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The Quantitative Assessment of *Pseudomonas Aeruginosa* (PA)14 Biofilm Surface Coverage on Slippery Liquid Infused Polymer Surfaces (SLIPS)

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Abstract

Slippery, porous polymeric antimicrobial surfaces for biofilm attachment inhibition of the clinical strain *Pseudomonas aeruginosa* (PA14) have been prepared. Porous BMA-EDMA, characterized for its hydrophobic properties, was infused with a slippery liquid creating a hydrophobic liquid interface and characterized by water contact angle and SEM. A low shear force bioreactor was used to prepare biofilms on these antimicrobial surfaces. Biofilm attachment was studied using fluorescence microscopy coupled with image analysis in ImageJ. While the literature presents that these slippery polymers work well as antimicrobial surfaces for several strains of *Pseudomonas aeruginosa*, it has been found to be strain dependent. This report demonstrates that slippery surfaces do not work well for the strain PA14, and biofilm covered >3.5 times more area as compared to the control glass surfaces.

Keywords: Biofilms, *Pseudomonas Aeruginosa* (PA14), Slippery Liquid-Infused Porous Surfaces (SLIPS), Antifouling

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Introduction

Biofilms are communities of microorganisms organized by extracellular polymeric substance matrices adhered to a surface, which are typically found at the interface between a liquid and a solid surface.[1] When biofilms are grown in the presence of media, biofilm adhesion is influenced by a number of factors, including surface roughness and hydrophobicity, media flow rate and composition, and even the bacterial cell surface.[1–4] Biofilms have been found to adhere to many different types of surfaces, including medical devices, such as in implants during surgery, which can cause severe medical complications.[2,5] They also infiltrate natural water systems, such as rivers and lakes, as well as water supply lines like water pipes introducing bacteria into drinking water which could lead to infections.[3,6,7] Therefore, there is a pressing need to design surface materials that have the ability to deter bacterial attachment. Early designs for these types of antibacterial surfaces either involved materials that leach biocides, or antibacterial metals such as silver.[8] However, there are concerns that these methods may induce more resistant bacteria and cause toxicity to other living organisms.[9,10] More recently, photocatalyic materials have been developed where semiconductors, such as zinc oxide and titanium dioxide absorb radiation energy forming superoxide and hydrogen peroxide that will kill bacterial cells.[9,11,12] Further, exploration in nano-topography changes has led to the development of surface modifications including polymer brushes[13] and nature-inspired nanopillars.[10]

While there are many possible lines of defense against biofilm development, prevention of initial adhesion has been a major focus in recent years, specifically with bio-surfactants and superhydrophobic surfaces.[9] Bio-surfactants are amphiphilic compounds produced by microorganisms as part of bacteria quorum-sensing, cell surface, and metabolic systems.[14] Their antimicrobial properties have been a significant topic of interest and several compounds have been identified and commercialized for the inhibition of biofilm growth.[15] Similarly, superhydrophobic surfaces - inspired by the lotus leaf - have been found to change the behavior of bacterial adhesion by the repellent nature of the surface.[16] Made up of nanostructures that reduce the surface contact area and attraction forces of the surface, these superhydrophobic surfaces cause “beading” or the “lotus effect” and are characterized to have a very large water contact

angle (≥150°).[17] Many methods have been developed to fabricate these surfaces, including the development of highly porous polymeric material such as poly(L-lactic acid) (PLLA).[17,18]

Continued inspiration from nature, the Nepenthes pitcher plant has led to the development of slippery liquid-infused porous surfaces (SLIPS) where a liquid interface forms a repellent surface. In these surfaces, the porous structure is used as the solid support but the pores can be infused with a liquid lubricant.[19] In this study, the macro sized porous polymer poly(butyl methacrylate-co-ethylene dimethyacrylate) (BMA-EDMA) is lubricated with perflouropolyether (PFPE) to form SLIPS as an antibacterial surface.

