Effect of Aligned Nanoscale Surface Structures on Microbial Adhesion

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Effect of Aligned Nanoscale Surface Structures on Microbial Adhesion

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Academic Abstract

Microbes in nature live collaboratively in adherent communities, known as biofilms. Biofilms can be contextually beneficial or detrimental. In medical implants, biofilms cause infections leading to additional healthcare costs of billions of dollars. Studies have found that micro/nanoscale surface topography can significantly alter (i.e., promote or hinder) the process of biofilm formation. The formation of biofilm starts with planktonic microbes attach to the surface. To further understand the biophysical underpinning of this process, the effect of aligned nanoscale surface structures on microbial adhesion was studied. To this end, aligned nanofiber coating with controlled fiber diameter and edge-to-edge spacing were manufactured using the Spinneret-based Tunable Engineered Parameters (STEP) techniques. The effect of surface topography on bacterial near-surface motility was studied. The experimental results showed that the bacterial attachment and near-surface motion can be greatly impacted by surface topography. Furthermore, the finding was applied to ureteral stents. The results showed that the aligned nanofiber can significantly reduce the biofilm formation process on ureteral stents.
Effect of Aligned Nanoscale Surface Structures on Microbial Adhesion

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General Audience Abstract

Many microbes in nature live in adherent communities known as biofilm. Biofilms contain individual microbes inside a polymeric matrix which protect them from environmental stressors such as antibiotics. Biofilms are a significant contributor to the infection of implantable medical devices, which leads to additional healthcare costs of billions of dollars annually in the U.S. alone. Studies have found that sub-micron scale surface topography can significantly alter progression of the biofilm formation process; however, the exact mechanism remains poorly understood. To further understand this process, the effect of surface topography, in form of aligned nanoscale surface structures, on microbial adhesion was studied.

The formation of microbial biofilm starts with swimming bacteria sensing the liquid-solid interface and attaching to the surface. Microbes are more likely to settle on a surface if the surface is favorable to attach. However, the decision-making process has not been fully understood. Our experimental results showed that the bacterial attachment and near-surface motion can be greatly influenced by surface topography.

Furthermore, the finding was applied to ureteral stents, which is a type of medical implants used to maintain the flow of urine in the urinary tract. Ureteral stents serve great medical purposes, but as foreign bodies, they also lead to urinary tract infection. The results showed that certain diameter and spacing of aligned fiber coating increased microbial attachment density, while others reduced the bacterial surface coverage by up to 80%, which provides directions for future studies.
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1. **Introduction**

1.1 **Motivation**

Microbes in nature live collaboratively in communities called biofilm. Biofilms can be contextually beneficial or detrimental. For example, dense biofilms with large microbial populations are favored in microbial fuel cells which generate electrical energy from chemical energy using the metabolic activities of microbes.\(^1\) The increase of the microbial population on the electrodes can increase power production by the fuel cells.\(^2\) But in many cases, biofilms are detrimental. On medical implants, biofilms lead to healthcare-associated infection (HAI). In the US alone, HAIs cost $96 - $147 billion directly and indirectly annually in the acute-care hospitals.\(^3\) Biofilm growing on the medical implants would ultimately lead to encrustations, causing difficulty for removal, additional surgeries, and discomfort for patients. Besides, bacteria in biofilms have phenotypic resistance to antibiotics which makes them recalcitrant to antibiotic treatment.\(^4\) Thus, there exists a crucial need to regulate the progress of biofilm formation for various applications.

1.2 **Biofilm Formation**

Biofilms are often formed at the solid-liquid interface. The schematic of biofilm formation is shown in Figure 1.1. In the nutrient environment, proteins and ions are adsorbed on the solid surface which forms a conditioning film that promotes microbial adhesion. The conditioning films change the physicochemical properties of the surface thus facilitating microbial adhesion and formation of biofilms. The process of biofilm formation can be described as the following steps: Initially, planktonic bacteria seek the surface and form the reversible attachment. After the secretion of adhesions and repositioning and deformation of the cell body, the attachment becomes irreversible. Then, the attached bacteria divide and grow to form microcolonies. Microcolonies build up the extracellular polymeric substance (EPS) and mature to biofilms. The matured biofilms
can be developed within several hours since the formation of conditioning films depends on the strain of the microbe and the nutrient source. This process cycles by translocation of the planktonic bacteria released from the matured biofilms, thus the population spreads.

Figure 1.1 Steps of biofilm formation

1.3 **Bacterial Motility**

Bacteria are more likely to settle on a surface if the surface is favorable to attach.\(^5,6,7\) Many strains of bacteria can actively translocate in the liquid environment for survival benefits such as searching for nutrients and moving away from toxins using motility. Studies show that bacterial motility enhances biofilm formation and surface coverage.\(^8,9,10\) Researchers also found that the defect in the organelles that are in charge of the motility can greatly impact the bacterial surface sensing and adhesion.\(^11,12\)

Unlike living in the macroscale environment where motions are largely due to inertia force, bacteria living in the microscale are unable to utilize the principle of momentum exchange for propulsion due to the domination of surface forces. Thus, in this environment with low Reynold’s number, motile bacteria had to develop unique strategies to translocate.\(^13,14,15,16\)

Bacteria utilize different types of appendages to achieve translocation in fluid or on surfaces. Variety in types, locations, length, and the number of appendages leads to various moving
strategies.\textsuperscript{15,16} In the liquid environment, the most common active translocation is achieved by swimming which is caused by the rotation of appendage propulsive organelle, called flagellum.\textsuperscript{13,17} Flagellum is a helical filament that is about 20 nm in diameter and several microns in length.\textsuperscript{13} Each flagellum has a rotational motor powered by proton motive force. Flagellum rotates and propels the bacterium through the medium. Bacteria may possess a singular of multiple flagella, which can be located at the poles or randomly on the body. The number and location of flagella lead to a variety of swimming patterns. But for all types of swimming powered by flagella, the motions are basically controlled by the change of rotational direction of flagella.

For peritrichous flagellated bacteria, such as \textit{Escherichia coli}, multiple flagella are randomly distributed around the cell body. When all flagella rotate counter-clockwise (CCW) and bundled together, the cell body moves forward (known as a \textit{run}). When one or more of the flagella rotate clockwise (CW), the flagellar bundle is disrupted. In absence of propulsion bacteria randomly reorient, due to Brownian motion (known as a \textit{tumble}). In presence of a chemo-effector gradient, the frequency of tumble will decrease when moving towards the preferential direction (\textit{i.e.}, in presence of a chemoattractant) and increase when moving away from the preferential direction (\textit{i.e.}, in presence of a chemorepellent).\textsuperscript{15,16}

For monotrichous strains such as \textit{Pseudomonas aeruginosa}, where each cell only has one polar flagellum, the translocation strategy is simpler. When flagella rotating CCW, the cell body moves forward, when rotating CW, the cell body moves backward.\textsuperscript{15,16} The cell body sometimes reorients when the flagellum switches the rotational direction, thus the bacterium changes its moving direction.\textsuperscript{18} Flagella are also known to be used as surface detectors and adhesins that help bacteria settle on surfaces.\textsuperscript{19}
Twitching is another type of moving strategy that exists when a bacterium is moving at a liquid-solid interface. Twitching depends on pili, which is another type of cell appendage that is much shorter than flagellum. Pili extends out and retract and drag the cell body along the surface using a retraction force of on the order of 100 pN.\textsuperscript{20} Mutants that are defective in pili have shown defect in microcolonies formation.\textsuperscript{9} Researchers also have found that pili play and role in sensing the environmental cues. For example, obstructing pilus retraction can initiate the physiological changes for bacterial surface attachment which is triggered when bacteria attach to the surface.\textsuperscript{11}

1.4 Strategies for Combating Biofilm Formation

Strategies for biofilm control can be categorized into two major categories: removal and prevention.\textsuperscript{21} Perhaps the most traditional way is to remove the biofilm using physical or chemical methods, such as using mechanical force, or antibiotics.\textsuperscript{22} However, for medical implants such as the urinary stent, biofilm removal often requires removal of the medical device from the patients’ body, which introduces additional pain and cost.\textsuperscript{23} Another traditional solution is to apply antibiotics to the surface (e.g., as a coating) which kills the microbes or stops their growth.\textsuperscript{22} This method can be effective but with a few disadvantages. First, the amount of antibiotics that the surface can carry is limited. Second, the killed microbes may adhere to the surface and serve as a nutrient source and attract more microbes to attach. Last but not least, the overuse of antibiotics can lead to the selection of the microbe strains with antibiotic resistance.

Thus, a more optimal solution to combat biofilm formation is to prevent or slow down its formation from the beginning using non-toxic methods to alter the physical properties of the surface. Perhaps, the most efficient way is to control the first step of biofilm formation, which is the reversible attachment, as mentioned above. Researchers have conducted studies to alter the
frequency of the reversible attachment. For example, one can chemically modify the surface by attaching functional groups to the surface, thus, change the physicochemical properties of the surface and change the frequency of occurrence reversible attachment, resulting fewer bacteria attached to the surface per unit time, thus slow down biofilm growth. Other solutions include using superhydrophobic or hydrophilic materials, and introducing microscale surface topography. However, the choice of material can be limited to specific applications and fabrication techniques. For example, the materials used for medical implants in the US are required to be approved by the US Food and Drug Administration (FDA).

Hydrophobic surfaces tend to have low surface energy and repel water. When bacteria attach to hydrophobic surfaces, the attachment tends to be weaker due to the low surface energy of the surface which results in low energy required to break the attachment bond. Thus, bacteria are less likely to remain strongly attached to the hydrophobic surface.

However, hydrophilic surfaces can also have good bacterial attachment resistance. The principle is the following. When a hydrophilic surface is submerged into bulk fluid, it forms strong attachment bonds with water. As a thin layer of water forms on the surface, there appear hydration force, which is a strong short-range repulsive force which repels the approaching bacteria. The anti-bacterial adhesion property can also be achieved by attaching polymer brushes to the surface to reduce the surface area available to bacterial contact thus reduce attachment. Besides, researchers have also attached charged functional groups to the surfaces to achieve bacterial antifouling. Bacterial membranes are negatively charged. By attaching the negatively charged functional group to the surface, the bacteria get repelled due to electrostatic forces. Positively charged functional groups are also studied and appeared as a more effective approach.
By attracting the bacteria to the brushes, the positively charged polymer branch can penetrate the cell membrane thus kill the bacteria.\textsuperscript{44,45,46}

1.5  \textbf{Antifouling Strategies Founded in Nature}

The control of reversible attachment can also be achieved physically by surface topographical modification.\textsuperscript{28,29,30,31,32,33,34,35} One type of topographies is highly aligned grooves.\textsuperscript{34,35} Nature has found this solution centuries ago. Mussels are known to have plentiful biofouling on their shells. However, there exist the blue mussel \textit{Mytilus galloprovincialis}, whose shells has significant less fouling. Under the microscope, it was observed that the surface of this strain is covered with uniformed ridges as shown in Figure 1.2 a). This antifouling feature disappears on the mutants without ridges.\textsuperscript{47} In this research, we mimic the uniformed ridges as shown in Figure 1.2 b) and study its effect on bacterial attachment.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1-2.png}
\caption{a) Surface topography of blue mussel \textit{Mytilus galloprovincialis}.\textsuperscript{47} b) Mimicked surface topography with aligned nanofibers. Scale bar = 1 \textmu m.}
\end{figure}

1.6  \textbf{Spinneret based Tunable Engineered Parameters (STEP) method}

The aligned fiber coating was produced using the Spinneret based Tunable Engineered Parameters (STEP) technique.\textsuperscript{48} (US Patent# 9,029,149, 9,753,023, and 9,902,932) The schematic of the STEP method is shown in Figure 1.3. A substrate was mounted on a rotational motor which
is on top of a motorized positioning stage moving at a defined rotational and translational speed. A spinneret filled with polymer solution was brought into contact with the moving substrate to form single polymer fiber and pulled back. By doing so, a single polymer fiber with consistent fiber diameter can be continuously drawn from the polymer solution and wrap around the substrate to achieve consistent spacing. Figure 1.3 shows a representative scanning electron microscopy (SEM) image of the aligned fiber coating on the substrate.

