0Construction of a Flow Through Channel for the Observation of Early Events in Biofilm Formation

Kylie Sumner
Department of Chemistry and Biochemistry
Worcester Polytechnic Institute

Major Qualifying Project
May 2021

Abstract

One major problem in biomedical sciences is the risk of infection associated with prosthetic implants. A majority of these infections are attributed to the formation and growth of bacterial biofilms on the prosthetics. Infections from medical implants, on average, cost the United States two billion dollars each year. A biofilm is a group of bacteria that are able to adhere tightly to surfaces by the excretion of extracellular polymeric substances (EPS). This coating also protects the bacteria and makes them more resistant to antibiotics. The early steps of biofilm formation include adhesion, aggregation and accumulation, and release. The early stages of biofilm formation begin as soon as a population of bacteria meets a surface, and the device that was designed for this work can monitor these steps to give insight into the mechanisms of biofilm adhesion and to research possible preventative measures. The device uses electrochemical impedance spectroscopy along with fluorescence microscopy to monitor the initial steps in biofilm formation. The device was designed and milled in-house and the flow inside the channel was modelled using computational fluid dynamics.

Introduction

Biofilm (Definition)

Biofilms are accumulations of microorganisms, such as bacteria, that form a complex structure and act as a community on a surface [1]. These biofilms are not just a clump of organisms, the microorganisms also excrete a polymer known as extracellular polymeric substances (EPS). This EPS is a highly elastic polymer that is responsible for attaching the microorganisms to the surface and encasing each cell in the community [2]. The biofilm creates a matrix in the EPS with channels for nutrient uptake [1]. Biofilms are found in fossil records that are over three billion years old, making it apparent that the ability to form biofilms is advantageous to the organism when growing in diverse environments. The formation of biofilms can protect the organisms from external threats like antibiotics, UV exposure, dehydration, salinity, phagocytosis, and others that are present across a range of environments [1].

Biofilm (Non-medical biofilms)

In nature, biofilms are used by microorganisms as a form of protection from external sources [1]. The microorganisms are able to better communicate in the biofilm matrix and the EPS is able to be adjusted by the cells according to the environmental stimuli [1]. Biofilms can form on many types of surfaces and environments, including most natural aquatic environments, water pump systems, medical implants, and living tissue [3].

One industry that has been affected by biofilms in particular is the food industry. The equipment in the food industry can be a source of bacterial contamination that can cause food to spoil or even spread disease. This contamination is often attributed to biofilm growth. With biofilms being able to form the EPS matrix that make them more resistant to typical antimicrobials, these biofilms can act as a persistent source of contamination. There are many different variables that contribute to the formation of biofilms. These include which bacteria is present, what the surface the bacteria are trying to attach to is like, and environmental factors. The environmental factors include the nutrients available to the bacteria and the temperature. It is known that bacteria can form biofilms on a range of different types of surfaces, including stainless steel which is commonly used in equipment in the food industry. In a test with different disinfecting agents that are commonly used in the food industry, it was found that there was a wide range of variability between planktonic bacteria and their biofilm counterparts. The idea that biofilms are more resistant to disinfectants was reinforced by these experiments. The cleaning compound that was found to be most effective against *Salmonella* species was 70% ethanol. The experiment was done with *Salmonella* species since they are a

pathogenic bacterium that are common contaminants for the industry. 70% ethanol works best against *Salmonella spp.* biofilms, but there is no biocide that works against all biofilms [4]. As with the formation of biofilms, there are many different variables that determine which biocides will work best in a specific situation. The type of bacteria, the type of surface, exposure time, concentration, pH, and whether the bacteria present any resistance are some of the variables that affect which biocide should be selected. There are preventative measures that can be taken in order to prevent the formation, maturation, and mixing of biofilms. One of these ways is to clean the food processing equipment frequently to avoid buildup and to not allow for the attached bacterial cells to mature into full biofilms. This would also prevent having biofilms with mixed species of bacteria since the equipment would, in theory, be cleaned before a new species could be introduced [4].

Another way to prevent biofilm growth is in the design of the equipment itself. If the equipment is designed to prevent laminar flow, the turbulence prevents the attachment of bacteria. The type of material that the processing equipment is designed from can also prevent biofilm formation [4]. The material that the equipment is made out of is made important once the laminar product flow is prevented and the machine is designed for regular cleaning or self-cleaning. There are different ways to modify surfaces to make them more hygienic. For example, stainless steel that was modified with polyethylene glycol showed an 81-96% decrease in the attachment and biofilm formation of *Listeria monocytogenes* in the food industry setting. Stainless steel and polyethylene terephthalate have also shown resistance to bacterial adhesion when modified by nisin, an antimicrobial peptide that is commonly used as a food preservative. The continual cleaning of the equipment is important for reducing contamination from biofilms. The cleaning process typically consists of the use of chemical disinfectants and physical methods. There are three different types of chemical disinfectants that can be used: oxidizing agents, surface-active compounds, and iodophors. The oxidizing agents that are used as disinfectants include chlorine-based compounds, ozone, hydrogen peroxide, and peracetic acid. The surface-active compounds include quaternary ammonium compounds and acid anionic compounds. The most commonly used disinfectant is chlorine because it is cost effective, easy to prepare, and easy to apply to the equipment. Physical methods are also being explored as a means of disinfecting food processing equipment. These methods include ionizing radiation, atmospheric plasma inactivation, ultrasound, and electric fields. These physical methods have also been combined with the chemical methods to have a high efficiency removal of bacteria from equipment [4].

In nature, biofilms can benefit plants. The rhizosphere is a complex microbiome that contains many different types of microorganisms, including bacterial species that form

biofilms. Some bacteria are observed to form biofilms on the roots of some plants. These biofilms are beneficial to the plant and allow for increased productivity and growth. The biofilms also have been shown to protect the plant from many different types of stresses, including infection, drought, changes in salinity, pH and temperature, and heavy metals. This data that shows benefits to biofilm growth within the rhizosphere has prompted the idea of biofilm-based fertilizers. These fertilizers could be an eco-friendly alternative to chemical fertilizers. Chemical fertilizers have had negative health effects on humans and the environment. Biofilm-based fertilizers aim to restore biodiversity in the soil and improve the soil quality where it is applied [5].

Biofilms are also used for wastewater treatment. One type of biofilm used for this application is algal biofilms. The algal biofilms can be used as a means of nutrient removal from wastewater and as a source of biomass. The use of algal biofilms is still being studied, but it appears to be a cost-effective means for wastewater treatment with the added benefit of algal biomass production. One potential problem with algal biofilm wastewater treatment is that the algae require many nutrients to grow efficiently. This is not a problem when they are being used in raw wastewater, but wastewater that has already been mostly filtered could need added nutrients for the algae to grow. The biomass that is produced by this process also has applications as biofuels, bioplastics, nutraceuticals, animal food, fertilizers, and personal care products [6].

Biofilms have also been used for bioremediation for industrial wastewater. Biofilms are chosen over planktonic bacteria for this process because of their high biomass and their ability to immobilize different compounds. The cells within the biofilm also act as a community and they communicate. The biofilm cells can also undergo gene transfer that can help the community to survive within the polluted environment while they process the pollutants. This idea has led to the idea of bioengineering cells that form biofilms for enhanced metabolic abilities for whichever pollutant they are designed to process [7].

Biofilm (Medical implications)

The formation of biofilms is a natural process that is a part of the life cycle of some microorganisms, including many bacteria [1]. This natural process can be detrimental to humans though because of the extracellular polymeric substance (EPS) matrix that encases the biofilm can make the bacteria resistant to antibiotics [8]. Biofilms are estimated to be 10,000 times more resistant to antibiotics than their planktonic counterparts [9]. They are also extremely resistant to host immune responses due to the lack of permeability of the EPS. Many human diseases are attributed to biofilms and, according to the NIH, about 80% of microbial infections involve biofilms [9]. There are many different manners that biofilms can cause infections. A few of these biofilm-related diseases include cystic fibrosis, dental plaque, chronic wounds, urinary tract infections

from catheters, cardiac valve infections, and prosthetic joint infections [9]. Staphylococcal biofilms are particularly abundant when it comes to biofilm-related infections [10]. Some of the specific infections that are commonly caused by staphylococci include: infective endocarditis, otitis media, urinary tract infections, cystic fibrosis, acute septic fibrosis, an endophthalmitis [10]. Though there are many staphylococci-related infections, they are even more common in medically implanted devices. The devices can include prosthetic heart valves, central venous catheters, urinary catheters, intrauterine devices, and prosthetic implants [10,11]. The bacteria may be introduced to the environment during implantation and then they can adhere to the nonbiological surfaces that are associated with these devices [10]. The adherence step in the biofilm formation is more common on nonbiological surfaces, which could be why these devices act as catalysts for infections [10]. In a study conducted by Anisha Fernandes and Meena Dias, 50 patients that received orthopedic prosthetic implants were examined for the presence of infection [11]. The type of bacteria causing the infection was determined after the infection was found. In this study 84% of the patients were recorded as getting an infection related to their prosthetic implant with *Staphylococcus aureus* being the most common pathogen. These infections were frequently treated with intensive antibiotic therapy, which did not work in some cases, leading to the prosthetic needing to be removed and replaced [11]. In the United States alone, it is estimated that antibiotic resistant infections cost the country over 2 billion dollars and that 23,000 Americans die from them each year [12]. This data shows the importance of studying the adherence of bacteria to the surfaces of medical implants. In particular, it is vital to gain a better understanding of the early timepoints of biofilm formation on these surfaces so that a way to prevent this growth can be discovered.