Pseudomonas aeruginosa (PA) is a Gram-negative bacterium with high thriving capabilities in a variety of environmental and nutrimental conditions.[20] PA is a common cause of community-acquired and hospital-acquired infections and found to be closely associated to injuries, such as severe burns, and immunocompromised hosts, such as in cystic fibrosis (CF)[5,20–22] AIDS, and cancer patients.[5,22] The formation of PA biofilms has been most seriously connected to CF, where lung infections are the leading cause of death for this disease.[5] In this work, the strain used is PA14 which is a human isolate and commonly used as a reference strain because it has been identified as a strain capable of infecting animals, plants, insects, and nematodes.[21]

Bioreactors are used in the study of biofilms to mimic real life conditions.[23] In this work, a drip flow reactor (DFR) was used to simulate PA growth in common infections such as catheters and lungs of CF patients. The biofilm growth conditions in DFR represent a low-shear environment where nutrients in media are flowing across cells that are attaching to a surface over time to form a biofilm with a high cell density.[24]

There is literature precedence of several different PA strains that were grown on the slippery BMA-EDMA surfaces using DFR. These reports demonstrated that many PA strains show a decrease of bacterial attachment, and thus an inhibition of biofilm accumulation. However, some bacterial strains show more attachment on superhydrophobic surfaces and an increase in biofilm mass, which may be due to the fact that different genotypes express different phenotypic attachment properties.[16,25] Therefore, we have studied the initial attachment and growth of the clinically relevant PA14 strain on the SLIPS surfaces in a drip flow reactor to determine whether this strain's biofilm formation can be inhibited on SLIPS.

Methods

PA14 growth

Bacteria were obtained from a frozen glycerol stock kept at -70 °C. The bacteria were scraped out of the vial using a sterile tip and streaked onto agar plate. The agar plate was placed in the incubator at 37 °C overnight for colony growth. An overnight culture was prepared in 20 mL of sterile minimal media (0.0478 M Na_2HPO_4, 0.022 M KH_2PO_4, 0.00856 M NaCl, 0.0374 M NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.2% Glucose) in an autoclaved 125 mL sidearm flask. A colony was picked from the streaked agar plate and the minimal media was inoculated. The culture was grown for 12-16 hours on a New Brunswick Scientific incubator shaker I2400 at 37 °C 180-220 rpm. The optical density was determined using a Spectronic 20D+ and the culture was grown until an OD of 0.2 was achieved.

The complete polymer modification procedures and characterization are addressed in the supplementary information (Figure S1 to Figure S4). Two BMA-EDMA-modified slides were obtained and immersed in 90% ethyl alcohol and laid flat under UV light for two hours. After which, the UV light was turned off and the slides were angled by 20° and infused with perflouropolyether (PFPE) Krytox oil and left for two hours to drain excess oil. Finally, the slides were laid flat and left under UV light overnight for a complete SLIPS modification. Unmodified glass slides used for biofilm growth comparison were flame sterilized using 90% ethyl alcohol.

Initial Attachment Measurements

PA14 with a genetic modification that allows for constitutive express of enhanced green fluorescent protein (EGFP) was used for initial bacterial attachment studies. The genetic modification of PA14/EGFP was performed at the Helmholtz Institute for Infectious Disease Research. Cultures were grown from glycerol stock cultures stored at -70 °C and maintained for three week periods on tryptic soy agar. Prior to measurements, liquid cultures were grown overnight in tryptic soy broth at 37 °C in an incubator with 180-200 rpm rotary shaking. The culture was transferred into M9 minimal media by centrifuging it at a low speed (1380xg) to pellet the bacteria and decanting and replacing the tryptic soy broth with M9 minimal media. Cultures were diluted in M9 minimal media to between 5×10^8 and 1.4×10^9 cells/mL.

Bacterial cultures were quantified initially using a Petroff-Hausser bacterial counting chamber and an Olympus BX60 fluorescence microscope. All cell counts were based on three images from different regions of the Petroff-Hausser counting grid. BMA-EDMA-modified glass slides and oil-infused BMA-EDMA-modified (SLIPS) glass slides were prepared and used for the initial attachment quantification. A drop (20.0 µL) of bacterial culture was placed on the slide surface and photographed from above and in profile. After 5.0 minutes, 2.00 µL of bacterial culture were removed from the drop without disturbing the slide surface using a micropipet. This culture was transferred to the Petroff-Hausser counting chamber. The number of attached cells was determined by the difference between the initial count and the count after the attachment period. Surface area of contact was determined by ImageJ analysis of the drop images. Briefly, the diameter of contact between the drop and the surface was determined using the images of the total drop diameter (top view) and the shape (profile image) of the drop. The area of contact between the drop and the surface was then calculated assuming a circular drop profile using area $=\pi r^2$. Three attachment trials were performed for each surface.