![Figure 1.3 Schematic of the STEP platform and a picture of aligned fiber coating taken by scanning electron microscopy (SEM).](image)

1.7 **Objective and Organization of the Thesis**

In this study, inspired but the topography of the blue mussel shell, we examine bacterial adhesion on nanofiber-coated surfaces with the long-term goal of hindering or eliminating biofilm formation on surfaces. The topic is approached in two perspectives.

Bacteria are more likely to attach to a surface if the surface is favorable to attach. However, the detailed procedure is not fully understood yet. So far, studies are conducted on exploring the effect of surface topography on biofilm formation, and bacterial motions at the
liquid-solid interface. The study of bacterial motion near-surface topography is rarely studied.

In Chapter II, the effect of aligned surface structure on microbial adhesion is studied experimentally by studying the effect of aligned nanofibers of controlled diameter and spacing on bacterial near-surface motility. Bacterial motility plays a significant role in reversible attachment. Thus, we hypothesized that controlling the bacterial near-surface motility affects biofilm formation.

In Chapter III, the effect of aligned surface structure on microbial adhesion is studied experimentally by comparing the bacteria surface attachment on the nanofiber-coated and uncoated surfaces. Two adhesion assay methods were performed and one method was selected for future study.

In Chapter IV, a summary of the key contribution of this thesis is presented. The limitation of the study and the possible future work directions are discussed.
2. Effect of aligned nanofibers on near-surface motion and reversible attachment of *Pseudomonas aeruginosa*

2.1 Abstract

Bacteria with motility have great abilities to seek favorable surfaces to attach and form biofilms. Bacteria often show unique moving patterns near solid surfaces due to the surface effect. Studies have found that surface topography in micro/nanoscale can significantly accelerate or decelerate the process of biofilm formation. However, the effect of surface topography on bacterial near-surface motions was barely studied. In this chapter, we studied the effect of aligned polystyrene nanofibers on near-surface motion and reversible attachment of *Pseudomonas aeruginosa PA14* on polystyrene films. The aligned fiber coating with controlled fiber diameter and edge-to-edge spacing were manufactured using Spinneret-based Tunable Engineered Parameters. The experiments were conducted with a self-made setup. Bacterial attachment and near-surface motion were quantified at the individual and population level using bright field optical microscopy. It was found that the 375nm diameter-0.7 μm spacing fiber coating had the greatest impact on the bacterial attachment and bacterial near-surface motions for both reversible attachment and biofilm formation. The 375nm diameter-0.7 μm spacing samples had fewer bacteria swimming near the surface. But once bacteria come close to the surface, the chance of attachment increased eight times compared to the uncoated samples. For future work, it is curious to study the reason behind this unique behavior. Besides, the results have shown that bacterial attachment and near-surface behavior can be affected by surface topography. Potentially, there may exist aligned fiber coating with certain diameters and spacings to reduce the bacterial adhesion which can be studied shortly.
2.2 Introduction

2.2.1 Biofilm formation by Pseudomonas aeruginosa

*Pseudomonas aeruginosa* is a Gram-negative bacterial strain that is capable of causing infections in plants, animals, and humans. The model strain studied here is *Pseudomonas aeruginosa* PA14, which is an opportunistic human pathogen that was clinically isolated from a burn wound in the 1990s.\(^{56}\) *P. aeruginosa* PA14 is rod-shaped with a single polar flagellum and multiple pili around the cell body as shown in the Scanning Electron Microscopy (SEM) image in Figure 2.1. *P. aeruginosa* PA14 was chosen because it has been extensively used as the model strain to study biofilm formation.\(^{57,58,59,60,61}\)

![Figure 2.1 Scanning Electron Microscopy (SEM) image for *P. aeruginosa* PA14](image)

Studies showed that the process of biofilm formation of *P. aeruginosa* can be accelerated or decelerated by changing environmental factors.\(^{62,63,64,65,66}\). Besides the widely studied chemical environmental cues such as the gradient of chemoattractant/repellent, mechanical cues like shear stress,\(^{62,63,64}\) surface stiffness,\(^{67}\) and surface topography\(^{28,29,30,31,32,33,34,35}\) are increasingly being...
recognized for having significant effects on bacterial community\textsuperscript{62,65,66} and the physical behavior and individual level\textsuperscript{64,54,55,68}. The details can be found in Chapter I and section 2.2.2

\textbf{2.2.2 Effect of Shear Stress on Biofilm Formation}

The majority of biofilms form at the solid-liquid interface. Shear stress exerted on the bacteria varies with the flow pattern and speed. One may think that increasing shear stress can easily overcome the adhesion force that was exerted by bacteria to anchor to the surface, thus reducing bacterial adhesion density. This phenomenon was reported in many studies that measured the adhesion force of individual bacterium\textsuperscript{69,70}.

In addition to the direct effect on the force balance, a study using a clinical isolate of \textit{P. aeruginosa} from the lung of a patient with cystic fibrosis showed that high shear can downregulate \textit{algD} (alginate biosynthesis) operon, which is involved in the biofilm formation process; thus more planktonic bacteria were observed under high stress condition\textsuperscript{63}. \textit{algD} operon was also upregulated under low shear condition, thus promoted biofilm formation\textsuperscript{63}.

However, researchers found that the residence time for individual \textit{P. aeruginosa} PA14 bacterium in the reversible attachment phase increases approximately linearly as the shear stress increases within a certain range\textsuperscript{64}. The same phenomenon was observed in strains with defected flagellum, pili, or extracellular matrix production, which suggest that the trend is due to shear stress only and response on the bacteria body\textsuperscript{64}. Besides \textit{P. aeruginosa}, researchers also found that increasing shear stress within a certain range can enhance the adhesion of \textit{Staphylococcus epidermis} \textsuperscript{71} and \textit{S. aureus} \textsuperscript{72}. 
2.2.3 Effect of Surface Topography on Biofilm formation

Studies showed that modifying surface topography can modify the physicochemical properties of the surface hence promote or hinder the biofilm formation.\textsuperscript{28,29,30,31,32,33,34,35} For hydrophobic surfaces, topographical modifications can change the hydrophobicity of the surface, resulting in superhydrophobic surfaces and reduce bacterial adhesion.\textsuperscript{28,33} One of the examples of gaining superhydrophilicity by modifying surface topography is lotus leaves. The microscopic grooves on the surface with a diameter and height of several micrometers make the leaves surface superhydrophobic thus, have antifouling property.\textsuperscript{21}

Many studies showed that bacterial adhesion depends on the feature size of the topography on the surface.\textsuperscript{30,31,32} Surface topography can also make the surface energetically unfavorable for microbes to settle.\textsuperscript{29,33,34,35} Researchers have reported different ways to manufacture the desired surface topography at the nano/micro-scale.\textsuperscript{32,34,48} When microbes detect energetically unfavorable surfaces, they are less likely to transition from the planktonic status to the adherent phenotype.

2.2.4 Near-surface motion

As mentioned earlier, \textit{P. aeruginosa} is monotrichous, meaning each bacterium has one polar flagellum. In bulk fluid, when the flagellum is rotating CCW, the bacterium moves forward; when rotating CW, the bacterium moves backward.\textsuperscript{15,16} Other types of swimming patterns such as circling and sudden change of directions are also observed in various studies.\textsuperscript{49,50}

For motile bacteria, researchers report that surface effects play an important role when bacteria are swimming near the solid boundary up to 20 μm.\textsuperscript{54,55,73,68} The near-surface region is defined as the boundary layer which depends on the property of the surface and the fluid. The propulsion of bacterial motion is due to viscous force. When bacteria swimming inside the
boundary layer, due to the gradient of shear stress, the viscous force becomes unbalanced in the
direction perpendicular to the direction of the flagellum, thus the bacteria curve.

For *P. aeruginosa*, both pili and flagellum can contribute to its unique moving pattern near
the surface. Pili, as described in Chapter I, is another type of surface appendage that contributes to
bacterial surface motility and biofilm formation. The extension and retraction of pili (~0.5 μm/s)\(^7^4\)
result in twitching motility, where the bacterium reorients its body multiple times within a fraction
of second.\(^7^4^,7^5^,7^6\) The other types of near-surface bacterial motions are largely attributed to the
rotation of the polar flagellum combined with the surface effect.

The motion types due to rotation of flagellum in bulk fluid such as swimming forward and
switching directions were observed and reported for *P. aeruginosa*.\(^4^9^,6^8\) Other types of motion due
to the combined effects of rotation of flagellum and the proximity to a solid surface such as circling
were observed in strains such as *E. coli, Caulobacter crescentus*, as well as *P. aeruginosa*.\(^5^4^,6^8^,7^7\)

2.2.5 Bacterial Surface Sensing

Bacteria have the ability to sense the surface and initiate attachment. Studies showed that
pili of *Caulobacter crescentus* can detect the force exerted on retraction to initiate the process of
its holdfast synthesis which is essential for surface attachment.\(^1^1\) The role of pili on surface
attachment was also reported for *P. aeruginosa*. Studies showed that pili serve as mechanical
sensors that can drive cAMP signaling when touching the surface.\(^7^8^,7^9^,8^0\)

Studies showed that flagella can also serve mechanical sensors. One of the first studies on
surface sensing conducted in 1988 in *V. parahaemolyticus* showed the flagellum is capable of
sensing the external force, which affected its motion. The performance of flagellum is coupled
with *laf*, which is the gene that enables swarming on agar. A more recent study showed that the flagella rotatory motor in *Caulobacter crescentus* also serves a sensor for surface sensing.

### 2.2.6 Innovation

Bacteria have preferences when attaching to surfaces. However, the detailed procedure is not fully understood yet. So far, studies are conducted on exploring the effect of surface topography on biofilm formation and bacterial motion at the liquid-solid interface. The study of bacterial motion near-surface topography is rarely studied. As described in chapter 1, the anti-adhesion strategies based on topographical modification target the reversible attachment stage of the biofilm formation process. We hypothesize that nanoscale surface topography alters the near-surface motion of bacteria, thereby affecting bacterial attachment strength and density. To test our hypothesis, we utilized the STEP technique to deposit polystyrene nanofibers of well-defined diameter and spacing on smooth polystyrene surfaces. These engineered surfaces enabled us to examine the effect of nanofiber diameter (i.e., topographical feature size) and spacing (i.e., the separation distance between topographical features) independent of one another.

In this research, the effect of aligned surface structure, in form of nanofibers, on microbial adhesion and bacterial near-surface motility of *P. aeruginosa* PA14 mKO was studied. Furthermore, the relationship among surface topography, bacterial near-surface motion type, and bacteria settlement are discussed.
2.3 Materials and Methods

2.3.1 Cell Culture

*Pseudomonas aeruginosa* PA14 mKO\(^1\) was streaked on 1.5% Lysogeny broth (LB, 1% tryptone, 0.5% yeast extracts, 0.5% NaCl) agar plate from frozen stock stored at -80 °C and incubated at 37 °C for 14 - 16 h. A colony was picked and used to inoculate 5 mL LB medium in a 12 mL glass test tube. The liquid culture was incubated at 37 °C and shaken at 150 rpm for 14 - 16 h to reach the stationary phase.