Biofilm (Detailed structure)

When a biofilm forms, the bacteria first adhere with very weak and reversible interactions with the surface [12]. They then will attach more securely with an irreversible step where the bacteria become sessile [12]. Once the bacteria are attached to a surface, they begin to excrete various polymers and they create an extracellular polymeric matrix. This matrix protects the bacterial cells from antibiotics or even host immune responses. These bacteria that are initially adhered to the surface can then proliferate and create microcolonies. These microcolonies are a thin layer of bacteria that act as the first layer of the biofilm. Once the microcolonies produce enough cells that there are many layers and channels can form between the typically mushroom-shaped cell masses, it is considered to be a mature biofilm [12]. As previously stated, the biofilm is encased in an extracellular matrix, but the exact composition of this matrix is variable between organisms [13]. It is known that the matrix typically contains eDNA, lipids, exopolysaccharides, and extracellular proteins [13]. The matrix has different compositions for different organisms along with different specific functions included with

it [13]. The extracellular matrix adds structural integrity to the community of bacteria by having proteins that act as scaffolding, it can act as a protective barrier, especially from water, and it can have components for specific interactions between the biofilm and the host in the case of infection. In any case, the extracellular matrix is extremely complex and well-organized, even having distinct channels for nutrient transport through the biofilm [13]. A cartoon diagram of a mature biofilm attached to the conductive polymer used in the experiments is shown in Figure 1. This figure shows a simplified visual for the complexity of biofilms and how they are not just a pile of slime, but they are highly complex in structure. The planktonic bacteria, EPS matrix, and the channels within the biofilm are represented in this diagram.

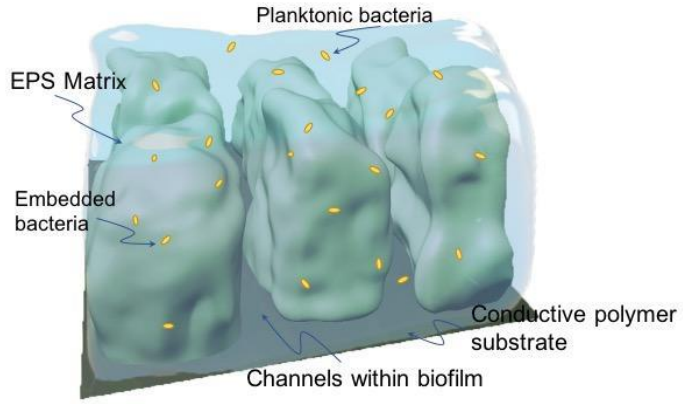

Figure 1. Diagram of Mature Biofilm on Conductive Polymer

The bacteria used for the MOBIC device experiments were *Staphylococcus aureus*. For *S. aureus*, it has been observed that they can form multilayer biofilms. This multilayered structure is contained within a glycocalyx that is mostly composed of teichoic acids and proteins (both staphylococcal and host). Another component of the EPS matrix for *Staphylococcus* biofilms is extracellular DNA (eDNA) and genomic DNA. Cells within the biofilm community undergo lysis in order to release their genomic DNA into the EPS matrix. One mechanism of the process is by an enzyme called autolysin that is upregulated in sessile *S. aureus* cells in biofilms. This enzyme can hydrolyze staphylococcal cells. There are also intracellular phage genes that are upregulated in the stressed environment within the biofilm. The main function of the eDNA in biofilms is to provide structure for the formation of the biofilm, so it must be released in early stages of its development. The formation of staphylococcal biofilms can be regulated by controlling the process of cell lysis for this reason [14].

When it comes to biofilm research, the most common way to collect biofilm data is from the formation of mature biofilms. The CDC bioreactor is a commercially available piece of equipment that is designed to measure biofilm growth. This apparatus requires the bacteria of interest to be inoculated in the “Batch Phase” for 24 hours before beginning the “Continuous Flow Phase” for another 24 hours. After the 24 hours of the “Continuous Flow Phase,” the number of bacteria in the bioreactor can be measured. The biofilms get homogenized to break up the bacterial communities, then the

homogenized sample is serially diluted, and these dilutions are plated on agarose plates and allowed to grow colonies. From the number of colonies that grow on each plate with a different dilution, the number of colony forming units (CFUs) can be calculated and then the amount of bacteria in the original sample can be determined [15]. This is different from the MOBIC bioreactor because the MOBIC device focuses on the initial attachment steps in biofilm formation, not the amount of bacteria present at the end of a specific time period. The CDC Bioreactor looks at mature biofilms after 24 hours while the MOBIC device will look at the initial attachment of bacteria to a surface in real-time in just a few minutes to a few hours. The bacteria in the CDC Bioreactor setup are in exponential growth while the MOBIC device will observe bacteria that remain at a practically constant concentration.

Biofilm (Mechanism of formation)

Bacteria can be described as in one of two states: planktonic or sessile. Planktonic bacteria are the single cells that are free-floating in the medium while the sessile bacteria are those that have accumulated on a surface to form a biofilm. The formation of biofilms is a multistep process that begins when the bacteria are in the planktonic state [16]. Figure 1 shows a diagram that represents the steps of biofilm formation. In this figure, the bacteria are visualized with flagella, but the process of biofilm formation remains the same for all bacteria that form biofilms.

The initial attachment of bacteria to a surface is a reversible process (step 2 in Figure 2). This attachment is induced by weak forces such as van der Waals interactions. On the bacterial cell itself, flagella or fimbriae allow for mechanical attachment to the surface [16]. The irreversible attachment step consists of stronger interactions between the bacterial cells and the surface (step 3 in Figure 2). In this step, the attached bacteria are significantly more resistant to both physical and chemical shear forces. The irreversible attachment step is also when the EPS matrix begins being excreted and flagella genes become downregulated [16]. For *S. aureus*, the cells use different cell wall anchored proteins, such as fibronectin-binding proteins, serine-aspartate repeat family proteins, clumping factors, collagen adhesin, Protein A, and others to attach to a variety of different possible host surfaces. These host surfaces can include fibronectin, fibrinogen, collagen, and cytokeratin [17]. Many of these host molecules can coat implanted devices which can allow for the *S. aureus* infections associated with these devices [17]. *S. aureus* can also attach to abiotic surfaces using hydrophobic interactions and electrostatics. This is where the charge of the material surface can influence the ability of bacteria to attach and thus form biofilms [17].

There is an intermediate step in biofilm formation that some sources call multiplication and others call it microcolony formation (step 4 in Figure 2). In this step, the attached

bacteria can multiply and create a thin layer of cells on the surface [16]. For the *S. aureus* biofilms, the multiplication process leaves daughter cells vulnerable to detachment if there is no extracellular matrix. The main functions of this step include the cell division and accumulation of the attached bacterial cells. There are many proteins produced by *Staphylococci* that facilitate the accumulation portion of this step in biofilm formation. Some of the cell wall anchored proteins have dual functions by facilitating the attachment and the accumulation of the cells on the surface. Polysaccharide intracellular adhesin (PIA) is another component of the extracellular matrix in these early stages of biofilm development. This is also when cell lysis is induced by some of the cell community members in order to release eDNA into the extracellular matrix [17]. After the multiplication step, there is an exodus step that is described by *Moormeier & Bayles* for *S. aureus* [17]. This stage is an early dispersal step that produces microcolonies and restructures the biofilm. This step is mediated by the degradation of eDNA [17].

The next step in the formation of a biofilm is maturation (step 5 in Figure 2). This is when the thin layer of EPS coated cells form more layers and create a mushroom-like shape. When a biofilm accumulates many layers, the cells within it organize themselves based on their needs. This means that anaerobic bacteria will migrate to the lower levels of the biofilm to avoid exposure to air and leave those surface layers for the aerobes. The cells communicate with each other and act as a community where different functions are completed by different cells in different locations within the biofilm. The mature biofilm continues to produce more of the components of the extracellular matrix as it grows [16]. *S. aureus* undergoes metabolic diversification during the maturation step of biofilm formation. This is a result of the production of microcolonies that have distinctly different metabolisms. One presented explanation for this development is that the biofilms produce a range of different types of metabolism as a means of preparing for different environmental stressors [17].

The final step in biofilm formation is the dispersal step (step 6 in Figure 2). This step can occur in a part of a mature biofilm, or the whole biofilm. In this step, planktonic bacteria are released from the mature biofilm. There are many reasons that a biofilm will release planktonic cells. These reasons include lack of nutrients, intense competition, or outgrown population. This release of planktonic cells will initiate biofilm development in a new location, thus beginning the cycle over again [16]. For *S. aureus*, the control of biofilm dispersal has been shown to be connected to Accessory gene regulator (Agr) quorum sensing. This mechanism is sensitive to cell density and autoinducers. Specifically, there is an octapeptide pheromone, auto-inducing peptide (AIP), that, upon reaching a certain concentration, will trigger a cascade and eventually initiate the P3 promoter [17].