Drip Flow

Four slides (two unmodified glass slides and two SLIPS-modified slides) was placed in petri dishes and 25 mL of PA14 culture (OD 0.2) were added to each petri dish. The slides were incubated at room temperature for a six hour attachment and incubation period.

Autoclaved peristaltic tubing was connected on a sterile surface using MasterFlex silicone tubing and Thermo Scientific tubing connectors. A waste container was added at the bottom to collect all runoff waste from the reactor (Figure 1). For cleaning, 1:128 CiDecon solution was ran through the tubing. Afterwards, 70% ethyl alcohol solution was run through the tubing. Finally, sterile water was run through the tubing.

The DFR was placed in a UV hood for a ten minute sterilization period. After the six hour incubation period, the slides were transferred from the petri dishes to the DFR in a sterile hood. The needles were placed into the designated holes at the top of the reactor to each reactor chamber as shown in Figure 1. A 10 L carboy with 10 L of sterilized minimal media was connected to the tubing. The peristaltic pump was turned on and set to a flow rate that was calibrated to 0.8 mL per minute. The reactor ran for eighteen hours.

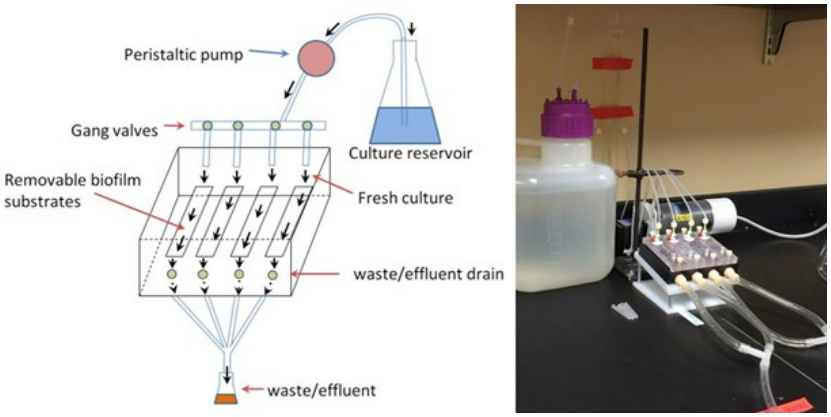

Figure 1: Schematic and laboratory set up of the drip flow reactor

Staining and Fixing

Detection of bacteria was accomplished with the DNA-specific stain 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI). Slides were removed from each reactor chamber, gently washed twice with fresh minimal media, and incubated for 25 minutes in a dark chamber with freshly diluted 10 µg/ml DAPI in sterile minimal media solution at room temperature. Bacteria were then fixed for 1 hour in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) and stored in a dark environment prior to imaging.

Fluorescence Microscopy

Biofilm growth was analyzed by epifluorescence microscopy on a Leica DMi8 inverted microscope at 400x magnification with a DAPI filter. Fluorescence images were captured using a cooled CCD camera (Leica DCF7000 T) in monochrome mode and LAS X software. Exposure times were <100 ms. At least 7 different fields were captured per sample.

Quantification of Biofilm Coverage

The area occupied by biofilm was quantified using ImageJ. A threshold was set for each image and a binary image was produced where all pixels with an intensity above the threshold were white, and those below it, black. The threshold value was adjusted to include visible bacteria and exclude background staining. The area covered by bacteria was calculated from the white pixels. Covered area and total area for all fields captured from a single sample were combined, with percent covered being defined as the combined covered area divided by the combined total area. A two-sided Student's t-test was used for statistical evaluation of the data sets.

Results and Discussion

Antimicrobial surfaces, SLIPS, were prepared by a previously described method with a few modifications and is described fully in the supplementary information (Figure S1).[25–27] Glass slides were modified with a porous polymer, BMA-EDMA, by two methods that controlled the height of the polymer surface. As previously described, Teflon tape was used to control the height when the polymer mixture was sandwiched between two silanized slides and polymerized by UV exposure.[26] However, the tape can be stretched producing varying height and doesn't allow for complete modification of the glass slide. A second method was developed using 15 µm silica beads, where height could be controlled to the diameter of the beads used and the polymer modification completely covered the entire slide. To complete the SLIPS modification, the polymer was infused with PFPE liquid forming a fluidic, hydrophobic interface. The modified slides were characterized with IR, SEM and water contact angle and confirmed a similar surface compared to previous reports (Figure S2-S3, Table S1).[25,27]

The principle behind the prevention of biofilm growth on these superhydrophobic surfaces is that bacterial attachment is inhibited by the very "slippery" liquid interface on top of the superhydrophobic polymer structure. To explore the inhibition of initial attachment of PA14 bacteria, an attachment assay was carried out by the quantification of unattached cells from a culture before and after a five minute contact period with a modified surface (Figure 2).

