon EOS Rebel T4i camera (Melville, NY). After image capture, the samples were placed into 15 mL Falcon tubes (Fisher Scientific, Pittsburgh, PA) and CV was dissolved away in 5–7 mL of 70% ethanol (v/v) in deionized, distilled water. 200 µL of the resultant solution was transferred in triplicate into a 96-well Falcon plate (Fisher Scientific, Pittsburgh, PA). Endpoint absorbance at 590 nm was measured using a Spectramax i3 – UV–vis spectrometer (Molecular Devices, Sunnyvale, CA). Absorbance was normalized to the product of the volume of ethanol and surface area exposed to biofilm formation.

Image analysis was performed as following using MATLAB (Mathworks, MA, USA). Photographs taken of the CV-stained samples were cropped to the region of interest (i.e., to substrate edges) and converted from Red-Green-Blue color model to hue, saturation, value. Cropped images (n = 3) were binarized based on an interval defined in hue/saturation/value and the biofilm coverage was defined as percentage of the binarized biofilm over the whole image area.

Adhesion Assay and Relative Adhesion Analysis: PDMS/infused PDMS samples were removed from culture aseptically and immediately dipped into a 15 mL Falcon tube (Fisher Scientific, Pittsburgh, PA) containing 10 mL of sterile 1× phosphate buffer solution (PBS) to remove planktonic bacteria. There was no clear difference in CFU counts of these planktonic bacteria between static and dynamic growth conditions, although the results were highly variable ($1.7 \times 10^7 \pm 3.7 \times 10^7$ cells mL^{-1} cm^{-2} for samples grown under static conditions and $3.1 \times 10^6 \pm 2.2 \times 10^6$ cells mL^{-1} cm^{-2} for samples under dynamic conditions). Each sample was then transferred to another 15 mL Falcon tube containing 10 mL of sterile 1× PBS and subsequently vortexed for 90 s at the highest setting (level 10) on a VWR Analog Vortexer (VWR, Radnor, PA) and sonicated for 180 s in a Branson B8510 Ultrasonic cleaner (Branson Ultrasonics Corp., Danbury, CT). One round of vortexing and sonication for these durations was considered one removal cycle, thought to reliably remove bacteria adherent to the surface.[27] This was repeated with all samples for a total of four cycles. Removal supernatant was serially diluted from 10^0 to 10^{-6} in 1.7 mL minicentrifuge tubes (VWR, Radnor, PA). 10 µL from each dilution was plated onto a LB 1.5% agar plate. Plates were incubated at 37 °C for 12 h. Colonies were counted and CFU count in CFU mL^{-1} cm^{-2} was calculated. When plotting normalized CFU data,

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each removal fraction mean count was normalized to the noninfused sample mean count and plotted as $\frac{CFU_i}{CFU_c} \pm \text{St. E.}$ where CFU_i represents the CFU count of the infused sample, CFU_c the CFU count of the corresponding control sample, and St. E the standard error.

Confocal Imaging: Aseptically removed PDMS samples of dimensions 2 cm × 1 cm × 0.5 cm were dipped once into 1× PBS to remove planktonic organisms. Samples were moved into 60 mm × 15 mm circular petri dishes (VWR, Radnor, PA), then 1× PBS was pipetted into each dish until the sample was submerged. Confocal images were captured using a 40× water dipping objective lens on an Upright Zeiss LSM 710 (Zeiss, Thornwood, NY).

Statistics: One-way analysis of variance (ANOVA) tests were used to test the hypotheses for image analysis and biofilm staining. Comparison of CFU counts were performed using square root-transformed data in a univariate ANOVA to compare between noninfused and infused counts at a given removal stage, with $P < 0.05$ considered significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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