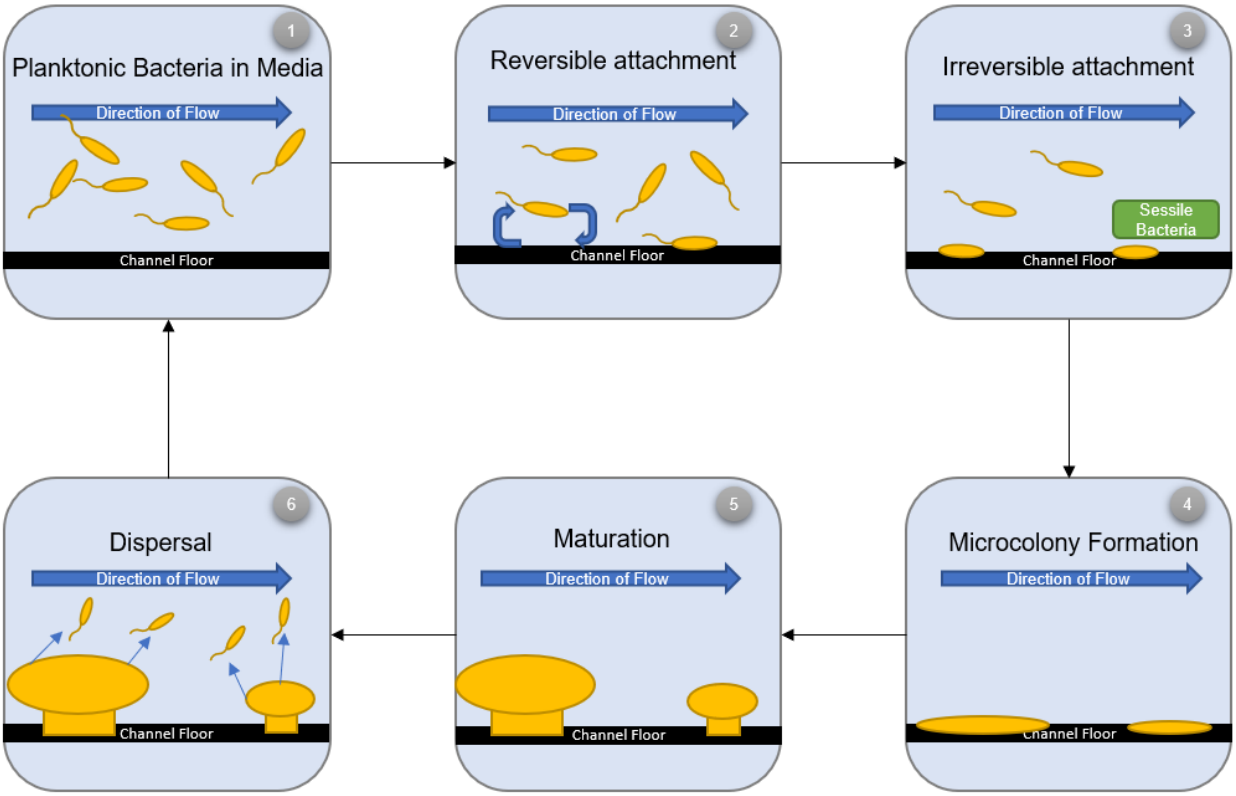

Figure 2. Diagram of Biofilm Formation

Biofilm (Organisms involved)

Biofilms are diverse and can even be shared by multiple different species [13]. There are mono-species biofilms, and even within these communities there are phenotypically different organisms [13]. Bacterial biofilms are the most commonly researched and known biofilms, probably due to the implications of infections in humans, but there are fungal and algal biofilms and protists can even be included in bacterial biofilms [13,18,19]. There are medically significant fungi that form biofilms [18]. A few of the medically relevant fungi that form biofilms are: *Candida*, *Aspergillus*, *Cryptococcus*, *Trichosporon*, *Coccidioides*, and *Pneumocystis.* Many of these fungi can form biofilms on biotic and abiotic surfaces, but they may also require the presence of hyphal cells, which are the branch-like structures that are formed by some fungi that initiate vegetative growth [18]. The majority of wildtype bacteria are capable of forming biofilms, but laboratory strains are typically selected for those which do not adhere to surfaces [20]. A few of the notable bacteria that regularly form biofilms are: *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas, Enterobacter cloacae, Salmonella, Streptococcus mutans,* and *Vibrio cholerae* [13].

Staphylococcus aureus are gram-positive bacteria that are cocci. These cocci group together into clusters and they have been “grape-like” in shape. The colonies of *S. aureus* tend to be yellow in color. They are capable of growing in both the absence and presence of oxygen. *S. aureus* is of high importance because it is one of the most common causes of bacterial infections in humans. The bacteria can be found on our skin and in our mucosal membranes. It has been estimated that as high as 50% of adult humans are colonized by *S. aureus.* The colonization of a person by *S. aureus* also depends on the population. People that work in healthcare, frequent needle users, people who are hospitalized, and people who are immunodeficient tend to have higher rates of colonization. When it comes to infection, *S. aureus* can cause invasive infections or toxin-mediated diseases. One of this bacteria’s means of avoiding detection by the host’s immune system is by forming biofilms. There are many different types of infections that are caused by *S. aureus*. Some of these include bacteremia, infective endocarditis, septic arthritis, pulmonary infections, meningitis, toxic shock syndrome, urinary tract infections, prosthetic device infections and other healthcare associated infections, like Methicillin Resistant *Staphylococcus aureus* (MRSA) [21].

Another bacterium that forms biofilms and is relevant in human health is *Escherichia coli* (*E. coli*). *E. coli* is a gram-negative bacillus that usually lives in the human digestive tract, but certain strains can cause a range of different diarrheal diseases. *E. coli* is typically spread through contaminated food or water. When biofilms are formed, the contamination is more difficult to remove from food products. *E. coli* also causes other medical ailments, like uncomplicated cystitis and other extraintestinal illnesses. These illnesses include pneumonia, bacteremia, and spontaneous bacterial peritonitis. The severity of *E. coli* diseases is a wide range with some mild cases and some that lead to renal failure and septic shock. *E. coli* can also cause infections on medical devices, like urinary catheters and ventilators because of its ability to form biofilms [22].

Pseudomonas species are gram-negative bacilli that can range from 0.5-0.8µm by 1.5-3.0µm. They are aerobic and use a single flagellum for motion. They normally live in soil, water, and vegetation, but they can also be isolated from samples from healthy humans too. They also have come to colonize within hospitals in sinks, food, taps, mops, and respiratory equipment. *P. aeruginosa* and *P. maltophilia* are particularly relevant within this genus because they cause 80% of opportunistic *Pseudomonas* infections. When *P. aeruginosa* infects patients with cancer, cystic fibrosis, or burns, there is a severe increase in fatalities. Some other infections caused by *Pseudomonas* bacteria are endocarditis, pneumonia, urinary tract infections, infections of the central nervous system, wound infections, eye infections, ear infections, skin infections, and

musculoskeletal infections [23]. *P. aeruginosa* can form biofilms, which makes it more pathogenic and drug resistant. Infections caused by the drug-resistant biofilms are prevalent in hospitals and have high mortality rates in hospitalized individuals. One infection that is caused by *P. aeruginosa* is chronic lung infections for patients that have cystic fibrosis [24].

Bacteria in the genus *Enterobacter* are gram-negative, rod-shaped, and are facultative anaerobes. Facultative anaerobes can undergo aerobic respiration when oxygen is present, but they can switch to fermentation when oxygen is absent. *Enterobacter cloacae* is one of the *Enterobacter* species that is well known because of its clinical significance. This bacterium causes opportunistic infections that are resistant to treatment. These infections are particularly prevalent in hospitals. *E. cloacae* is one of the most common of the *Enterobacter* species that only causes nosocomial infections. This bacterium can form biofilms and secrete cytotoxins, which are expected to contribute to the antibiotic resistance of the pathogen. *E. cloacae* can cause bacteremia, endocarditis, osteomyelitis, septic arthritis, soft tissue infections, lower respiratory tract infections, urinary tract infections, and intra-abdominal infections. Medically implanted devices have also been shown to become contaminated by *E. cloacae* [25].

Salmonella spp. are rod shaped, gram-negative bacteria that are facultative anaerobes. They use flagella for their motility. *Salmonellae* infect both humans and animals; in humans, Salmonellosis is usually food poisoning, but can be more serious, presenting as enteric fever. Infections of *Salmonella* depend on a few factors of the bacterium. The ability for the bacterium to invade cells, if the bacteria have a lipopolysaccharide coat, if it can replicate intracellularly, and if the bacterium can produce toxins. These properties are considered virulence factors and they are required for the *Salmonella* to be fully pathogenic. *Salmonella* is typically transmitted through contaminated food. Since this bacterium can form biofilms, disinfecting contaminated foods is difficult due to the inherent resistance that biofilms have against physical and chemical cleaning practices. The bacteria can survive in meat of animals, such as chickens, turkeys, pigs, and cows, and then it can be spread to humans when undercooked meat is consumed. Typhoid fever and other generic fevers are also caused by *Salmonella* and are mostly transmitted with person-to-person contact and water contaminated with human feces. Use of antibiotics in farm animals and overuse in humans puts selective pressure for the *Salmonella* to become antibiotic resistant [26].

Streptococcus mutans is a gram-positive, coccus shaped bacteria that is found in the human mouth. This bacterium can form biofilms and has an impact on human health. *Streptococcus mutans* play a large role in tooth decay in humans. This bacterium forms

multispecies biofilms within the mouth. The tooth decay is caused by *S. mutans* ability to produce glucans and acid. They produce enough acid to overcome the buffering capacity of saliva. Because of their production of acid, they are well-suited for low pH environments and are able to outcompete other bacterial species in the mouth. Once the *S. mutans* adheres to the teeth and creates biofilms with other microorganisms, the matured biofilm allows for the bacteria to access deeper tissues and begin dissolving tooth enamel. Once a cavity is formed in a tooth, there is a protected area for further biofilms to form and mature, progressing the tooth decay [27].