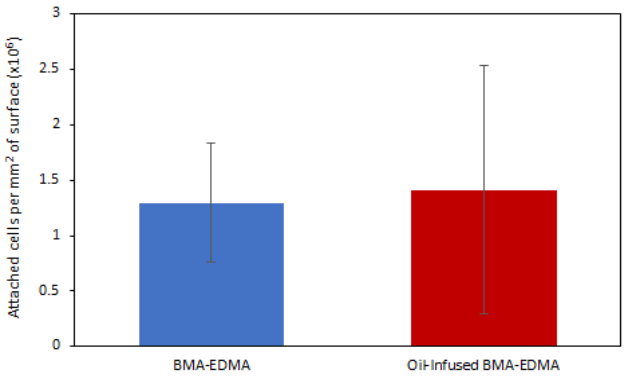

Figure 2: Average number of attached PA14 (EGFP) cells per mm² of the surface after 5 minutes for a 20 µL drop of culture (5 x 10^8 to 1.4 x 10^9 cells/mL) on BMA-EDMA and oil-infused BMA-EDMA (SLIPS) surfaces. Averages are based on three independent trials and error bars represent standard deviation.

Comparing BMA-EDMA-modified and oil-infused BMA-EDMA-modified surfaces (SLIPS), initial attachment of bacteria was found to be 1.3 (±0.5) and 1.4 (±1.1) million cells per mm^2, respectively, demonstrating no significant difference in initial attachment behavior (Figure 2). In a previous investigation of biofilm formation on SLIPS surfaces, BMA-EDMA-modified surfaces exhibited similar or greater biofilm growth compared to the growth on unmodified glass surfaces for multiple strains of PA. However, with the exception of PA49, SLIPS-modified surfaces had less biofilm growth.[25]

To further study the antimicrobial properties of SLIPS, biofilms of strain PA14 were grown on both SLIPS-modified and unmodified glass slides in the low-shear environment of a DFR. Slides were seeded using a log-phase culture, after which minimal media was allowed to flow down the slides at a rate of 0.8 mL min^{-1} for 18 hours (Figure 3). Afterwards, slides were removed from the reactor and gently rinsed with minimal media to remove the presence of lingering planktonic bacteria. The biofilm on each slide was stained with DAPI and chemically fixed.

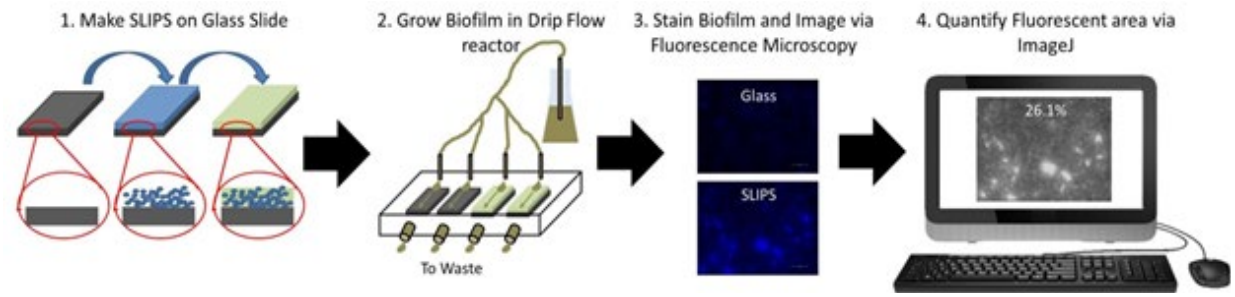

Figure 3: Schematic of the biofilm study performed. Glass slides were first modified to a slippery surface as a BMA-EDMA porous polymer followed by infusion with oil, perfluoropolyether [PFPE] (1). A biofilm of PA14 was grown in a DFR for 18 hr (2). For biofilm quantification, the slides were stained with DAPI and imaged by fluorescence microscopy (3). Using ImageJ, images were quantified to calculate the surface coverage of the biofilm (4).