2.3.2 Substrate Preparation

NO.1 cover glass slides (thickness: 0.13-0.17 mm) were cut into 20 mm × 3 mm substrates using an automatic dicing saw. The glass substrates were cleaned by sequential rinsing with acetone and deionized water (DI) and stored in isopropyl alcohol. Before use, the glass substrates were cleaned again with 100% ethanol then, air-dried and treated in an Ozone Cleaner (Bioforce Nanosciences UV Ozone Cleaner ProCleaner™ Plus), for 15 minutes.

2.3.3 Polystyrene Film Deposition

The glass substrates were coated with polystyrene film (MW: 2M g/mol) on one side using a custom casting system. The polystyrene solution used for casting was prepared by dissolving 4 wt.% polystyrene (MW: 2M g/mol) in xylene. To ensure, full dissolution of polystyrene in xylene, the solution was used a minimum three days after preparation. After the casting, the substrates were placed in a vacuum chamber for an hour to remove the solvent (xylene) residue. The coated

---

\(^1\) The PA14 mKO strain was constructed to express Kusabira orange fluorescent protein by a chromosomal modification. The modified strain was provided by George O’Toole (Dartmouth College). The fluorescence expression was not utilized in this study.
substrates were cleaned with ethanol and DI water, then air-dried. The caste substrates were ready to use as the unmodified control (hereafter referred to uncoated, meaning without fiber coating) samples in all experiments.

2.3.4 Nanofiber Coating

Aligned polystyrene nanofibers, with controlled fiber diameter and edge-to-edge spacing, were deposited on glass substrates with the coated with thin polystyrene film using the Spinneret-based Tunable Engineered Parameters (STEP) technique. The diameter and spacing of the aligned fibers were confirmed using optical microscopy and scanning electron microscopy (SEM), as shown in Figures 2.2 and 2.3.

Figure 2.2 Optical microscopy images of the sample types used in this study. $D_f =$ fiber diameter, $S =$ fiber spacing, all scale bars are 50 μm. a) Uncoated polystyrene film, b) $D_f =$ 375 nm and $S = 0.7$ μm. c) $D_f =$ 375 nm and $S = 1.4$ μm. d) $D_f =$ 481 nm and $S = 0.7$ μm.

Figure 2.3 Scanning electron Microscopy images of the-fiber coated samples. a) $D_f =$ 375 nm and $S = 0.7$ μm. b) $D_f =$ 375 nm and $S = 1.4$ μm. c) $D_f =$ 481 nm, and $S = 0.7$ μm. All scale bars are 2 μm.
2.3.5 Experimental Device

The experiments were conducted in custom-made glass-bottom 60 mm-diameter Petri dishes. A 0.6-inch diameter hole was drilled at the center of the dish. A 20 mm × 20 mm No. 1 glass slide was attached to the bottom of the Petri dish to cover the drilled holes using a waterproof adhesive. The glued glass-bottom Petri dish was left at room temperature for three days for the adhesive to fully cure.

2.3.6 Biofilm Medium

The experiments were conducted using M63 medium (0.3% KH₂PO₄, 0.7% K₂HPO₄, 0.2% (NH₄)₂SO₄) supplemented with 1mM MgSO₄, 0.2% glucose, and 0.5% casamino acids. The medium was sterilized by filtration through a 0.22 μm pore size filter.

2.3.7 Sample Assembly

The fiber-coated samples were placed into the glass-bottom Petri dishes with the fiber-coated side facing up and attached to the Petri dishes using Polydimethylsiloxane (PDMS). The assembled Petri dish, shown in Figure 2.4, was placed on a heat plate at 70 °C for 4 hours for PDMS to cure. The assembled device was placed in a UV crosslinker (UVP Crosslinker, Analytic Jena, Thuringia Germany) under UV-light of 254 nm wavelength with the light intensity of 11.5 mW/cm² for 10 min for sterilization.

2.3.8 Experiment Setup

The optical microscope enclosure was brought to 37 °C. Then, 7 mL of culture medium was to the sample assembly prepared in 2.3.7 and placed in the microscope with 7 ml culture
medium. The optical density of the overnight culture in LB was measured at a wavelength of 600 nm (OD$_{600}$). The overnight culture was diluted to an OD$_{600}$ of 0.024 in M63. A 1 mL aliquot of the diluted culture was drawn into a 3-mL syringe and introduced into the Petri dish to achieve a final OD$_{600}$ of 0.003.

![Figure 2.4 The schematic of the experimental setup.](image)

### 2.3.9 Imaging

The initial time point (t = 0 min) was defined as the moment that the diluted culture was fully introduced to the Petri dish from the syringe. Time-lapse bright-field microscopy images were recorded using an inverted optical microscope (Zeiss AxioObserver Z1) with a high-speed camera (AxioCam HSma camera) and 40× objective. The videos were recorded at 61 fps for 1 hour. Right after the one-hour imaging, another video was recorded at 30 seconds per frame for 20 – 22 hours.
2.3.10 Image Processing

The 1-hour high frame rate videos were exported as image sequences from Zen software at 20 fps for each video. The exported images were divided into 5-minute intervals and imported into ImageJ for bacterial tracking. The tracking was done using ImageJ plugin MtrackJ \(^82\). Each bacterium was tracked for at least three frames (0.10 sec) The motion types and other relevant parameters for each bacterium were categorized and calculated.

For the long-term videos recorded at 2 frames per minute, the number of bacteria at specified timepoints were manually counted.

2.3.11 Statistical Analysis

Pairwise t-tests assuming non-equal variance were used to determine the statistically significant differences. The null hypothesis was rejected using the criteria of \( p < 0.05 \). The difference between the quantitative results was tested using Cohen’s delta analysis.

2.4 Results and Discussion

2.4.1 Bacteria Size Measurement and Surface Topography Parameter Selection

The size of individual \( P. \ aeruginosa \) PA14 bacterium is measured using optical microscopy. The cell has an average length \( (L_c) \) of 1.97 \( \mu \text{m} \pm 0.4 \mu \text{m} \) and a diameter \( (D_c) \) of 0.71 \( \mu \text{m} \pm 0.1 \mu \text{m} \) when incubating in M63 culture medium. The effect of surface topography was studied using two factors: fiber diameter, \( D_f \), and edge-to-edge fiber spacing, \( S \) as shown in Figure 2.5. The fiber diameters tested are 375 nm and 481 nm. The tested spacings are 0.7 \( \mu \text{m} \) and 1.4 \( \mu \text{m} \). The choice of diameter and spacing values was guided by a mathematical model previously developed in our lab.\(^{34}\) The model uses the ratios of fiber diameter to bacteria diameter, fiber spacing to bacteria
diameter, and the stretching modulus to work of adhesion as the input to determine the optimal choices of fiber diameter and spacing that maximizes changes in the total energy of the cell as it transitions from the planktonic to the adherent state. We have previously shown that for the non-motile *Candida albicans* this optimal surface topography leads to the minimum microbial attachment density on the surface. Since *P. aeruginosa* is rod shaped, the equivalent diameter was calculated assuming consistent volume. The equivalent diameter for *P. aeruginosa* PA14 mKO grown in M63 medium was 1.34 μm. The Young’s modulus of bacteria is 100 MPa. The cell wall thickness was 12.8 nm. Thus, the stretching modulus is 1280 mN/m. The polar and dispersive component of the bacteria was 14.7 mJ/m² and 24 mJ/m². The polar and dispersive component of the polystyrene film was 2.4 mJ/m² and 37.5 mJ/m². Thus, the work of adhesion for *P. aeruginosa* PA14 attaching to the surface is 72 mJ/m². Overall, the non-dimensionalized ratio is 17.8.

The effect of fiber diameter is tested by comparing the results of \( D_f = 375 \text{ nm} \) and \( D_f = 481 \text{ nm} \) while using \( S = 0.7 \mu \text{m} \). The effect of fiber spacing is tested by comparing the results of \( S = 1.4 \mu \text{m} \) and \( S = 0.7 \mu \text{m} \) while using \( D_f = 375 \text{ nm} \).

![Figure 2.5 Parameters of bacteria and aligned fiber coating.](image)
2.4.2 Long-term Bacterial Attachment

The central question of this study was to determine how aligned nanoscale patterns affect bacteria attachment. Thus, we first examined bacteria attachment outcomes over long timescales (1 – up to 24 hours). The result of bacterial attachment on different types of substrates are shown in Figure 2.6. There exists a visible difference in the number of attached bacteria between the $D_f = 375 \text{ nm}$, $S = 0.7 \mu\text{m}$ sample and the rest of the samples.
The areal density of bacteria attachment is shown in Figure 2.7. The $D_f = 375 \text{ nm}$, $S = 0.7 \mu\text{m}$ had significantly more attachment at 1, 2, and 4 hr incubation. In contrast, the $D_f = 375 \text{ nm}$, $S = 1.4 \mu\text{m}$ sample had the lowest attachment within the same timeframe. The attachment density of the $D_f = 375 \text{ nm}$, $S = 1.4 \mu\text{m}$ samples and the uncoated samples were comparable throughout the measurement period. Due to the difference in attachment density observed among the samples at
the long-term timepoints, we hypothesized that the surface topography may also affect the short-term attachment (0 – 60 min).

2.4.3 Short-term Bacterial Attachment Analysis

There are three possible outcomes for each tracked bacterium: (i) moving out of focus (i.e., exiting the focal plane by swimming upward), (ii) moving out of the imaging field (i.e., swimming out of the imaging field while parallel to the substrate), and (iii) attaching (i.e., ceasing to move until the end of the tracking period).

The settlement outcome vs. sample types was first determined as a function time, at three time points of 0 – 5, 25 – 30, and 55 – 60 min, as shown in Figure 2.25 in Appendix. The statistical analysis showed that the settlement outcomes were not time-dependent. Thus, the data from all time points were combined for further analysis.

Figure 2.8 shows the percentage of each possible settlement outcome for the 4 sample types. The 375 nm diameter-0.7 μm spacing samples have an eight times higher chance of bacteria
attachment, compared to all other sample types studied. The comparison between 375 nm diameter-0.7 μm spacing samples and 481 nm diameter-0.7 μm spacing samples showed that change of fiber diameter dramatically increased the bacteria attachment (p < 0.0001). The comparison between 375 nm diameter-0.7 μm spacing samples and 375 nm diameter-1.4 μm spacing samples showed that the change of fiber spacing also significantly changed the bacteria attachment (p < 0.0001). The high percentage of attached bacteria for 375 nm diameter-0.7 μm spacing samples agreed with the results of the long-term analysis results shown in Figure 2.7.

Besides, uncoated samples have a significantly lower percentage of bacteria that move out of focus compared to 375 nm diameter-0.7 μm spacing samples (p < 0.01).