Vibrio cholerae is a gram-negative bacterium that uses a single flagellum for motility. There are hundreds of serogroups of *V. cholerae*, with some being pathogenic and others not. *V. cholerae* is well-known in human medicine because it causes the diarrheal disease, cholera. Cholera is a potentially fatal diarrheal disease that is caused by the toxin-producing *V. cholerae.* Cholera can cause fatal dehydration due to the high volumes of watery stools induced from the infection. It has been shown that the fatality rate can be as high as 50% in high-risk populations. The populations that are at the highest risk for cholera infections are those with poor sanitation and hygiene and those affected by natural disasters. The *V. cholerae* bacteria have specialized adherence factors so that they can attach to the microvilli surface. This surface attachment allows them to form biofilms. They can then excrete cholera enterotoxins into the epithelial cells of the intestine. These toxins cause adenylate cyclase to be forced “on,” which then causes excess cAMP to be produced. The excess cAMP causes excess chloride, bicarbonate, and water to be secreted [28].

Biofilm (Methods to monitor biofilm)

One method that is used to monitor biofilms is the CDC Biofilm Reactor. This device is available commercially for the use in measuring mature biofilm growth. This device was designed as a way to grow biofilms under high shear conditions. This bioreactor is designed with eight independent rods that each contain three coupons. These coupons are able to be removed from the bioreactor at different timepoints in order to measure the biofilm formation at multiple timepoints. The shear conditions are added to the apparatus by a magnetic stir bar that is controlled externally by a stir plate. The process of using the CDC bioreactor requires a lot of time and more steps than the MOBIC device. There is a “Batch Phase” for the CDC bioreactor in which the reactor is inoculated with a previously prepared inoculum culture. 10^8 cfu/mL are added to the reactor during this phase. The stirring also begins at this point, with the speed of the stirring determining the shear stress inflicted upon the bacteria. Once the bacterial culture is added and the stirring has begun, the reactor needs to sit for 24 hours. At this point there is 500mL of sterile batch media, and it is not a continuous flow. The next phase is the “Continuous Flow Phase,” when the continuous flow nutrient broth is

continuously pumped into the reactor and the waste is pumped into a waste container. At this point, the reactor is supposed to sit for another 24 hours. During this phase, samples can be taken from the coupons previously mentioned in order to have data at different timepoints. The sampling process utilizes homogenization as a means of breaking apart the biofilms that formed over the time period. Once the sample is homogenized, serial dilutions are made from the disaggregated sample. These dilutions are plated on agar plates and incubated at 35°C for 18-24 hours. The colony forming units (CFUs) can be calculated from the growth on the plates. The log density for each coupon is then calculated using the following equation:

$$LOG_{10}\left(\frac{cfu}{cm^2}\right) = LOG_{10}\left[\left(\frac{\left(\frac{mean\ cfu}{plate}\right)}{vol.of\ sample\ plated}\right) * \left(\frac{volume\ scraped\ into}{surface\ area\ scraped}\right) * (dilution)\right]$$

Once the density of biofilm is calculated for each coupon, the average for each timepoint can be taken in order to find the mean biofilm density [15].

Many different types of microscopy are used for the monitoring of biofilms. This includes light microscopy, fluorescence microscopy, confocal laser scanning microscopy, atomic force microscopy, scanning electron microscopy, and more. These types of microscopy provide different data regarding the biofilm being analyzed [29].

Light microscopy can be used as a method to count cells. The cell counting by light microscopy could be used to determine the cell count in a biofilm. In order to use this method without any homogenization, the biofilm would have to be very thin. If the biofilm has a complex, three-dimensional structure, the sample will need to be homogenized before counting the cells. Light microscopes can be much cheaper than fluorescence microscopes, but fluorescence microscopes can provide more information about the sample. In particular, using fluorescence allows for the bacteria cells to easily be distinguished from miscellaneous cell debris within the sample. There are also live/dead stains that can be applied to the bacterial cells in order to easily tell the live cells apart from the dead cells for a viability count. Light microscopy cannot be used to differentiate between live and dead cells apart from apparent morphological changes. There are also fluorescent stains that can mark different parts of the cell. 4′,6-Diamidino-2-phenylindole dilactate (DAPI) is a fluorescent stain that localizes near DNA within the cells. There are also lipophilic dyes that stain the cell membrane. There is even a stain that specifically targets the matrix proteins within biofilms [29].

Confocal scanning laser microscopy (CLSM) is a method of microscopy that can create three-dimensional images of biofilms. CLSM works by having confocal optics that can exclude unwanted light and can focus on a small volume of the sample at a time. The microscope scans across the sample and creates two-dimensional slices of the samples that are eventually compiled to create the final three-dimensional image. CLSM can also use fluorescent markers in the biofilm because it can use multiple excitation lasers. These microscopes are expensive (hundreds of thousands of dollars to begin) to purchase and run and they take training to be able to get accurate results from them [29].

There are many different methods for the indirect quantification of biofilms. These methods use the quantification of different cellular components to infer the number of bacteria that were present. Dry mass is a common indirect quantification method that is used for biofilms. For the dry mass analysis, a mature biofilm is either dried in an oven until there is no water left in it, or it is precipitated from solution using ethanol if the surface is temperature sensitive. The dry biomass is the total mass of the dried sample on the substrate minus the mass of the substrate after the biomass was scraped off. The dry mass is then normalized to the growth area that the wet biofilm had or to the volume of the wet biofilm in order to find the biofilm density. Though this method is easy and cost effective, the dry mass includes the mass of the other components of the biofilm, not just the bacterial cells [29].

Crystal violet assays are also used for the indirect quantification of biofilms. This method stains the bacterial cells using crystal violet, a stain that can permeate the cell membrane in both gram positive and gram-negative bacteria. When the cells are stained and subsequently washed and decolorized, the samples can be analyzed in a spectrometer. The absorbance of the samples should be read between 530 and 600nm. This absorbance relates to the number of cells that were present in the sample [29].

Biofilms have also been characterized qualitatively by the use of different techniques. The main technique for this category is scanning electron microscopy (SEM). The result of this method is a detailed image of the topography of the biofilm sample. These images give insight into the interactions that occur in biofilms, the organization and morphology of them, and how they form. One major disadvantage of using SEM is that the sample being analyzed cannot be living. In this method, the sample is kept under high vacuum after many different preparatory steps. Since this preparation is difficult, environmental scanning electron microscopy (ESEM) was created. In this technique, the sample is able to be untreated and does not need to be dehydrated or be in a vacuum. The resolution of ESEM is not as high as that of SEM since the electrons have to travel through gasses to reach the sample. The sample is also likely to be damaged by the

electrons being shot at it, so a live sample is still not able to be examined. Even so, the examined sample can still be wet and does not need to undergo the rigorous preparations that are required for SEM [29].

One method for the real time monitoring of biofilms is the use of long period grating (LPG) optical fiber sensors. This was done by Kurmoo *et al.* in experiments for monitoring the growth and formation of biofilms on endotracheal tubes (ETTs). They were able to do this by incorporating the LPG optical fiber sensors into the ETTs. For the experiment, over the span of 24 hours, the shift in the wavelength of the LPG attenuation bands was monitored. The biomass of the biofilm was also determined after 24 hours by using confocal fluorescence microscopy. Once the biomass and the wavelength shift were acquired, they could be compared. The hope for this method is that medical devices, like ETTs, could have these sensors for early detection of biofilm growth on the device. This would allow for the device to be removed earlier than when an active infection needs to be present and could prevent these infections in patients [30].

Fluid Dynamics

A Swiss physicist and mathematician named Daniel Bernoulli founded a principle that is important to the study of fluid dynamics. This principle is Bernoulli's principle, and it relates the fluid speed and elevation to the fluid pressure. This idea is used to examine the reason that a fluid flows. Since the law of conservation of energy still applies, the changes in flow speed are either going to be caused by the increase in potential energy or from the internal energy that creates the fluid pressure. This means that elevation can cause flow due to the change in potential energy. Pressure differences within the fluid can also cause flow because the high-pressure zones will want to move to areas of lower pressure. Another part of Bernoulli's principle is that fluids that are flowing faster have lower pressure. Bernoulli's equation is an application of the conservation of energy in fluid dynamics. The basic idea of Bernoulli's equation is that the energy density is equal to a constant or that it is equal at two different points. Bernoulli's equation is presented below. In this equation, the first term is pressure, the second is kinetic energy per volume, and the third is potential energy.

$$P_1 + \frac{1}{2}\rho V_1^2 + \rho g h_1 = P_2 + \frac{1}{2}\rho g h_2$$

Fluid dynamics is the study of fluids in motion. The main topic within fluid dynamics is how fluids flow. As was stated previously, fluids will flow from elevation, like flowing downhill, or from pressure. The mass flow rate of a fluid must remain the same during

flow. This means that the amount of mass that passes one point must also pass the next point. The equation that describes this relationship is called the Continuity Equation, presented below. In this equation, ρ represents density, *A* represents cross-sectional area, and v is velocity.

$$\rho_2 A_2 v_2 = \rho_2 A_1 v_1$$

There are two types of flow that can be understood using Bernoulli's principle. The first is turbulent flow. Turbulent flow is disorganized and has vortices, eddies, and wakes. This erratic behavior of the flows makes it difficult to predict. This type of flow is more common with low viscosity, high flow speeds, and higher volume for the fluid to flow through. The speed and direction of the fluid changes constantly in turbulent flow. Turbulent flow is much more common than its counterpart, laminar flow.