Growth of PA14 biofilm was measured by detection of DAPI using fluorescence microscopy. However, biofilm growth in a DFR exhibits some heterogeneity due to minute fluctuations in how media is able to flow down the slide. Thus, in an effort to quantify the growth of the biofilm across the entire slide, multiple non-overlapping fields were systematically imaged along each slide. At least 7 fields were imaged per slide, accounting for >0.5mm^2 of the slide (an average of 19.4 fields were imaged per slide accounting for >1.4mm^2). It was necessary to apply a threshold for each image (Figure 4) to visualize biofilm growth and calculate the area of the slide that was covered by biofilm. For the accurate determination of biofilm coverage, all fields on a single slide were combined to get an average biofilm coverage across the slide. This procedure allowed for the calculation of percent biofilm coverage by taking the combined covered area divided by the combined total area.

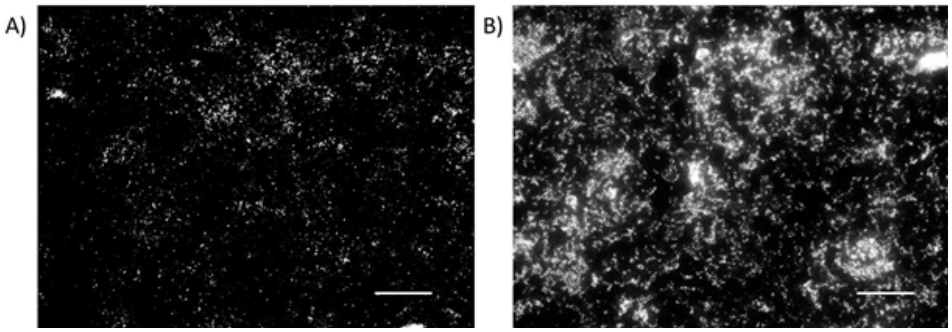

Figure 4: Fluorescence microscopy images after 18 hr PA14 growth in DFR stained with DAPI (white). A) Glass surface with 6.6% surface coverage. B) Slippery BMA-EDMA surface with 22.8% surface coverage. Scale bars are set at 40 μm.

Contrary to previous reports from Li et al., slides modified with SLIPS were found to have significantly more biofilm growth than unmodified glass slides ($p < 0.02$).[25] An average of 24.1% (±17.8) of SLIPS-modified slides (n=9) were occupied by PA14 biofilm, compared to only 6.7% (±6.9) in unmodified glass slides (n=10) (Figure 5). The heterogeneity of the biofilm samples was likely responsible for the large stand deviations in Figures 4 and 5. This was addressed by sampling each slide multiple times to obtain an accurate average of the biofilm coverage.

To confirm that DAPI only stained the biofilm, blank SLIPS-modified and unmodified slides were stained, but neither slide was found to have any significant DAPI fluorescence (data not shown). Furthermore, minimal changes in water contact angle (< 4°) showed that the SLIPS modification was minimally impacted by the continuous flow of media (Table S1), which suggests that surface stability was not a factor in the bacterial adherence or growth.

It is not currently clear why our results do not match prior studies as reported by Li et al.[25] But it is clear from our results that the clinical isolate PA14 must attach to the SLIPS surface by a slightly different

mechanism, and is actually enhanced by the slippery surface. It is also unclear which of the genetic abnormalities harbored by PA14 might be responsible for this different behavior. The results demonstrate that biofilms may have different preferences of surfaces when it comes to attachment. In fact, some strains may prefer to grow on hydrophobic surfaces such as SLIPS. Our results question the universality of the antimicrobial properties of SLIPS.

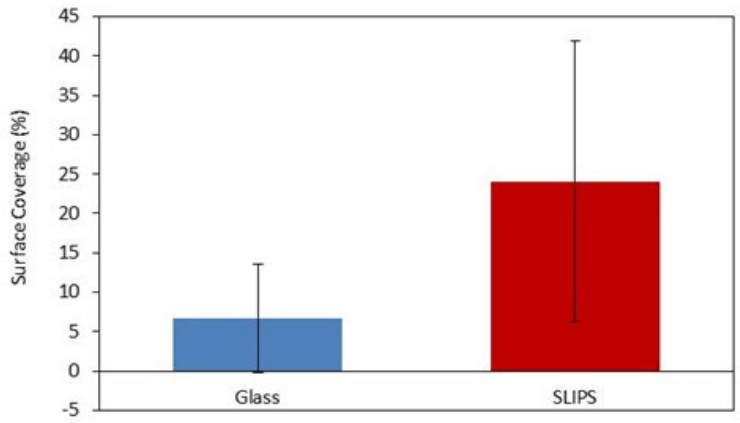

Figure 5: Comparison of area occupied by PA14 biofilm grown on SLIPS-modified and unmodified glass slides. Percent coverage was calculated by taking the area occupied by biofilm in all images taken from a single slide divided by the total area imaged. 10 total SLIPS-modified and 9 total unmodified glass slides were imaged.