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>N</th>
<th>Attached</th>
<th>Move out of Focus</th>
<th>Move out of Imaging Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>375 nm, 0.7 μm</td>
<td>216</td>
<td>59%</td>
<td>14%</td>
<td>27%</td>
</tr>
<tr>
<td>375 nm, 1.4 μm</td>
<td>1015</td>
<td>6%</td>
<td>39%</td>
<td>54%</td>
</tr>
<tr>
<td>481 nm, 0.7 μm</td>
<td>704</td>
<td>7%</td>
<td>35%</td>
<td>58%</td>
</tr>
<tr>
<td>Uncoated</td>
<td>1542</td>
<td>9%</td>
<td>25%</td>
<td>66%</td>
</tr>
</tbody>
</table>

Figure 2.8 Settlement vs. Sample Types. Bacteria used for each type is a combination of at least two repeated experiments.

2.4.4 Types of Near-surface Motions

The bacterial attachment mentioned in section 2.4.3 occurred during the reversible attachment phase in the process of biofilm formation. Bacterial motility is an important factor when bacteria are approaching the surface. Thus, we asked if the surface topography also affects bacterial near-surface motility.

To quantitatively analyze the results, different types of near-surface bacterial motion were first identified. The types of motion can be broadly divided into three groups: (i) motion due to the
rotation of flagellum in the bulk fluid, (ii) motion due to the rotation of flagellum near a surface, and (iii) motion due to the extension and retraction of pili. There also exist distinguishable motion types in each category, thus, results in a total of seven types of categorized motion.

Motion due to the rotation of flagellum can be further divided into three categories: *Forward, Forward & Backward, and Sharp Turn.*

The *Forward motion* shown in Figure 2.9 is defined as bacteria movement in one direction and a straight line. This type of motion is commonly observed far from the surface where the surface effect is negligible. However, it may also be observed near solid surface boundaries. One of the possible reasons is that the flagellum connects to the bacterial body using a universal joint. The flagellum is invisible under the optical microscope. Thus, the situation of the bacteria body is in the near-surface region but the flagellum is not can happen.

![Figure 2.9 Forward motion in time series. All scale bars are 10 μm.](image)

The *Forward & Backward motion* is defined as when a bacterium is moving straight and then move in the opposite direction within a fraction of second. The demonstration is shown in Figure 2.10. This motion is due to the change of direction of flagella rotation and has been reported in different studies.\textsuperscript{49,50,51,52}

![Figure 2.10 Forward & Backward motion in time series. All scale bars are 10 μm.](image)
The *Sharp Turn motion* is defined as when a bacterium moving straight makes a sudden sharp turn to another direction which is around 90 degrees (between 60 to 120 degrees) as shown in Figure 2.11. This motion has been reported in different studies and is due to the reorientation of the bacteria body due to effects such as Brownian motion. Different from the *Forward & Backward motion*, the *Sharp Turn motion* involves another stage where the bacterium flicks, the cell body reorients in a random direction to move towards.

![Figure 2.11 Sharp Turn motion in time series. All scale bars are 10 μm.](image)

Motion due to surface effect can be divided into three categories: *Circular, Tight Turn, and Wide Turn*.

The *Circular motion* is defined as when a bacterium is turning with a constant radius as shown in Figure 2.12. The radius is relatively small with a typical radius of around 20 μm and the tracked curve is more than 360 degrees. This *circular motion* occurs at the liquid-solid interface due to surface effects and has been reported many times in different studies.

![Figure 2.12 Circular motion in time series. All scale bars are 10 μm.](image)

The *Tight Turn motion* shown in Figure 2.13 is defined when a bacterium is turning with a visible constant radius, then move straight. The radius of turning is relatively small (~20 μm), and the tracked curve is around 180 degrees (150 – 270 degrees). This motion is similar to the *Circular motion* with the only difference being the magnitude of the angle of turning.

![Figure 2.13 Tight Turn motion in time series. All scale bars are 10 μm.](image)
The Wide Turn motion shown in Figure 2.14 is defined as when a bacterium is turning with a visible consistent radius. The radius is usually larger than 60 μm, which is half of the size of the imaging field. As a result, the turning curve is less than 180 degrees.

The twitching motion shown in Figure 2.15 is defined as when a bacterium is moving in a random direction at a relatively low speed (< 40 μm/s). This motion is caused by the extension and retraction of pili. The extension and retraction happens within a fraction of one second, which allows bacteria to change its direction of motion as mentioned in section 2.2.4.
The bacterial motion on each sample type was analyzed separately for three periods of (0 – 5, 25 – 30, and 55 – 60 min (Appendix). The results from different time points did show a significant difference, thus the data from all the periods were aggregated for further analysis. Some bacteria moved in more than one type of motion. Figure 2. A2 showed that more than 60% of bacteria only had one type of motion. Thus, the further discussed are built on the bacteria that only performed a single motion type.

Figure 2.16 shows the percentage of each motion type for each sample type. The 375 nm diameter-0.7 μm spacing samples show a significantly lower percentage of _Sharp Turn_ compare to the 481 nm diameter-0.7 μm spacing samples (p < 0.01). The 481 nm diameter-0.7 μm spacing samples also showed a significantly higher percentage of _Sharp Turn_ compare to the uncoated samples.

Besides, the 375 nm diameter-1.4 μm spacing samples showed significantly different for the percentage of _Wide Turn_ motion compared to the 375 nm diameter-0.7 μm spacing samples and the uncoated samples (p < 0.01).

<table>
<thead>
<tr>
<th>Sample_Type</th>
<th>375 nm, 0.7 μm</th>
<th>375 nm, 1.4 μm</th>
<th>481 nm, 0.7 μm</th>
<th>Uncoated</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>216</td>
<td>1015</td>
<td>704</td>
<td>1542</td>
</tr>
<tr>
<td>Forward</td>
<td>58%</td>
<td>36%</td>
<td>36%</td>
<td>49%</td>
</tr>
<tr>
<td>Forward &amp; Backward</td>
<td>7%</td>
<td>14%</td>
<td>11%</td>
<td>11%</td>
</tr>
<tr>
<td>Sharp Turn</td>
<td>10%</td>
<td>11%</td>
<td>17%</td>
<td>10%</td>
</tr>
<tr>
<td>Circling</td>
<td>0%</td>
<td>1%</td>
<td>1%</td>
<td>2%</td>
</tr>
<tr>
<td>Tight Turn</td>
<td>5%</td>
<td>5%</td>
<td>4%</td>
<td>5%</td>
</tr>
<tr>
<td>Wide Turn</td>
<td>13%</td>
<td>29%</td>
<td>27%</td>
<td>18%</td>
</tr>
<tr>
<td>Twitching</td>
<td>6%</td>
<td>4%</td>
<td>3%</td>
<td>5%</td>
</tr>
</tbody>
</table>

Figure 2.16 Motion Type vs. Sample Types. Data were obtained from a minimum of two independent experiments.
2.4.5 Motion Type vs. Settlement

Since the percentage of motion type data showed a significant difference, we then inquired if the motion type affected the bacterial settlement. Figure 2.17 shows the percentage of motion type performed by the attached bacteria for each sample type. It showed that for 375 nm diameter-0.7 μm spacing samples, before the bacterium attached to the surface, 65% of bacteria were doing the forward motion. Bacteria with the forward motion had the highest chance or the second-highest chance to attach for all sample types. Among all the sample types, bacteria attached were the least likely to exhibit the Circling motion before attachment.

In addition, twitching motion is the most or the second most likely motion that leads to attachment. However, this is not the case for 375 nm diameter-0.7 μm spacing samples. Besides twitching, wide turn motion is also likely to lead to attachment in all types of samples. Thus, we wonder if the quantitative parameters such as speed and duration of the Forward, Twitching, and Wide Turn motion are any different among different types of samples.

The relation between motion type and bacteria swimming direction was also studied as shown in Figure 2. A3, Appendix. The type of motion is independent on the bacterial orientation with respect to the fibers (for details, see Appendix)
One interesting observation emerged when comparing the sample size of tracked bacteria for each sample. As mentioned, the bacterial motions are tracked at three different time intervals. For uncoated, 481 nm diameter-0.7 μm spacing, and 375 nm diameter-1.4 μm spacing samples, the number of bacteria tracked at each 5-minute time interval is always more than 100. The sample size of 375 nm diameter-0.7 μm spacing samples is significantly lower than the others, as shown in Figure 2.18. With the same inoculation procedure and the same inoculated culture density, the total number of bacteria tracked at 5-minute time interval for 375 nm diameter-0.7 μm spacing samples over four replicated experiment was 55. In other words, the 375 nm diameter-0.7 μm spacing had fewer bacteria moving near the surface, but once the bacteria enter the near-surface area, the probability of attachment is 59%.

The bacterial attachment density after 1-hour inoculation on the 375 nm diameter-0.7 μm spacing was significantly higher compared to the other samples. Thus, the low sample number could be due to the high probability of attachment. In other words, bacteria are more likely to attach to the surface of the 375 nm diameter-0.7 μm spacing samples. Thus, bacteria were less likely to move along the substrate.

![Table: Percentage occurrence of various motion types performed by attached bacteria for each sample type.](image)

<table>
<thead>
<tr>
<th>Attached</th>
<th>375 nm, 0.7 μm</th>
<th>375 nm, 1.4 μm</th>
<th>481 nm, 0.7 μm</th>
<th>Uncoated</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>127</td>
<td>65</td>
<td>51</td>
<td>139</td>
</tr>
<tr>
<td>Forward</td>
<td>65%</td>
<td>42%</td>
<td>22%</td>
<td>52%</td>
</tr>
<tr>
<td>Forward &amp;</td>
<td>3%</td>
<td>8%</td>
<td>10%</td>
<td>6%</td>
</tr>
<tr>
<td>Backward</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sharp Turn</td>
<td>8%</td>
<td>3%</td>
<td>6%</td>
<td>4%</td>
</tr>
<tr>
<td>Circling</td>
<td>0%</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
</tr>
<tr>
<td>Tight Turn</td>
<td>8%</td>
<td>3%</td>
<td>8%</td>
<td>6%</td>
</tr>
<tr>
<td>Wide Turn</td>
<td>9%</td>
<td>12%</td>
<td>18%</td>
<td>7%</td>
</tr>
<tr>
<td>Twitching</td>
<td>6%</td>
<td>31%</td>
<td>35%</td>
<td>22%</td>
</tr>
</tbody>
</table>

Figure 2.17 Percentage occurrence of various motion types performed by attached bacteria for each sample type.
Because the 375 nm diameter-0.7 μm spacing samples lead to the most attachment, the number of bacteria that already attached since the beginning of the tracking period is added to the number of bacteria tracked as shown in Figure 2.19. Since the 375 nm diameter-0.7 μm spacing samples had significant attachment over time, when comparing the number of bacteria that appeared at each time interval, the differences among sample types become smaller. This may explain the observation mentioned in Figure 2.18, where the number of bacteria tracked on the 375 nm diameter-0.7 μm spacing samples was reduced due to the significant number of bacteria attaching to the surface.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>0 min</th>
<th>25 min</th>
<th>55 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>375 nm, 0.7 μm</td>
<td>55</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>375 nm, 1.4 μm</td>
<td>180</td>
<td>219</td>
<td>250</td>
</tr>
<tr>
<td>481 nm, 0.7 μm</td>
<td>128</td>
<td>171</td>
<td>151</td>
</tr>
<tr>
<td>Uncoated</td>
<td>299</td>
<td>234</td>
<td>256</td>
</tr>
</tbody>
</table>

Figure 2.18 Number of Bacteria Tracked at Each Time Interval.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>0 min</th>
<th>25 min</th>
<th>55 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>375 nm, 0.7 μm</td>
<td>71</td>
<td>122</td>
<td>149</td>
</tr>
<tr>
<td>375 nm, 1.4 μm</td>
<td>150</td>
<td>198</td>
<td>195</td>
</tr>
<tr>
<td>481 nm, 0.7 μm</td>
<td>163</td>
<td>212</td>
<td>240</td>
</tr>
<tr>
<td>Uncoated</td>
<td>136</td>
<td>186</td>
<td>219</td>
</tr>
</tbody>
</table>

Figure 2.19 Number of Bacteria Appeared at Each Time Interval.