Laminar flow only exists in special circumstances. In laminar flow, the quantities that describe the flow remain constant. This includes the pressure and velocity of the fluid. Laminar flow occurs in layers that move smoothly together. This layered nature of the fluid keeps from the mixing and disorder that is seen within turbulent flow. Laminar flow is more common with low speed, high viscosity, or a small space to flow through.

There are many examples of flow that can change from laminar to turbulent. One of which is a kitchen faucet. The water can be turned on to be a smooth flow where it is laminar, but if the water gets turned up higher, the flow becomes turbulent. Information about the point where the flow changes from one type of flow to another can be given by the Reynolds Number. The formula for the Reynolds Numbers results in a dimensionless number that relates to fluid flow. When the number is less than 2,300, the flow is laminar and turbulent when it is over 4,000 [31].

For this experiment, fluid dynamics apply to the flow within the MOBIC channel. The flow within the channel will affect the movement of the suspended bacterial cells and will impact the interactions between the bacteria and the surface of the channel. Since this MOBIC device is designed to model a biological setting to observe bacterial attachment to medically implanted devices, the flow within the channel should be laminar. Blood vessels use laminar flow and in order to have the channel be as similar to a human body as possible, the location within the channel that is used for observation and measurements should have laminar flow.

Methods

Growth of Bacteria

The bacteria that were used in these experiments was *Staphylococcus aureus.* The bacteria were obtained from ATCC (ATCC 19685, Manassas, VA) and it was grown in a medium that contained 3% weight by volume of Tryptic Soy Broth (TSB, BD, Franklin Lakes, NJ). Before the bacteria were needed, they were kept in a -80°C freezer. The day before an experiment, $3x10^5$ cfu/mL were incubated at 37°C in a 50mL tube. This allowed for the bacteria to be in the exponential growth phase during the experiment.

Polymer Synthesis

The conductive polymer for the bottom part of the channel was made from elastomer (Sylgard 184), hardener, and carbon nanofiber at a 1: 0.1:0.045 ratio. 0.36g of carbon nanofibers were added to 12g of elastomer in a 5-dram vial. The vial was mixed on a vortex to ensure that the carbon nanofibers were well incorporated in the elastomer. It was then sonicated for 15 minutes. After the sonication step, 1.2g of hardener were added to the vial and the mixture was vortexed again. The mixture was then poured into a plastic petri dish and spread out until it was 0.5cm thick. The petri dish was degassed for 5 minutes at 25inHg and heated for 45 minutes at 70°C. The resulting polymer was conductive with a resistance of about 10k ohms/cm from the measurements by the voltmeter probes.

Carbon nanofibers are formed when graphene layers stack or roll to form different shapes. These graphene layers can be arranged on cones, cups, or plates. They can also be rolled into a perfect cylinder, which is then called a carbon nanotube. Carbon nanofibers can be a range of different sizes, with diameters usually between one and one-hundred nanometers with some reaching 500 nanometers [32].

The carbon nanofibers that were used for the creation of the conductive PDMS were produced by the floating catalyst method and were provided by Sigma Aldrich. The nanofibers that were obtained from Sigma Aldrich were said to be hollow cylinders with an outer diameter of 125-150nm and an inner diameter of 50-70nm. The length of the carbon nanofibers raged from 50-100µm.

Biofilm (Methods to monitor biofilm)

The device that was designed for these experiments will use simultaneous fluorescence microscopy and electrochemical impedance spectroscopy to monitor early timepoints of biofilm formation. Understanding the initial attachment of bacteria to surfaces will allow

for research into prevention of this attachment. If bacteria are unable to attach to surfaces, they are unable to develop into mature biofilms and cause infections that are resistant to being treated with antibiotics. As was shown in the previous section, there have been many methods used to monitor and observe biofilms in their mature state, but few for the initial attachment phase. It is this initial attachment phase of biofilm formation that will allow for preventative changes in biomedical materials to prevent the infections that come with biomedical implants.

The impedance spectroscopy will use three electrodes, with one being the conductive PDMS in the bottom of the channel. The PDMS within the channel was made conductive by the use of carbon nanofibers so that it could be used as an electrode. The stainless-steel inlet manifold forms the counter electrode, and the outlet manifold forms the reference electrode. The outlet manifold is silver plated to form a silver pseudo reference electrode. The outlet was designated as the reference so that silver, which is a known bactericide did not contaminate the bacterial culture interacting with the surface.

Impedance Spectroscopy (Description of the technique)

Electrochemical Impedance spectroscopy (EIS) is a technique that is used in many different applications in microbiology, food processing screening, quality control, and many more. For the MOBIC device, potentiostat EIS will be used. A potentiostat is a piece of equipment that controls the voltage between two electrodes and measures the current. In this type of EIS, a sine-wave voltage is applied to a sample and the induced current is measured. Once the current is measured, the complex impedance can be calculated. The basic idea behind EIS is that an alternating voltage is applied across the reference and working electrodes and the resulting current is measured between the working and the counter electrodes. Changes in this current will be indicative of changes in the system, such as bacterial attachment in the MOBIC channel.

There are three common setups for EIS that use two, three, or four electrodes respectively. The simplest is the two-electrode setup, where the voltage is applied and the current is measured from the same two electrodes, the working and counter electrodes. The impedance for this setup will be affected by the sample interface at both electrodes.

In the three-electrode setup, an additional reference electrode is added. The counter electrode is now where the current will be measured while the voltage is applied between the working and reference electrodes. For this setup, only the contribution of the surface interface of the working electrode. For the MOBIC channel, this is the setup that is being used. It was designed in such a way that the inlet can act as the counter

electrode, the outlet can act as the reference electrode, and the conductive PDMS at the bottom of the channel can be the working electrode. This allows for the attachment of bacteria to the PDMS surface to be measured using EIS as well as changes to the surfaces such as the deposition of EPS or other materials.

The four-electrode setup uses an additional electrode that is called the working sensing electrode. The sine-wave test current is applied to the working electrode and the counter electrode, the voltage is measured between the working sensing electrode and the reference electrode. The impedance for this setup is independent of the interfaces between the electrodes and the samples because there is no current drawn at the working sensing electrode and the reference electrodes [33].

There are two kinds of plots that can be created from the data produced in EIS. The first is a Bode plot. In a Bode plot, the log of the absolute value of the impedance is plotted against the log of the frequency. The phase shift is also plotted against the log of frequency on the same plot. The Bode plot has frequency information, unlike the Nyquist plot. This means the plot can show which frequency a specific data point was made at.

Since the impedance complex number contains components that are real and imaginary, these components can be plotted against each other. In a Nyquist plot, the real part is plotted on the X-axis and the imaginary part is plotted on the Y-axis. The impedance can be represented as a vector with the length benign the absolute value of Z. The angle that is made by the impedance vector and the X-axis is called the phase angle. The Nyquist plot does not have frequency information because for any given point on the plot, the frequency at which the data was collected cannot be determined [33,34].

Electrochemical impedance spectroscopy has been used for the real-time monitoring of biofilm formation. Ben-Yoav *et al.* proposed a method of using EIS for monitoring biofilms. In the experiments carried out by Ben-Yoav *et al.*, *Escherichia coli* (*E. coli*) was pumped through a flow channel [35]. The flow channel for these experiments was created from an elliptic chamber. The flow channel was carved out of a transparent polymer. There are two electrodes that go in the top and bottom grooves of the channel apparatus. These electrodes are glass plates that are coated with indium-tin-oxide (ITO). The bottom electrode is the working electrode, the top electrode is the counter electrode, and a silver-silver chloride wire reference electrode is in the inlet of the chamber. Once the chamber was ready, phosphate buffered saline (PBS) was pumped through the channel. The bacteria could then be pumped into the system and be observed through an inverted optical microscope. For three hours, images were taken

of the bottom electrode for use in determining the coverage of bacteria on the electrode. The impedance spectroscopy portion of their analysis was also done during this time. The frequency range that was used to determine the electrical properties of the bacteria was 0.1Hz to 0.4MHz. The voltage was applied to the electrodes at variable frequencies with a 50mV amplitude by an impedance analyzer. To analyze the attachment of the bacteria to the electrodes, the potential used was 50mV and the frequency range was 100mHz to 400kHz. The authors found that there was an increase in optical coverage for longer deposition periods. The impedance data showed variability between different polarization values of the deposition electrode. The authors also proposed a model of the equivalent circuit that shows the interactions that occur within the channel. They then fitted their EIS data to this equivalent circuit [35]. In our process of designing the MOBIC device to use EIS, we believe that the equivalent circuit presented by Ben-Yoav *et al.* is incorrect and will be further explored in the discussion.

Flow Through Cell

The MOBIC channel that was designed for these experiments was designed based on the previous MOBIC channel in Solid Works. The 3D model was then milled in house from a block of polycarbonate. The new device was designed in such a way that the same stainless-steel fittings and custom coverslips from the first channel would fit the new one. The flow within the device was then analyzed using ANSYS/FLUENT, a software that is used for computational fluid dynamic analysis. This software predicts fluid flow by using the finite volume method. In these methods, the region of the flow is discretized into a finite set of volumes and the general conservation equations get solved for these finite volumes. The bacteria were then able to be pumped through the channel using a calibrated KD Scientific (KD Scientific, Holliston MA) KDS 100 syringe pump.