Conclusion

In this study, we have analyzed the antibacterial property of slippery liquid-infused porous BMA-EDMA surfaces (SLIPS) in a drip flow reactor. The results show that *P. aeruginosa* (PA14) had a greater amount of biofilm growth on the SLIPS in comparison to the glass surfaces which contradicts prior reported growth of PA14 on BMA-EDMA SLIPS surfaces by Li et al.[25] and other SLIPS studies.[19] Characterization of the surfaces confirmed that modifications of the SLIPS slides were similar to those reported. This bacterial adhesion behavior was further confirmed by verifying the continuous stability of the SLIPS by water contact angle before and after the 18 hr incubation period. While our work does contradict previous research, it reinforces the strain dependent bacterial adhesion behavior and the need for further investigations.

Acknowledgements

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Supplemental Information

Polymer procedure

The modification of glass slides with was based on the procedure by Li et al with a few modifications is demonstrated in Figure S1.[25] The glass slides were washed in deionized water (ddH_2O), dried in air, and immersed in 1.0 M NaOH for 1 hr. The slides were rinsed with ddH_2O and immersed in 0.2 M HCl for 30 mins, followed by washing with ddH_2O and drying with N_2. The activated glass surface was functionalized with 3-(trimethoxysilyl)propyl methacrylate in ethanol (20% vol) adjusted to pH of 5 with acetic acid. A small volume of the solution was applied for 30 mins between two activated slides and reapplied for another 30 mins. The functionalized plates were washed with acetone and dried under a flow of N_2.

The BMA-EDMA polymerization mixture was prepared by mixing 4.0 g of 1-decanol, 2.0 g of cyclohexanol, 2.4 g of BMA, 1.6 g of EDMA, and 0.04 g of DMPAP. Two methods were used to further modify the glass surface with the BMA-EDMA polymer. Using general Teflon tape, the polymerization mixture was injected in between the two silanized glass slides separated by two Teflon strips that were placed vertically on the slides. This controlled the polymer height to be 72 (±5) µm. The slides were held vertically, while injecting the mixture to allow gravity to pull the mixture downward. When about ¾ of the plates were filled with the mixture, the slides were tilted horizontally and injecting of the mixture continued until the mixture covered the entire surface in between the slides. The photopolymerization was initiated by irradiating the filled slide-pair with UV light at 254 nm for 1 hr. The slides were carefully opened using a razor blade resulting in one slide with the polymer modification that was used for biofilm growth analysis. After the polymerization was complete, the slides were washed in methanol and dried in air. The second method of modification with the BMA-EDMA used 15 µm Silicycle silica beads placed on each corner and middle edge of each slide to control the height of the polymer by the diameter of the beads. Then 70 µL of the mixture was applied to a slide with a second slide sandwiched on top. The slides were then irradiated in the same manner for 1 hr and opened with a razor blade.