2.4.6 Quantitative Description of the Motion Types

Six parameters were selected to quantitatively describe the kinematics of individual bacteria over each sample type. The schematic of the 2D bacterial motion is shown in Figure 2.20.
The following parameters are defined and quantitated:

Duration, $\Delta t$, represents the tracking period of each bacterium. It is defined as the time interval between when the bacterium was first observed in the field of view until one of the three settlement outcomes occurred (i.e., moving out of the field of view, moving out of focus, and attached).

$$\Delta t = t_f - t_0$$

Distance, $l$, is defined as the summation of the distances the bacterium traveled during each time step over the entire duration:

$$l = \sum_{n=1}^{f} \sqrt{(x_n - x_{n-1})^2 + (y_n - y_{n-1})^2}$$

Displacement, $d$, is defined as the magnitude of the displacement vector which connects the first and last tracked point for each bacterium.

$$d = \sqrt{(x_f - x_0)^2 + (y_f - y_0)^2}$$
Persistence was defined as the displacement to distance ratio, $P$. This parameter represents the directionality of the bacterial motion.

$$ P = \frac{d}{l} $$

Average speed $V_{avg}$ is calculated by dividing distance $l$ by the duration $\Delta t$. It represents the average instantaneous velocity of the bacterium during the tracking.

$$ V_{avg} = \frac{l}{\Delta t} $$

Angle $\alpha_n$ is measured at each tracked point. The average change of angle of each bacterium $\alpha_{avg}$ represents the average of angle change through the tracking.

$$ \alpha_{avg} = \frac{\sum_{n=2}^{f} \alpha_n}{n - 1} $$

Since the quantitative parameters are interrelated to each other, the motions of bacteria on top of different types of samples were compared using persistence, duration, and average speed.

The kinematic parameters were then analyzed for each categorized motion types on each sample type. Figure 2.21 showed the kinematic parameters for bacteria on each sample type performing Forward motion. The comparison showed that for the Forward motion on 375 nm diameter-0.7 μm spacing samples, the duration is shorter and the persistence is higher than the bacteria on other sample types.
Figure 2.2 showed the kinematic parameters for bacteria on each sample type performing Twitching motion. The comparison showed that for the Twitching motion on 481 nm diameter-0.7 μm spacing samples, the persistence is higher than the bacteria on 375 nm diameter-0.7 μm spacing samples and 375 nm diameter-1.4 μm spacing samples. (p < 0.05)

<table>
<thead>
<tr>
<th>Twitching</th>
<th>375 nm, 0.7 μm</th>
<th>375 nm, 1.4 μm</th>
<th>481 nm, 0.7 μm</th>
<th>Uncoated</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>20</td>
<td>15</td>
<td>31</td>
</tr>
<tr>
<td>Persistence</td>
<td>0.70 ±0.14</td>
<td>0.64 ±0.24</td>
<td>0.40 ±0.21</td>
<td>0.59 ±0.24</td>
</tr>
<tr>
<td>Duration (s)</td>
<td>2.081 ±1.349</td>
<td>3.164 ±3.229</td>
<td>3.611 ±2.546</td>
<td>3.102 ±2.856</td>
</tr>
<tr>
<td>Average Speed (μm/s)</td>
<td>19.0 ±9.6</td>
<td>19.1 ±11.8</td>
<td>22.8 ±9.3</td>
<td>20.7 ±8.86</td>
</tr>
</tbody>
</table>

Figure 2.22 Quantitative parameters for bacteria on each sample type performing Twitching motion.

Figure 2.23 showed the kinematic parameters for bacteria on each sample type performing Wide Turn motion. The comparison showed that for the Wide Turn motion on 375 nm diameter-0.7 μm spacing samples, the persistence is higher than other samples.

Wide Turn motion.
The speed of the bacteria performing Forward, Twitching, and Wide Turn motions that attached are plotted in Figure 2.24. Bacteria performing Forward motion on the uncoated, and the 375 nm diameter-0.7 μm spacing samples showed an increase in speed before attaching, so did the bacteria Twitching on the 375 nm diameter-0.7 μm spacing samples, the 375 nm diameter-1.4 μm spacing samples, and the 481 nm diameter-0.7 μm spacing samples, and the bacteria doing Wide Turn motion on the 481 nm diameter-0.7 μm spacing samples.

<table>
<thead>
<tr>
<th>Wide Turn</th>
<th>375 nm, 0.7 μm</th>
<th>375 nm, 1.4 μm</th>
<th>481 nm, 0.7 μm</th>
<th>Uncoated</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>8</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Persistence</td>
<td>0.88 ±0.07</td>
<td>0.83 ±0.08</td>
<td>0.86 ±0.14</td>
<td>0.85 ±0.13</td>
</tr>
<tr>
<td>Duration (s)</td>
<td>0.736 ±0.277</td>
<td>0.708 ±0.410</td>
<td>1.268 ±0.857</td>
<td>1.274 ±1.027</td>
</tr>
<tr>
<td>Average Speed (μm/s)</td>
<td>52.9 ±16.2</td>
<td>37.5 ±11.0</td>
<td>50.1 ±13.4</td>
<td>42.1 ±15.8</td>
</tr>
</tbody>
</table>

Figure 2.23 Quantitative parameters for bacteria on each sample type performing Wide Turn motion.

Figure 2.24 Speed of bacteria doing Forward, Twitching, and Wide Turn motions before attaching.
2.5 Conclusions and Future Work

In this study, the effect of aligned nanofiber coating on the bacterial attachment and the near-surface motion was investigated.

First, it was found that the 375 nm diameter-0.7 μm spacing samples had significantly more attachment during the long-term imaging (1 to up to 24 hours). This behavior was also observed during the short-term high frame rate imaging, where bacteria were 8 times more likely to attach to the 375 nm diameter-0.7 μm spacing samples are eight times than all the other sample types.

Seven types of near-surface bacterial motion were identified and categorized to test if the aligned surface topography affects near-surface motion. Statistical comparisons of categorical results showed that the 375 nm diameter-0.7 μm spacing samples had a significantly higher percentage of bacteria in the Forward motion.

Bacteria moving in different types of motion have various possibilities to attach to the surface. Forward motion among all sample types the most or the second most likely to lead to attachment. The other two types of motions that are the second and the third most likely leading to attachment are twitching and wide turn motion.

From quantitative analysis, the Forward motion for the 375 nm diameter-0.7 μm spacing samples had the highest persistence and shortest duration. For bacteria moving in Twitching motion, on the D the 481 nm diameter-0.7 μm spacing, bacteria had significantly lower persistence than on the 375 nm diameter-0.7 μm spacing samples and the 375 nm diameter-1.4 μm spacing samples (p < 0.05)

The potential relationship between the hydrodynamics of the Forward motion near the fiber-coated surfaces and the attachment outcomes will be further explored in future work.
Interestingly, significantly fewer bacteria were observed on the 375 nm diameter-0.7 μm spacing samples. However, after the number of bacteria that already attached since the beginning of the tracking period is added to the number of bacteria tracked, the difference of number of bacteria observed at each time interval among sample types became smaller. In addition, the bacteria that do make it near the surface of this sample time, have eight times higher probability to attach to the surface compared to the other sample types. This observation suggests that the 375 nm diameter-0.7 μm spacing fiber coating ultimately increased the bacteria attachment on the surface.

For future studies, the results should be double tested by performing the following studies. First, additional replicate experiments are needed for the 375nm diameter-0.7 μm spacing samples to achieve a comparable sample size with other samples to further confirm the statistical significance of the results. It would also be insightful to repeat the pairwise comparisons with samples of another diameter and spacing such as studying the effect of fiber diameter using 375 nm diameter-1.4 μm spacing samples and 481 nm diameter-1.4 μm spacing samples, and studying the effect of fiber spacing using 481 nm diameter-1.4 μm spacing and 481 nm diameter-0.7 μm spacing samples. This will ensure that the conclusions reported herein are limited to the particular dimensions used in this study.

In addition, the nanofiber coating process can also be improved to achieve more uniform aligned fiber coating. The tracking was performed manually in this study due to the challenges of tracking bacteria over textured surfaces in bright-field microscopy images. An automated tracking program would significantly speed up this type of study, enabling examination of larger parameter space.
The mechanism of the effect of aligned nanoscale fiber coating on the near-surface motions remains unclear. By further studying, one may develop a more tunable method to control the bacterial near-surface motion, thus, control the irreversible attachment.
Appendix A

One possible factor that may contribute to bacteria motion and attachment is the duration of that the bacteria interaction with the surface. Thus, first, we tested the influence of time on the motion of bacteria and bacteria settlement outcomes. Bacteria tracking was carried out at three 5-minute time intervals: 0 to 5 min, 25 to 30 min, and 55 to 60 min, with $t = 0$ min being the moment that bacterial culture was introduced onto the substrate. Figure 2.25 shows the percentage of different types of settlement for each sample type at different time points. Statistics showed that there is no significant difference among time points for each sample. Thus, tracking from all three intervals are considered as one data group for further analysis.

![Figure 2.25 Settlement vs. Sample Types as a function of time. Bacteria used for each type is a combination of at least two repeated experiments.](image-url)
Figure 2.26 showed the probability of the number of motions for each substrate type. As a result, the analyses were carried out for the bacteria that only performed one type of motion.

Figure 2.27 shows the percentage of different motion types for each sample type at different time points. Statistic showed that there is no significant difference among time point for each sample. Thus, tracking from all three intervals were considered as one data group for further analysis.
For bacteria swimming on the fiber coated samples, the directions of bacteria motion were categorized based on its swimming direction according to the direction of respect to fibers as shown in Figure 2.28. The categories are: At an angle with respect to the fibers, cross fibers (moving perpendicular to the direction of fibers), follow the fiber direction (moving parallel to the fibers) and N/A (the direction of the fiber cannot be categorized). Type of motion is not dependent on the bacteria orientation with respect to the fibers.