The channel will be used for real time monitoring of the initial attachment of biofilm-forming bacteria to a surface. Through the simultaneous use of fluorescence microscopy and electrochemical impedance spectroscopy, the interactions and attachment of bacteria to the surface of the floor of the channel can be analyzed.

Fluorescence Microscopy

Fluorescence microscopy is used for many applications within biological sciences. It is widely used because there are a range of different fluorescent probes that can bind different cell structures with high selectivity [36]. This allows for different components of a cell to be marked within one sample or just to easily tell dead cells from live ones [36]. For this experiment, the bacteria that were pumped through the flow channel were stained with a live/dead dye (Molecular probes, Live/Dead assay Propidium Iodide and Syto 9) and observed as they proceeded through the channel. Propidium Iodide and

Syto 9 both fluoresce when they are near nucleic acids. The difference between the two is their ability to permeate the cell membrane. Propidium Iodide cannot permeate the membrane of a live cell, but it can easily enter dead cells. Syto 9 can pass through the membrane of live cells easily [29]. The flow channel was designed to have a coverslip on the top so that a fluorescent microscope could be used to view the cells within the channel in real-time. The live bacteria in the flow channel were also used to create a velocity profile of the cells in order to determine how many of them interacted with the floor of the channel. This interaction is vital for the formation of biofilms and the use of the flow channel, fluorescent microscopy, and electrochemical impedance spectroscopy will allow for the analysis of the earliest attachment of bacterial cells.

Analysis

The analysis of the data will use the data collected from the electrochemical impedance spectroscopy (EIS) and that from the fluorescence microscopy. The EIS will give insight into the changes in relative impedance which could vary with the flow rate in the channel and the material the floor of the channel is made of. The fluorescence microscopy will provide velocity data for each level of depth within the channel, allowing for analysis of whether the bacteria are interacting with the floor of the channel.

Results and Discussion

Description of Flow Through Cell

The new MOBIC (Microhydrodynamics of Biofilms in Channels) flow channel that is designed for measuring changes in resistance as bacteria adhere to the surface is based on the MOBIC used in the previous experiment, where there were narrower channels and impedance spectroscopy was not used. The differences are that the new MOBIC has only two channels, the channel width is 1 cm, and there is a recessed area in the bottom of the channel for conductive polymer. The rest of the dimensions remain the same and the two MOBIC devices will use the same stainless-steel fittings and custom coverslips. There will be a small hole inside of the channel where a copper wire will be fed prior to pouring the conductive polymer to act as a connecting point for the electrode. Figure 3 shows an image of

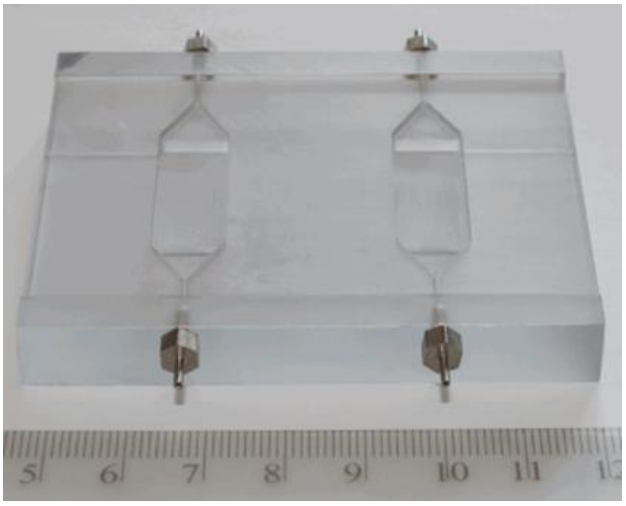

Figure 3. Image of MOBIC Channel

the completed channel. In this image, it is seen that there are two flow channels within the same apparatus so that two trials can be run. The top of the channel has a space that fits custom coverslips that were designed for the previous MOBIC design. Once the channel was successfully milled and the stainless-steel fittings were installed into the polycarbonate channels, ANSYS/FLUENT was used to analyze the flow within the channel. This analysis is shown in Figure 4. The flow within the channel can be seen to be laminar between approximately -2mm and 2mm from the center of the channel on the figure. This means that the center portion of the channel has laminar flow with no edge effects. The same laminar flow is observed over the three flow rates that cover two orders of magnitude. This is significant because this is the portion of the channel that will be being analyzed for both impedance spectroscopy and for fluorescence microscopy. This removes the variable of turbulence from the analysis and the interactions between the surface and the bacteria will be due to initial adhesion steps in biofilm formation. Laminar flow also makes the MOBIC device more similar to the environment that is being modelled, the human body. The flow within blood vessels is laminar and this device was designed to analyze the attachment of bacteria to biomedical implants from the bloodstream.

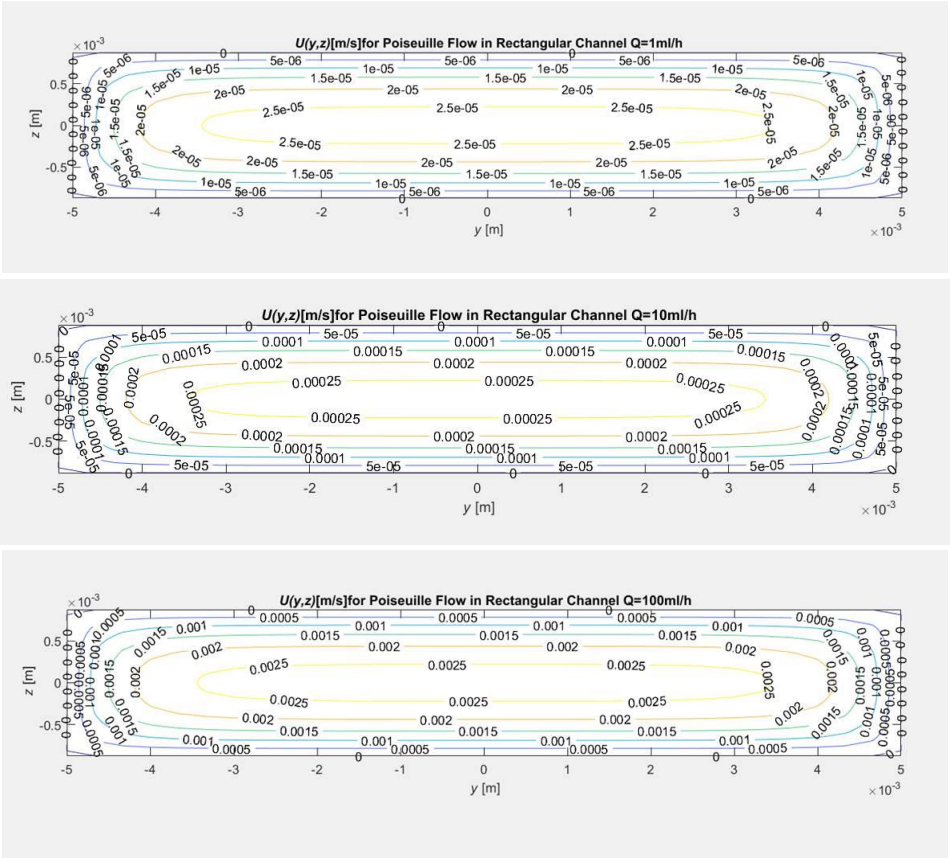

Figure 4. Flow Analysis of Inside the Channel at Three Flow Rates

This device will be used to monitor early timepoints in biofilm formation. This will be done using impedance spectroscopy and fluorescence microscopy simultaneously. The data collected will show the interactions between the bacterial cells and the surface within the channel. Eventually, the surface in the channel can be changed out to test the adherence of bacteria of materials with a range of different surface chemistries.