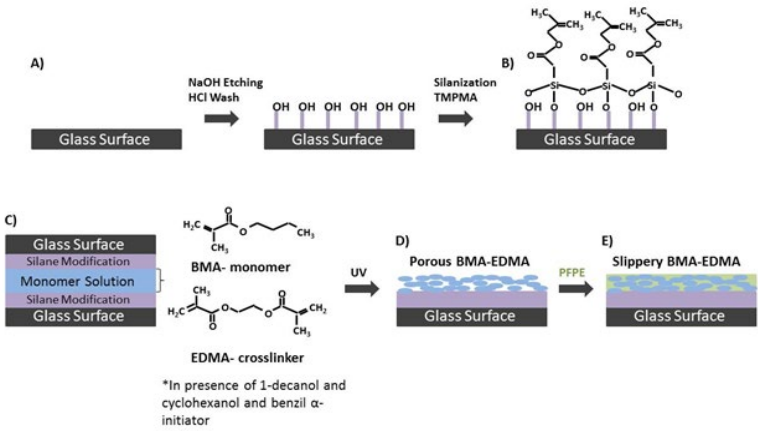

Figure S1: Surface modification schematic. First, the glass surface is subjected to NaOH etching exposing hydroxyl groups (A). The surface is then treated with 3-(trimethoxysilyl)propyl methacrylate [TMPMA] which binds to the hydroxyl groups to create functional groups for polymer adhesion (B). Two silanized glass slides are sandwiched with the modified surface toward the center and the monomer solution placed between the glass surfaces for UV polymerization (C). This leaves a porous polymer (BMA-EDMA) attached to the surface of the glass slide (D) which can be infused with perfluoropolyether [PFPE] to create a slippery surface (E).

Characterization

The polymer sample was sputter coated with a metal alloy using a Technics Hummer sputter coater. SEM images (Figure S2) were obtained with a Hitachi S-3000N microscope at 20.0 kV accelerating voltage. The height of the polymer was determined using Image J. IR spectroscopy using Perkin Elmer Spectrum 100 was used to characterize the completion of the polymerization (Figure S3).

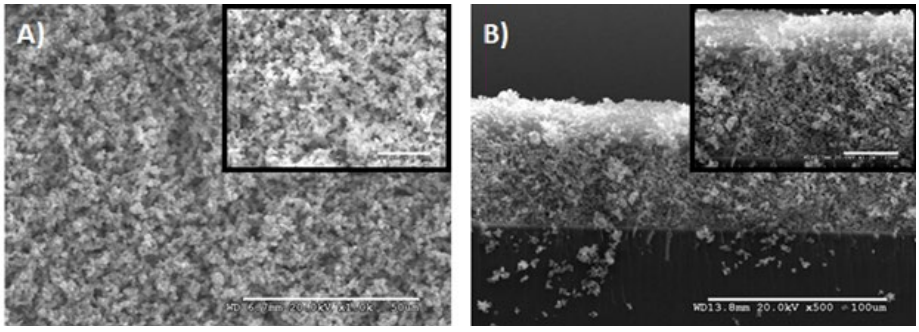

Figure S2: SEM imaging showed the porous polymer structure from the top (A and inset) and side (B and inset). Image scale bars are 25 and 50 μm for Image A and B, respectively, and inset scale bars are 25 μm.

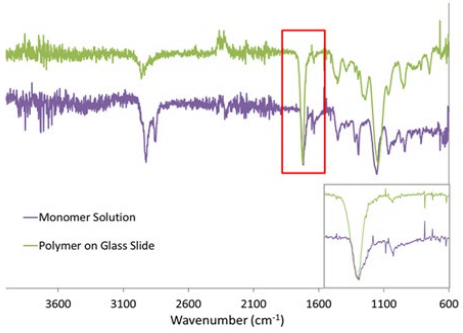

Figure S3: The Fourier Transform Infrared Spectroscopy (FTIR) analysis shows distinct structure of monomer solution components vs polymer. In the 1500-1800 cm^{-1} region (red box inset), it was observed that the peak for C=C at ~1650 cm^{-1} decreased in the polymers compared to the monomer solution confirming polymerization and removal of excess monomer solution by methanol.

Static water contact angles were determined using the literature procedure described by Lamour et al. using a lens focal point of 45-50 mm (Table S1).[28] The procedure involved pipetting 5 µl of deionized water onto the sample surface, obtaining a photo of the magnified drop, and measuring the contact angle using ImageJ Contact Angle plugin and manual points procedure. The "Slippery BMA-EDMA after DFR run" sample was obtained by treating a Slippery BMA-EDMA modified slide in the drip flow reactor as described in the drip flow procedure (see article methods section) with modifications of replacing media with distilled water and no bacteria was introduced.

Surface	Contact Angle (°) (Average ± STD)
BMA-EDMA	137.0 ± 17.4
Slippery BMA-EDMA	111.2 ± 4.3
Slippery BMA-EDMA after DFR run	107.97 ± 13.2

Table S1: Contact angle measurement of polymer vs oil-infused polymer and post run infused polymer. The polymer and infused polymer were found to have comparable contact angle to those found by Levkin et al.[27] The contact angle of the infused polymer after the DFR run was found to decrease insignificantly ($P > 0.05$, student t-test) confirming the stability of the infusion in the low shear environment throughout the biofilm growth period.

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