Figure 2.27 Motion Type vs. Sample Types as a function of time. Bacteria used for each type is a combination of at least two repeated experiments.
<table>
<thead>
<tr>
<th>Motion Type</th>
<th>Sample Type</th>
<th>N</th>
<th>At an angle</th>
<th>Cross fibers</th>
<th>Follow fiber direction</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>375 nm, 0.7 μm</td>
<td>125</td>
<td>63%</td>
<td>21%</td>
<td>15%</td>
<td>1%</td>
</tr>
<tr>
<td>Forward</td>
<td>375 nm, 1.4 μm</td>
<td>366</td>
<td>67%</td>
<td>11%</td>
<td>21%</td>
<td>1%</td>
</tr>
<tr>
<td>Forward</td>
<td>481 nm, 0.7 μm</td>
<td>253</td>
<td>74%</td>
<td>12%</td>
<td>14%</td>
<td>0%</td>
</tr>
<tr>
<td>Forward &amp; Backward</td>
<td>375 nm, 0.7 μm</td>
<td>16</td>
<td>56%</td>
<td>25%</td>
<td>0%</td>
<td>19%</td>
</tr>
<tr>
<td>Forward &amp; Backward</td>
<td>375 nm, 1.4 μm</td>
<td>145</td>
<td>52%</td>
<td>10%</td>
<td>9%</td>
<td>30%</td>
</tr>
<tr>
<td>Forward &amp; Backward</td>
<td>481 nm, 0.7 μm</td>
<td>77</td>
<td>44%</td>
<td>14%</td>
<td>19%</td>
<td>22%</td>
</tr>
<tr>
<td>Sharp Turn</td>
<td>375 nm, 0.7 μm</td>
<td>22</td>
<td>64%</td>
<td>0%</td>
<td>18%</td>
<td>18%</td>
</tr>
<tr>
<td>Sharp Turn</td>
<td>375 nm, 1.4 μm</td>
<td>113</td>
<td>54%</td>
<td>13%</td>
<td>17%</td>
<td>16%</td>
</tr>
<tr>
<td>Sharp Turn</td>
<td>481 nm, 0.7 μm</td>
<td>123</td>
<td>69%</td>
<td>3%</td>
<td>16%</td>
<td>11%</td>
</tr>
<tr>
<td>Tight Turn</td>
<td>375 nm, 0.7 μm</td>
<td>11</td>
<td>64%</td>
<td>0%</td>
<td>18%</td>
<td>18%</td>
</tr>
<tr>
<td>Tight Turn</td>
<td>375 nm, 1.4 μm</td>
<td>47</td>
<td>51%</td>
<td>15%</td>
<td>2%</td>
<td>32%</td>
</tr>
<tr>
<td>Tight Turn</td>
<td>481 nm, 0.7 μm</td>
<td>30</td>
<td>50%</td>
<td>17%</td>
<td>3%</td>
<td>30%</td>
</tr>
<tr>
<td>Twitching</td>
<td>375 nm, 0.7 μm</td>
<td>12</td>
<td>33%</td>
<td>8%</td>
<td>25%</td>
<td>33%</td>
</tr>
<tr>
<td>Twitching</td>
<td>375 nm, 1.4 μm</td>
<td>38</td>
<td>42%</td>
<td>16%</td>
<td>13%</td>
<td>29%</td>
</tr>
<tr>
<td>Twitching</td>
<td>481 nm, 0.7 μm</td>
<td>23</td>
<td>17%</td>
<td>9%</td>
<td>13%</td>
<td>61%</td>
</tr>
<tr>
<td>Wide Turn</td>
<td>375 nm, 0.7 μm</td>
<td>29</td>
<td>59%</td>
<td>21%</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>Wide Turn</td>
<td>375 nm, 1.4 μm</td>
<td>291</td>
<td>69%</td>
<td>19%</td>
<td>8%</td>
<td>4%</td>
</tr>
<tr>
<td>Wide Turn</td>
<td>481 nm, 0.7 μm</td>
<td>191</td>
<td>64%</td>
<td>20%</td>
<td>11%</td>
<td>5%</td>
</tr>
</tbody>
</table>

Figure 2.28 Motion Type vs. Direction for each sample type
3. Effect of aligned nanoscale structure on Uropathogenic Escherichia coli biofilm formation on the surface of ureteral stents

3.1 Abstract

Microbes in nature live collaboratively in communities called biofilm. Biofilms can be detrimental in a variety of applications. In medical implants, biofilm causes infections leading to additional health costs of billions of dollars. The ureteral stent is a type of medical device used to maintain the natural flow of urine in the urinary tract. Ureteral stents serve great for medical purposes. But as foreign bodies, they also lead to urinary tract infections (UTIs).

Studies have found that surface topography in micro/nanoscale can significantly accelerate or decelerate the process of biofilm formation. In this chapter, the effect of aligned polyethylene oxide (PEO) nanofiber on the adhesion of urinary pathogenic *E. coli* on clinical used ureteral stents was studied. The aligned PEO fiber coating with controlled fiber diameter and edge-to-edge spacing were produced using Spinneret-based Tunable Engineered Parameters. The experiments were conducted in a self-made setup. The surface coverages on urinary stents were quantified every 24 hours of incubations for three days using fluorescent microscopy imaging. After three days of incubation, the biofilms were removed from each stent and quantified using colony counting. The comparison between fluorescent microscopy and colony counting showed that fluorescent microscopy is a better method for future studies.

The results showed that the aligned PEO nanofiber significantly affected the biofilm formation process on the ureteral stent. Some types of aligned fiber coating accelerated the biofilm formation. However, there exist types of aligned fiber coating with certain diameter and spacing which is capable of reducing the bacterial surface coverage, which provides directions for future studies.
3.2 Introduction

3.2.1 The State of Art of Ureteral Stents

Implantable medical devices often remain inside the human body fully or partially after surgery with a duration ranging from few days to the lifetime. Medical devices are desirable for their medical purpose. However, as foreign bodies, the implants often lead to infections that cannot be easily treated by antibiotics.

The ureteral stent is a type of medical device placed in the urinary tract to allow the kidneys to drain urine. Ureteral stenting is largely performed before and after the removal of kidney stones. The ureteral stent is placed along the ureter with one end placed inside the kidney and the other end in the bladder. The stents are hollow and are about several millimeters in outer diameter. When the kidney stone is blocking the ureter but the patient is unable to go through the surgery of kidney stone removal, the ureteral stent can be used as an emergency treatment to drain urine. The stent is also used after the surgery of kidney stone removal. Because the ureter tends to swell after the surgery and the placement of the stent ensures the natural flow of urine.

The ureteral stent was invented as a treatment for ureter injuries to restore the natural flow of urine by J. P. Herdman in 1948. The commonly used double-J stents were designed by Roy Finney in 1978 which the two hooks at the end help lock the stent in place as shown in Figure 3.1 a). The design has been used extensively since then.
Ureteral stents can be categorized into polymeric and metallic stents based on their base materials. Metallic stents were developed due to its rigidity which can extend the indwelling time up to 12 months by preventing the stents and ureters from collapse. However, there are issues related to the biocompatibility and stability of metallic stents. Metallic stents are not suitable for patients that are experiencing certain types of other medical conditions such as cancer since it may deteriorate the conditions.

Polymeric stents are the most commonly used nowadays due to their flexibility and stability. The commonly used polymers include polyurethane, silicone, polystyrene, and polyethylene. The disadvantages of polymeric stents are mostly material dependent. Silicone has good anti-adhesion property and is less stiff. Thus, silicone stents have good performance once placed in place. But the relatively low stiffness leads to difficulties during insertion as the stents can hardly going through the swelled ureter. Polyethylene has good anti-adhesion property and has a low friction coefficient, but it is structurally unstable in the urine leading to collapses. Polystyrene
has relatively low biocompatibility. Polyurethane is stable, biocompatible, and has a low friction coefficient, but it leads to more bacterial adhesion and encrustation compared to other materials.

Recently, biodegradable stents are also being investigated. Biodegradable stents have the potential to serve for drug delivery as the stents degrade. However, due to low rigidity and low absorption rate, biodegradable stents are still undergoing in vitro testing.\(^{93}\)

3.2.2 Current Strategies to combat stent-induced UTIs

Even though the use of the stenting technique is relatively matured, there remain several stent-associated complications, which can lead to significant morbidity. Stent-induced UTI is one of the stent-associated complications that tend to have a significant impact on patients.\(^{94}\) Approximately 70-80\% of UTIs are catheter or stent associated, leading to $3.5 billion in healthcare cost per year in the U.S. and has the potential of secondary bloodstream infections.\(^{95}\) Bacterial attachment leads to colonization and, further, leads to mineralization and encrustation as shown in Figure 3.1 b, c)\(^{96,97}\). The encrusted stent cannot be removed from the insertion path, thus, requires surgery for removal.\(^{23}\)

Strategies of biofilm control can be categorized into two major groups: removal and prevention.\(^{21}\) Perhaps the most traditional way is to remove the biofilm using physical or chemical methods, such as using mechanical force, or antibiotics.\(^{22}\) However, for medical implants such as the urinary stent, biofilm removal often requires removal of the medical device from patients’ body, which introduces additional discomfort and cost.\(^{23}\) Thus, the solution to this problem is limited to prevention. To delay the onset of biofilm formation, researchers have conducted studied to alter the efficiency of the first step of biofilm formation which is the reversible attachment.

Engineers and doctors have come up with various strategies to prevent stent induced UTIs. For metallic stents, magnesium and its alloys have been shown to greatly decrease the viability of
*E. coli* on the surface. Sliver or sliver compound stent coatings also appeared to provide good antimicrobial properties. However, sliver coated stent has a higher risk of stent-associated complications such as argyria due to its instability leading to a short indwelling time. The short indwelling time may lead to multiple insertions thus lead to higher costs.

Antibiotic coatings are, perhaps, the most commonly used strategies for UTIs. The antibiotic coatings can be divided into two categories as bacteriostatic coating, which arrests the growth of the microbes and bactericidal, which kills the microbes attached to the surface. Commonly used antibiotics include levofloxacin, ciprofloxacin, nitrofurantoin, trimethoprim, and ampicillin. Antibiotic coatings are effective for stents for only a couple of days. For stents with long indwelling time, the antibiotic coatings may promote the development of antibiotic resistance phenotype. Although the biodegradable stents provide opportunities for a gradual release of antibiotic and extended function, the problem with the uropathogen developing antibiotic resistance cannot be ignored. For example, research in 2018 shows that 62.6% of the uropathogen isolated from UTIs have ampicillin resistance in Turkish children under the age of eight. As a summary, antibiotic coatings can be an efficient treatment method, but its use should be limited to slow down the emergence of antibiotic resistance traits among uropathogen. Besides, the killed microbes remain on the surface and serve as a nutrient source, further promoting microbial attachment.

### 3.2.3 Innovation of the Presented Study

Considering the limitations of the solutions mentioned above, innovative methods are needed to combat stent-induced UTIs. Researchers have developed non-toxic physical surface modification methods to alter the adhesive properties of surfaces. Specific solutions include using
superhydrophobic and hydrophilic coatings, and introducing micro/nanoscale surface topographical features.

However, there seems to be no study conducted on the effect of surface topography on UTIs on the clinical ureteral stents. To fill the technological gap, the effect of surface topography on ureteral stents were studied in this research. Due to the limitation of materials that can be used for medical implants, polyethylene glycol/oxide (PEG/PEO) is selected to be used in this study. PEG and PEO are the same synthetic polyether with different molecular weights. PEGs refer to polymer chains with molecular weight smaller than 100,000, while PEOs refers to the ones with higher molecular weight.

The modification proceeded in two directions. The first approach is to attach PEG/PEO brushes onto the surface to make the surface hydrophilic. The method and results of this approach are reported in the Appendix. The second approach is to use PEG/PEO to develop micro/nanoscale surface topography. However, the first approach had a serious problem in the functional period (see Appendix). In this study, the surface topographical modification in the form of nanofibers of well-defined diameter and spacing was achieved using the Spinneret-based Tunable Engineered Parameters (STEP) technique described in Chapter I.