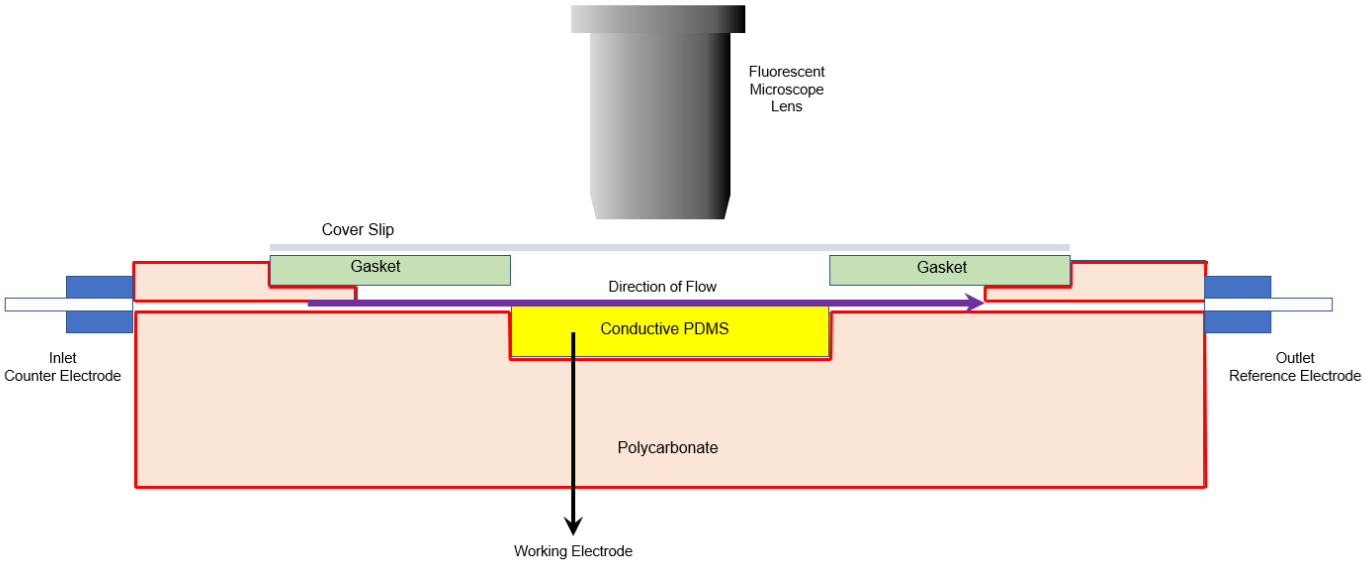

Figure 5. Schematic of MOBIC Device

A schematic of the MOBIC device can be seen in Figure 5. This figure shows a cross section of a channel with the direction of flow going left to right. The polycarbonate is the portion of the channel that had to be milled in the in-house machine shop. The inlet and outlets are the stainless-steel fittings that will be the counter and reference electrodes respectively. The outlet will be made into a reference electrode by electrochemically plating it with sliver/silver chloride. The conductive PDMS can also be seen in the figure in the bottom of the channel. There is a cover slip on top of two gaskets on the top of the channel. This is where the fluorescent microscope will view the inside of the channel. A microscope lens is shown in the diagram to represent this. The inlet is the counter electrode for the EIS, the outlet is the reference electrode, and the conductive PDMS is the working electrode. More details about the flow through the MOBIC array can be seen in Figure 6. Figure 6 shows the temperatures of the flow samples, how it is monitored, and the direction of the flow. The media and bacteria are kept at 4ºC to keep the bacteria from proliferating before entry into the channel. The

channel is heated to 37 ºC because that is the average body temperature of a human being. This is significant because the MOBIC device was created to model the biofilm attachment that occurs within the human body after a medical device is implanted. Having the temperature of the device be at body temperature makes the device a better model for the attachment that occurs in the body.

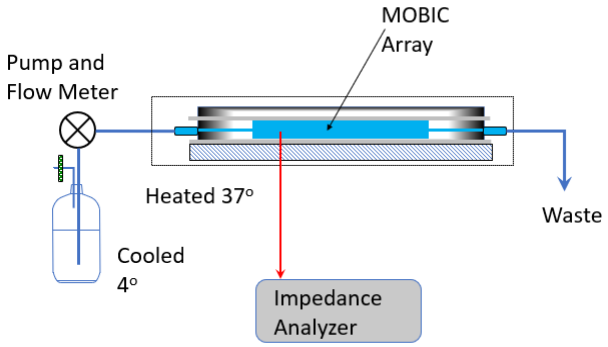

Figure 6. Schematic of MOBIC Pump System

The Bacteria

Staphylococcus aureus was the organism that was chosen for use in these flow-channel experiments. It is a nearly spherical bacteria that has a diameter of approximately 1µm. Figure 7 shows a fluorescence micrograph of *S. aureus* that has been stained with a live dead stain (propidium iodide, Syto 9). The green cells shown are live and the red cells are dead. Though this image was not taken in our laboratory, similar ones were [37]. Many bacteria have the ability to produce biofilms, but for the specific research into infections on medical implants, *S. aureus*, being the most prominent infectious agent in these infections, is the obvious choice [38]. *S. aureus* is a round, gram-positive strain of bacteria with the ability to grow in both aerobic and anaerobic conditions due to its use of respiration and fermentation [39]. The bacteria within the genus of *Staphylococcus* are able to adhere to surfaces and create colonies that grow into biofilms [39]. In the case of medical implants, *Staphylococci* presents an overwhelming problem: infection. Two-thirds of pathogenesis in orthopedic implants are caused by *Staphylococci* [39]. For the flow-channel experiments, the focus is to explore the growth of biofilms in a

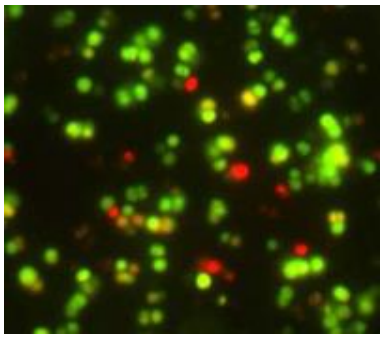

Figure 7: Example of Dead/Live Stained *S. aureus* [37]

setting similar to that of an orthopedic implant and since Staphylococcus aureus commonly forms biofilms in this setting, it was the choice organism for the experiments.

Conductive polymer

In the bottom of the MOBIC channel, there is a recess for conductive PDMS to be cast into. PDMS stands for polydimethylsiloxane. It is a member of the silicone polymer family and is formed by the hydrolysis of Me_2SiCl_2. The Me_2SiCl_2 is created by the Muller-Rochow reaction by high purity SiO_2 and CH_2Cl_2. PDMS has different uses at different molecular weights. In its lower molecular weight form, it is a liquid that is commonly used as a lubricant, an antifoaming agent, or hydraulic fluids. Its higher molecular weight form is a soft rubber or resin. In this form, it has been used in caulks, sealants, and soft lithography [34].

The PDMS in the MOBIC device is of higher molecular weight so that it is a rubbery solid. The samples that are used are made conductive using carbon nanofibers. These carbon nanofibers were mixed into the elastomer portion of the PDMS mix. In order to ensure that the carbon nanofibers were evenly dispersed throughout the mixture, it was thoroughly vortexed until it appeared homogeneous. The hardener could then be added and the whole mixture was thoroughly vortexed again. Once the three ingredients were combined together in their proper proportions (described in the Methods section), the conductive PDMS was ready to be cast.

Creating conductive PDMS using carbon nanofibers has been done before by many other scientists, including Chen *et al.*. In particular, this process is becoming popular in the research into flexible electronic devices. One experiment, conducted by *Chen et al*., tested the physical strength of combination of cellulose nanofiber and carbon nanofibers in PDMS. The authors of this paper observed that the creation of conductive PDMS by the use of composite films created a highly conductive polymer that was not mechanically strong. They decided to use cellulose nanofiber with the carbon nanotubes to see if a nano-network would form and add to the conductive polymer's strength. They created their cellulose nanofiber/carbon nanotube film using mixing and vacuum filtration. This film acted as a template for the development of the nanocomposite by the immersion process. The authors used carbon nanotubes that were well dispersed in water as a means of dispersing the carbon nanotubes in the mixture. They used a 1:1 ratio of the cellulose nanofiber and carbon nanotube. The film that was created using these molecules was shown to have high electrical conductivity that showed that the carbon nanotubes were well dispersed. In order to immerse the film into PDMS, the water in the film had to be exchanged for *n*-hexane. The pre-polymer was then allowed to seep into the pores of the film created by the three-

dimensional nano-network. The curing agent was then added, and the sample was heated. Different amounts of PDMS were used for different trials and the results were analyzed.

The authors used field emission scanning electron microscopy as a way to analyze the differences in the surfaces that they made after they were set. What they found was that plain PDMS showed a completely smooth surface. When the cellulose nanofiber and carbon nanotube film with PDMS was observed in the same manner, it was found that the nanofiber networks could be seen. When the concentration of PDMS was lower, the nanofiber network could easily be seen with little fiber aggregations. With the higher magnification, the PDMS elastomer could even be seen between the nanofibers. When the concentration of PDMS was higher (greater than 71.3%) the surfaces of the nanocomposite began to appear smooth instead of containing pores. For this concentration, it seemed that the PDMS completely filled the pores of the nano-network which gave it the smooth surface. The carbon nanotubes could still be seen with the field emission scanning electron microscopy because they show up as bright objects due to their conductivity compared to the non-conductive cellulose nanofibers. The conductivity of the PDMS with the nanocomposites was highly dependent on the concentration of the PDMS. It was observed that there was a large decrease in conductivity between 1 and 10% wv PDMS samples. This decrease was explained by the PDMS entering the pores of the nano-network, limiting the contact with the electrodes. Even with this decreased conductivity, the conductive PDMS was able to be used in place of a wire to power an LED. The conductive PDMS was also flexible and was able to be rolled and folded. Because it was still conductive and had the desired flexibility, the authors deemed this discovery a success and believe that this nanocomposite could have many applications in electrical devices [24].

The conductive PDMS made by Chen *et al.* was flexible and highly conductive [24]. This is not necessary for the application of the MOBIC device. This is because the PDMS in the device will be inserted into the channel and will not need to be bent or folded. The PDMS in the channel does not need to be highly conductive because, even though it is desirable, the EIS experiments can handle substrates with high resistance. This is because the changes to surface impedance will not be affected by the resistance of the PDMS since it is constant.

Impedance spectroscopy

The novel MOBIC device will use electrochemical impedance spectroscopy as one of two ways to monitor the initial attachment of bacteria in the formation of biofilms. The device was designed to be a three-electrode EIS system with the three electrodes built into the device. The inlet of the MOBIC device is the counter electrode, the outlet is the

reference electrode, and the conductive PDMS is the working electrode. The frequency that is going to be used will be 0.1Hz. The machine that will be used for this process is the Gamry Potentiostat 600.

Previous work

In the introduction section, many ways to monitor biofilms were discussed. The CDC Biofilm Reactor was one method that was described in detail in the introduction. The CDC Biofilm Reactor is able to be used to measure biofilm formation after a period of time, but the method requires homogenization of the samples and cannot be used for real-time biofilm analysis [15]. The MOBIC device that was designed can analyze biofilm formation in real time and it only takes a few minutes to an hour to collect the electrochemical impedance spectroscopy and fluorescence microscopy data. The MOBIC device is much more efficient timewise and had the added benefits of real time monitoring and having a constant concentration of the bacterial culture.