3.2.4 Choice of Bacterial Culture

The cause of UTIs can be both Gram-positive and Gram-negative bacteria and fungi. The strain used in this study is Escherichia. coli CFT073 (ATCC 700928), a clinical isolated UPEC E. coli CFT073 strain. This strain was chosen since 70% of UTIs are caused by urinary pathogenic E. coli (UPEC). E. coli CFT073 is a rod-shape, Gram-negative strain that is non-motile (when grown in Nutrient Broth).
3.3 Materials and Methods

3.3.1 Cell culture

The wild type *E. coli* CFT073 was transformed with a plasmid with BBa_J04450 (iGEM Foundation, Cambridge, MA), which is chloramphenicol (CAP) sensitive and expresses red fluorescent protein constitutively to enable fluorescent imaging. The culture was streaked on 1.5% Nutrient Broth agar plate (NB, beef extract: 3 g/L, peptone: 5 g/L and 15 g/L Bacto agar) with CAP (25 μg/ml) from frozen stock stored at -80 °C and incubated at 37 °C for 22-24 hours. One normal colony (round shape and typical size) was picked and used to inoculate 10 mL NB medium in a 125 mL baffled flask. The culture was incubated at 37 °C and shaken at 150 rpm for 22-24 hours to reach the stationary phase of growth. From optical microscopy, bacteria with transformed plasmid in the overnight culture in Nutrient Broth have the length ($L_c$) of $1.48 \pm 0.34 \mu m$ and the diameter ($D_c$) of $0.79 \pm 0.09 \mu m$.

3.3.2 Substrate Preparation

The urinary stent used in this study was a type of black silicone stent from Cook Medical (G15146). The stent was cut into 5-mm long pieces and placed on 22-mm long glass capillary (Sutter Instrument, BF120-60-10) as shown in Figure 3.2. The substrates are then sonicated for 60 seconds and rinsed with DI water, ethanol, and air-dried.

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2 Trevor Nier and Hajar Chokhmane conducted the dynamic retention essay experiments in section 3.3.
3.3.3 Nanofiber Coating

The assembled substrates were coated with aligned polyethylene oxide (PEO, MW: 900k) fibers of controlled fiber diameter and edge-to-edge spacing using the Spinneret-based Tunable Engineered Parameters (STEP) technique. Representative images of the fiber-coated stents are demonstrated in Figure 3.3. The PEO fibers are dyed with fluorescein to enable fluorescent microscopy as shown in Figure 3.3 d).

![Figure 3.2 Substrate setup. Urinary stent on glass capillary](image)

To manufacture aligned PEO fiber coating using the STEP method, a solution of PEO was prepared. Briefly, 0.005 g fluorescein was added to 1 mL of DI water. Subsequently, 1 mL of 100% ethanol was added to the fluorescein-water mixture. Lastly, 0.1g PEO (MW: 900k) was added into the water-ethanol solution and stirred at 100 rpm overnight using magnetic stirrer under shading. The crosslinking agent pentaerythritol triacrylate (PETA), at concentrations specified below, was
added into the solution the following day. The mixture was stirred at 100 rpm for 24 hours under shading. After stirring, the mixture is ready to use for the STEP technique.

PEO is soluble in water; thus, it will dissolve in the water-based culture medium. Thus, PETA was added when making the PEO solution. After deposition of fiber on stents, the substrates were placed onto a custom crosslinking apparatus which rotated the substrates at 15 rpm inside an ultraviolet (UV) crosslinker (UVP Crosslinker, Analytic Jena, Thuringia Germany) for 15 mins under UV-light of 254 nm wavelength with light intensity of 11.5 mW/cm². The crosslinking device is shown in Figure 3.4. The crosslinking time was determined iteratively (see section 3.4.1) such that the PEO fibers become insoluble in the culture medium after cross-linking.

![Arms to slide glass tube with mounted stent onto](image)

Figure 3.4 Custom crosslinking apparatus.

### 3.3.4 Sample Assembly

The substrates were placed into the glass-bottom well plate and attached using Polydimethylsiloxane (PDMS). The assembled well plate was placed at the room temperature for 48h for PDMS to cure. Before use, the well plate was placed in the UV crosslinker for 10 mins for sterilization under UV-light of 254 nm wavelength with a light intensity of 11.5 mW/cm². The assembled experimental setup is shown in Figure 3.5.
3.3.5 Inoculation

The overnight *E. coli* culture was washed twice at 3000×g for 8 minutes and resuspend in nutrient broth (NB). The washed culture was then diluted to an OD$_{600}$ = 0.03 using NB. A 2 ml aliquot of the diluted culture was added into each well. The well plate was then parafilm and placed on the Belly Button Shaker (IBI Scientific, Vernon Hills, IL) in the incubator at 37 °C. After 2 hours of incubation, the initial bacterial suspension was diluted out through the addition of 2 ml of fresh NB medium into each well and removal of 2 ml medium from each well.

![Figure 3.5 Adhesion Assay Experimental Setup. a) Glass-bottom well plate on Belly Button Shaker (IBI Scientific, Vernon Hills, IL) b) Substrates inside self-made glass-bottom well plate c, d) Assembled substrates](image)

3.3.6 Imaging

Fluorescence imaging was done after 24, 48, and 72 hours of incubation as shown in Figure 3.6. Before each imaging, 2 mL of fresh NB medium was added into each well and 2 mL medium was aspirated out from each well to dilute the planktonic bacteria. The well plate was placed on an inverted optical microscope (Zeiss AxioObserver Z1) at 37 °C. The bottom surfaces of each
stent sample were brought in focus and images were taken with an AxioCam MRm camera and a 40x objective. Fluorescent images for PEO fibers with fluorescein was taken at band-pass 450-490 nm excitation wavelength with a 500-550 band-pass emission filter. Fluorescent images for bacteria with red protein was taken at band-pass 525-575 nm excitation wavelength with a 570-640 nm band-pass emission filter. Representative images of the fiber-coated and unmodified after 24 hr incubation with bacterial culture are shown in Figure 3.6.

![Figure 3.6](image.png)

Figure 3.6 a) fiber-coated stent where green represents aligned nanofiber and red represents bacteria. b) uncoated stent. Images were taken after 24 hours of incubation with bacteria.

### 3.3.7 Image Processing

The fluorescence microscopy images were exported from the microscope image acquisition software ZEN 2 (blue edition) using the best fit black and white values for each image as 8-bit grayscale tiff files. The images were converted into binary images using appropriate local and global threshold values. A representative set of a grayscale image and the corresponding binary image are shown in Figure 3.7 using a custom MATLAB GUI image processing routine previously developed in our lab. The number of white pixels in each image was recorded to quantitate bacterial surface coverage.
3.3.8 Colony Counting

After the last day of imaging (i.e., the 24 hr time point), each substrate was carefully removed from the glass capillary support, washed in phosphate-buffered saline (PBS), and placed into a 15 mL test tube with 5 mL of PBS buffer. To remove the bacteria from the stent surface, the test tube was then sonicated (frequency: 40 kHz, Power input: 80 W) in a water bath for 30 seconds and kept on a vortex mixer at 1000 rpm for 30 seconds. The sonication and vortexing steps were repeated three times. Subsequently, the suspension of bacteria in PBS was diluted at the optimal dilution factor using serial dilution and plated on 2% Lysogeny broth (LB; 1% tryptone, 0.5% yeast extracts, 1% NaCl) agar plates. The plating for each sample was done at the least two dilution factors. The plates were incubated at 37 °C for 22-24 hours. The number of colonies was counted for each plate and was used to calculate the colony-forming unit (CFU)/mL of the original suspension and ultimately stent surface coverage in CFU/µm².

3.3.9 Statistical Analysis

Statistical analyses were performed using individual t-tests between each pair of samples assuming unequal variance. A significant difference was claimed if the p-value was less than 0.05.
3.4 Results and Discussions

3.4.1 PEO Fiber Manufacturing

Recall that PEO is soluble in water, thus, soluble in the water-based culture medium. To increase the stability of aligned PEO fibers, PETA, a UV active crosslinking agent was added to the PEO solution used for the STEP technique.

The effect of PEO to PETA ratio (w/w) was tested by maintaining the concentration of the PEO constant while changing the concentration of PETA. The PEO to PETA w/w ratio of 10:1, 10:1.5, 10:2, and 10:2.5 were tested. The effect of the PEO concentration on fiber diameter and solubility was studied using SEM and fluorescence microscopy, respectively. As shown in Figure 3.8, the SEM images showed that as the concentration of PETA increased, the PEO fiber diameter also increased.

![SEM images of PEO fibers made from solutions with PEO to PETA w/w ratio of a) 10:1; b) 10:1.5; c) 10:2; d) 10:2.5. f) Fiber diameter vs. PEO to PETA w/w ratio measured from SEM images (n = 28, 27, 28, 30 for cases a, b, c, and d, respectively).]

Figure 3.8 SEM images of PEO fibers made from solutions with PEO to PETA w/w ratio of a) 10:1; b) 10:1.5; c) 10:2; d) 10:2.5. f) Fiber diameter vs. PEO to PETA w/w ratio measured from SEM images (n = 28, 27, 28, 30 for cases a, b, c, and d, respectively).
After fiber deposition, the fiber-coated samples were placed into a UV crosslinking unit (UVP Crosslinker, Analytic Jena, Thuringia Germany). The appropriate crosslinking period was determined iteratively. The crosslinking time was first tested for the fibers made from the solutions with the lowest PETA concentration (PEO to PETA w/w ratio of 10:1). Fibers were crosslinked at 254 nm wavelength and 11.5 mW/cm\(^2\) intensity for 5, 10, 15, and 20 mins each and incubated in culture medium to test the stability. Fluorescence microscopy images showed that the samples that were crosslinked for 10 mins and longer maintained the aligned coating after 98 hours of incubations. Microscopy images of the samples that were crosslinked for 10 mins are shown in Figure 3.9. To ensure all fibers are properly crosslinked, all the samples used in the experiments were crosslinked for 15 minutes.

![Figure 3.9 Aligned PEO fiber coating with PEO to PETA w/w ratio of 10:1 and 10 mins UV crosslinking treatment.](image)

### 3.4.2 PEO Fiber Coating Parameters

To study the effect of aligned PEO fibers on bacterial surface coverage compared to the uncoated samples, adhesion assay was performed according to the methods described in section 3.3.5. Each experiment included triplicates of the uncoated sample (control) and one type of coated samples. The parameters of the fiber coating in each experiment are listed in Table 3.1.
3.4.3 Analysis of Bacterial Surface Coverage

The bacterial surface coverage for each substrate was measured every 24 hours. The results are shown in Figure 3.10-3.12. Overall, there exists a significant difference between the percentage of surface coverage of uncoated samples and all types of fiber-coated samples. For stents coated with fibers at $D_f = 320$ nm, $S = 1.39$ μm, after 24 hours of incubation, the surface coverage was on average 49% less, compared to the uncoated samples (p<0.001) as shown in Figure 3.10.

<table>
<thead>
<tr>
<th>Stent Material</th>
<th>Fiber Diameter ($D_f$, nm)</th>
<th>Fiber Spacing ($S$, μm)</th>
<th>Duration (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicone</td>
<td>$320 \pm 50$ (n = 3)</td>
<td>$1.39 \pm 0.56$ (n = 3)</td>
<td>1</td>
</tr>
<tr>
<td>Silicone</td>
<td>$420 \pm 45$ (n = 3)</td>
<td>$1.03 \pm 0.40$ (n = 3)</td>
<td>3</td>
</tr>
<tr>
<td>Silicone</td>
<td>$673 \pm 209$ (n = 3)</td>
<td>$1.66 \pm 0.7$ (n = 3)</td>
<td>3</td>
</tr>
<tr>
<td>Silicone</td>
<td>$675 \pm 146$ (n = 3)</td>
<td>$1.4 \pm 0.63$ (n = 3)</td>
<td>3</td>
</tr>
<tr>
<td>Polyurethane</td>
<td>$350 \pm 145$ (n = 3)</td>
<td>$0.9 \pm 0.45$ (n = 3)</td>
<td>3</td>
</tr>
</tbody>
</table>
For stents coated with fibers at $D_f = 420 \text{ nm}$ and $S = 1.03 \mu\text{m}$, after 24 hours of incubation shown in Figure 3.11, the surface coverage of bacteria was 72% higher than that of the uncoated samples. However, after 48 hours of incubation, the surface coverage of the coated samples dropped to 56% less compared to the uncoated samples. After 72 hours of incubation, the surface coverage between coated and uncoated samples are approximately the same (2% of difference).
For fiber-coated samples with $D_f = 675 \text{ nm}$ and $S = 1.40 \mu \text{m}$, after 24 hours of incubation shown in Figure 3.12, the surface coverage of bacteria was 57% less compared to uncoated samples. After 48 hours of incubation, the surface coverage difference remained unchanged where the coated samples have 58% less surface coverage compared to uncoated samples. After 72 hours of incubation, the coated samples had 45% less surface coverage compared to the uncoated samples.
In addition, it was observed that the surface coverage of the fiber-coated samples was more consistent across the triplicate samples, compared to the uncoated samples, nonetheless statistically significant difference amongst the triplicates was not observed for any of the sample types.

**3.4.4 Colony Counting Results**

The effect of aligned PEO fiber coating on the biofilm formation of the ureteral stents was also measured through colony counting at the end of the duration of incubation, as described in the method section. The colony counts on each plate were converted back to CFU/mL based on the dilution factor used for each sample. The bacteria coverage in CFU/mL for each sample was normalized to the average CFU/mL of the uncoated samples in the same set of experiments at the same time points.

The results of the normalized bacterial coverage from colony counting are shown in Figure 3.13. For fiber coated samples with $D_f = 320$ nm and $S = 1.39$ μm, the aligned fiber coating decreased the bacterial attachment by 28% compared to the uncoated sample after 24 hours of incubation. For fiber coated samples with $D_f = 675$ nm and $S = 1.40$ μm, the aligned fiber coating increased the attachment by 23% after 72 hours of incubation. For fiber coated samples with $D_f = 420$ nm and $S = 1.03$ μm, the aligned fiber coating increased the bacterial attachment by 87% compared after 72 hours of incubations.
3.4.5 Comparison of Surface Coverage and Colony Counting Results

The surface coverage analysis and colony counting were performed to assess the accuracy and repeatability of each procedure. Table 3.2 shows the comparison between the results from surface coverage and colony counting by showing the percentage difference of the bacterial coverage of the coated samples to the uncoated samples with respect to the uncoated samples. The negative sign indicates that the aligned PEO fiber coating decreased the bacterial attachment on the stents. The surface coverage results from the last day of incubation of each set of the experiment were expected to agree with the results of colony counting in the trend. However, the results from the two methods did not match.

<table>
<thead>
<tr>
<th>Experiment #</th>
<th>$D_r$ (nm)</th>
<th>$S$ (µm)</th>
<th>From Surface Coverage</th>
<th>From Colony Counting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$T = 24$ h</td>
<td>$T = 48$ h</td>
</tr>
<tr>
<td>No. 1</td>
<td>420.8</td>
<td>1.03</td>
<td>0.72</td>
<td>-0.56</td>
</tr>
<tr>
<td>No. 2</td>
<td>674.5</td>
<td>1.4</td>
<td>-0.57</td>
<td>-0.58</td>
</tr>
<tr>
<td>No. 3</td>
<td>319.8</td>
<td>1.39</td>
<td>-0.49</td>
<td></td>
</tr>
<tr>
<td>No. 4</td>
<td>673.6</td>
<td>1.66</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After comparing the method of measurement and the variance of results, it was concluded that the procedure of colony counting had lower repeatability. Thus, the result of colony counting...
remains questionable. In light of this finding, the decision was made that the surface coverage analysis using fluorescence microscopy images provided more accurate results. This method will be carried on for future studies.

3.4.6 Preliminary Results for Bacterial Surface Coverage of Polyurethane Ureteral Stents

The commercially available ureteral stents are made of a variety of materials, as discussed in 3.1. Future research will be conducted to study the effect of aligned PEO fibers on ureteral stents made of different materials using the established adhesion assay and surface coverage analysis. To demonstrate the generalizability of the established experiment and analysis methods, an experiment was conducted with a type of polyurethane stent (Cook Medical, G23317) with aligned fibers of $D_f = 350 \pm 145$ nm and $S = 0.90 \pm 0.44 \mu$m.

The results, presented in Figure 3.14, showed that there exists a significant difference between the bacterial surface coverage of the coated and uncoated samples at 24 and 72 hours of incubation. At 24 hours of incubation, the aligned fiber coating increased the bacterial attachment by 95%. However, at 72 hours of incubation, the aligned fiber coating reduced the attachment by 49%.
3.5 Conclusions and Future Work

The bacteria attachment comparison using surface coverage quantification and colony counting showed that the aligned fiber coating can significantly alter the bacterial attachment. In most cases, the topography-induced change in attachment density was sustained over the 72 hr period of the experiment. The goal of this study was to determine the aligned fiber coating that reduces the bacterial attachment. Consistent with our prior work in this area, the bacteria attachment varies with fiber diameter and spacing. After 24 hr incubation, fiber coating of $D_f = 675$ nm and $S = 1.40$ μm and $D_f = 320$ nm and $S = 1.39$ μm decreased bacteria attachment by 57% and 49%, whereas fiber coating of $D_f = 420$ nm and $S = 1.03$ μm increased the bacteria attachment by 72%.

For future studies, the surface coverage quantification from fluorescence microscopy imaging will be used. This method appeared to contain fewer errors and is more cost-effective. However, the confocal imaging showed that the biofilms were already grown to a thickness of 10
μm that cannot be ignored after 24 hours of incubation. Thus, in future studies 3D imaging and image processing methods that account for the biofilm thickness need to be developed.

Another possible improvement of methods could be alternating the material of the fibers. PEO with a lower molecular weight (PEG) can be used to form brushes on the surface of the fibers and provide another level of prevention of attachment. This was explored through the use of Pluronic® coating, as described in the Appendix, and showed promising results on reducing bacteria attachment. However, the stability of the Pluronic coating in biofluids needs to be carefully investigated. Additionally, other materials such as polyurethane can also be used to produce aligned fiber coating. However, when selecting the materials, one needs to keep in mind the biocompatibility of the potential materials.

Overall, the results showed that the aligned fiber coating can alter the bacterial surface coverage. Thus, it has the potential to reduce the attachment. Future experiments will be conducted to study the effect of aligned fiber coating on stents made from different materials. Furthermore, there are also potentials to apply the aligned fiber coating using other types of polymers such as PEG and PU.
Appendix B

Prior to the study of aligned PEO fiber coating on urinary stents, the preliminary studies were conducted to test the effect of PEO coating on 2D substrates.

Materials and Methods

2D Substrate Preparation
Polystyrene sheet with 0.125 mm thickness (Goodfellow) was cut into 20 mm by 3 mm substrates to serve as the 2D substrates. The substrates were sonicated for 60 seconds and rinsed with DI water and ethanol, then air-dried.

PEG Coating on 2D Substrate
Pluronic F108 \[ (PEG)_{129} - (PPG)_{43} - (PEG)_{129} \] is selected to attach to the 2D substrates to serve as the PEG brushes. Pluronic is a kind of synthetic copolymer with PEG groups on the two ends and Polypropylene glycol (PPG) at the center. The schematic is shown in Figure 3.15. Pluronic is chosen because of its physicochemical property of being amphiphile (both hydrophilic and hydrophobic). Thus, Pluronic can be easily anchored to the hydrophobic substrates while forming the hydrophilic PEG brushes.

![Schematic of Pluronic](image)

Figure 3.15 Schematic of Pluronic.

Pluronic Anchoring
Pluronic F108 was dissolved in DI water to make 1 wt.% Pluronic solution. 1ml of Pluronic solution and 2 μl of toluene was added in each of the 2-ml vials. The 2D substrates are added in
each vial and submerge in the solution at room temperature. After 12 hours, the Pluronic solution is removed and 1 ml of PBS is added in each vial to wash the substrate. The substrates are ready to use for the experiments.

The rest of the experiments were conducted following the same procedure as described in Chapter III.

**Results**

The normalized cell attachment densities for the surfaces treated with Pluronic and untreated are shown in Figure 3.16 below. Pluronic treated surface had significantly fewer bacteria coverage compared to the untreated samples.

![Bacteria 16-hour Assay](image)

Figure 3.16 Surface coverage of untreated surface and Pluronic treated surface.

However, the problem exists since the Pluronic was not anchored on the surface permanently. The effect of Pluronic coating disappears after changing the culture medium. Thus,
this approach cannot be achieved with a long-term effect so far. As a result, the experiment was carried on by studying the effect of surface topography.
5. Conclusions and Future Work

Studies have found that surface topography in micro/nanoscale can significantly accelerate or decelerate the process of biofilm formation. However, the causation of the effect is not entirely known. In this research, the effect of aligned nanoscale surface structure on microbial adhesion was studied.

First, the effect was studied at the individual cell level by studying the effect of aligned fiber coating on individual bacterium adhesion decision making and near-surface motile behavior. Aligned fiber coating can significantly alter the bacterial attachment for both the short-term and long-term. It was found that the 375 nm diameter-700 nm spacing fiber coating had the greatest impact on the bacterial attachment and bacterial near-surface motions for both reversible attachment and biofilm formation. The 375 nm diameter-700 nm spacing fiber coating had visibly more attachment during the long-term (up to 24 hr.) imaging and was eight times more likely for bacteria to attach compared to all the other sample types (within 1 hour).

Aligned fiber coating showed significant effects on the kinematics of near-surface motion. Interestingly, significantly fewer bacteria approach the 375 nm diameter-700 nm spacing fiber coating. However, those who do are more likely to attach. The difference in number became significantly less once adding the number of bacterial already attached to the surface prior to the tracking period.

Future work will focus on the hydrodynamic and biological underpinnings of near-surface motility as well as the differences in the number of bacteria approaching the surface. Besides, the results have shown that bacterial attachment and near-surface behavior can be affected by surface topography. Potentially, there may exist aligned fiber coating with certain diameters and spacings to reduce the bacterial adhesion which can be identified experimentally.
Furthermore, the study extended to the surface of ureteral stents, a type of medical implants that serves great for medical purposes and causes infections as foreign bodies. The effect of aligned polyethylene oxide (PEO) nanofiber on the adhesion of uropathogenic *E. coli* on clinically-used ureteral stents was studied. The results showed that aligned PEO nanofibers coating can significantly alter the bacterial attachment sustainably over the 72 hours of the experiment.

Some types of aligned fiber coating accelerated the biofilm formation. However, the aligned fiber coating has the potential to reduce bacteria attachment density for both silicone and polyurethane stents over the 72 hours of the experiment. For silicone stents, the 657 nm diameter-1.4 μm spacing fiber coating decreased the surface coverage by 57% after 24 hours, 58% after 48 hours, and 45% after 72 hours. For polyurethane stents, the 350 nm diameter-0.9 μm spacing fiber coating increased the surface coverage by 95% after 24 hours, decreased the surface coverage by 16% after 48 hours, and 49% after 72 hours.

For the future study to develop anti-adhesion coating for medical implants, one may consider introducing Pluronic coating or other similar brush coatings, after solving the problem of its instability in biofluids. The aligned nanofiber coating can also be produced using materials such as polyurethane or silicone, which matches the materials that are commonly used for ureteral stents.
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