Real time monitoring for biofilms has also been done. An example that was provided in the introduction section was the use of long period grating (LPG) optical fiber sensors [30]. The implementation of these sensors into endotracheal tubes (ETTs) allowed for real time monitoring of biofilm growth. Kurmoo *et al.* also used this sensor to test for anti-biofilm coatings that could be used in medical devices, such as ETTs. They did find a polymer that showed anti-biofilm properties and it proved to prevent the formation of biofilms for up to 24 hours [30].

The simultaneous use of impedance spectroscopy and microscopy for the monitoring of biofilms has been done prior to the invention of this MOBIC device. One instance was an experiment executed by Estrada-Leypon *et al.* to investigate the limitations of commercial interdigitated microelectrodes (IDμE). The authors were interested in how these devices would be able to measure biofilm growth in flow conditions with their own microfluidic device. They used bright field microscopy to observe the bacterial attachment to the IDμE with images being taken each minute for the construction of a time lapse. They also measured the changes in impedance, switching between a two and four-electrode system. The frequency sweep for the impedance was between 100 Hz and 1 MHz. The impedance measurements took about one minute each and they were taken every 10 minutes. The authors also calibrated the impedance measurements with a baseline solution measurement. Three channels were prepared for the experiment, one of which acted as a negative control. Media was pumped through the channels for two hours in order for the conditioning layer to form on the electrodes, then the bacteria was seeded into the experimental channels, then the bacteria were allowed to grow with a constant inflow of fresh media. Analysis of the

viability of the bacteria was done after the channel experiment by fluorescent staining. The biofilms on the electrode were also observed using scanning electron microscopy as the endpoint of the experiment. The results of the study show that the attachment of bacteria and their development into a mature biofilm on the IDuE could be seen by optical microscopy and their estimated thickness for their dried biofilm using SEM to be approximately 100um. The authors observed changes in impedance due to biofilm growth after five hours and they observed that using the four-electrode method allowed for better detection of biofilms. The use of their four-electrode model also allowed them to track morphological and structural changes in the biofilms by fitting the impedance measurements to the Cole model. The Cole model is an empirical complex nonlinear model that the four-electrode EIS data was able to be fitted to [40]. These experiments were primarily looking at the maturation of and already matured biofilms. The maturation of biofilms is a long process, leading to the authors collecting data for 25 hours [40]. The MOBIC device will be looking at the early timepoints of biofilm formation, which will only take from a range of minutes to an hour.

In our MOBIC device, the focus is on the initial attachment of bacteria to the surface and not on the growth of mature biofilms. Our setup will still simultaneously use impedance spectroscopy and microscopy, though there are many differences from the aforementioned experiment. One major difference is that Estrada-Leypon *et al.* used reflected light microscopy and we will use fluorescence microscopy. The authors were looking for visual signs of attachment with their microscopy while ours will allow us to track live cells at different depths within the channel to create a velocity gradient of the cells. Estrada-Leypon *et al.* further analyzed the mature biofilm after the flow experiments were finished by measuring viability and using SEM. The eventual amount of mature biofilm produced will be measured using crystal violet staining so that the initial changes monitored to the surface can be correlated with the eventual mature biofilm formed. The goal for the MOBIC is to monitor the earliest timepoints in the formation of biofilms. Both experiments use impedance spectroscopy setup to measure the changes in impedance with the attachment of bacteria to the electrode.

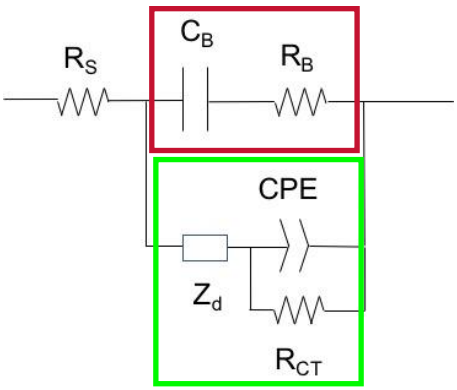

Figure 8: Equivalent Circuit [41]

In the methods section, the equivalent circuit proposed by Ben-Yoav *et al.* was introduced. The model that they proposed can be seen in Figure 8. In this figure, there is a red box and a green box. The red box encompasses the parts of the circuit that

describe the electrical properties of the bacterial biofilm they analyzed. The green box contains the components that describe the electrical properties of the channel surface. In the circuit diagrams (Figures 8 and 9), R_S is the resistance of the solution, R_B is the resistance of the biofilm, C_B is the capacitance of the biofilm, R_{CT} is the charge transfer resistance, Z_d is an anomalous diffusion type impedance boundary element, and CPE is the constant phase element of the interface between the solution and the electrode. The constant phase element of an equivalent circuit acts as a non-ideal capacitor that is double layer. This equivalent circuit model has these components in parallel while it is our belief that they should be in series with each other. This is because at the earliest points in biofilm formation, which is what we are analyzing with EIS, the biofilm has not been formed and thus, the resistance of the biofilm should be very low, and the capacitance is non-ideal because it is incomplete and therefore has a relatively low resistance as described by the charge transfer resistance in parallel with the constant phase element.

As the biofilm forms, it is our understanding that it should be in series with the components of the system. Figure 9 shows our proposed model for the equivalent circuit. In this figure, the same components that were included in Figure 8 were rearranged to have them in series instead of parallel. With the formation of a biofilm, the parallel system should not progress because the current should travel in series through the surface and the biofilm. This would mean that the circuit would change over a span of time while the biofilm forms, which makes it difficult to model with one simple equivalent circuit. Since this change over time will have an impact on the model that can be used to interpret EIS data for biofilm formation, comparing different circuits and continuing the investigation into the electrical interactions that occur is vital for the progression of EIS to monitor biofilm formation at early timepoints.

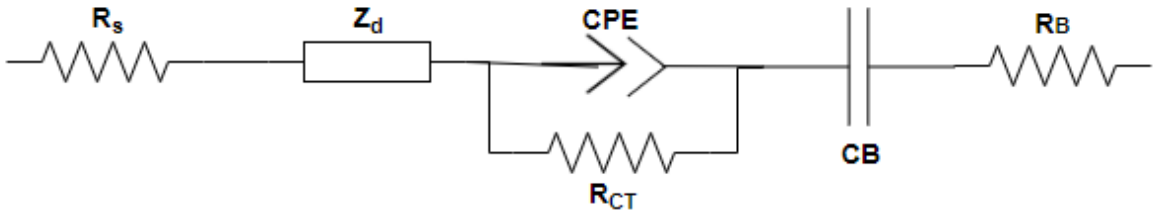

Figure 9: Equivalent Circuit for Biofilm on a Surface [41]

Correlation

It is known that the attachment of planktonic bacteria to a surface is the first step in biofilm formation. This is why the MOBIC device observes the early timepoints of biofilm

adhesion. If the bacteria are unable to attach to or interact with the surface within the channel, no biofilm will be able to form since the cells will not change to their sessile state. When the cells interact reliably with the surface, it is more likely to allow for the formation of a mature biofilm. Mature biofilms are the cause of the chronic infections that occur frequently with medical implants. Preventing the formation of mature biofilms will save lives and money throughout the world. This experiment will allow different surfaces to be tested for their ability to prevent the formation and maturation of biofilms in a modelled biological setting. For the electrochemical impedance spectroscopy (EIS), it is expected that if more bacteria are attached to the surface and the density of the biofilm is higher, the impedance measurements will be higher.

Future work

The next step would be modelling the flow in the channel with particles. These particles would represent bacteria in the channel for the modelling. This would allow for analysis of how particles interact with the channel surfaces. The experiments that should be conducted in the future are listed in Table 1.

Table 1. Table of Future Work

Experiment	**Goal**	**Expected Results**
Modelling with Particles	Gain insight into the flow dynamics and interactions of bacteria in channel	Fluid dynamics analysis model showing bacterial interactions.
Measure *S. aureus* Attachment	Record data for simultaneous EIS and fluorescence microscopy for use as a baseline	Attachment of *S. aureus* to channel floor. Higher impedance and lower velocity when bacteria attach.
Flow Rate	Record changes in bacterial attachment when the flow rate in the channel is varied	Higher impedance with higher flow rate.
Apply Novel Drugs	Find new drugs that can clear biofilms in the channel	Compounds that show antibiofilm properties will decrease impedance.
Changes in Attachment on Different Surfaces	The attachment of bacteria on a range of surfaces should vary and allow us to find if any are resistant to attachment	The rate of attachment will be lower with antibiofilm materials. Lower amount of total biofilm, lower impedance.

The first experiments that should be done using this novel MOBIC device would be to simply test the setup with *S. aureus*. This experiment would use the polycarbonate channel and the conductive PDMS as the floor of the channel. These experiments would be able to show that the simultaneous use of fluorescence microscopy and impedance spectroscopy can be used to successfully analyze the early timepoints of the initial attachment step in biofilm formation. We have previously used fluorescence microscopy to monitor biofilm adhesion in the original MOBIC device and with this new device we will be able to use EIS and fluorescence microscopy for more detailed analysis of the interactions of the bacteria with the surface of the channel. It is expected that as more bacteria adhere to the channel surface, the impedance will increase. The fluorescence microscopy will allow for a velocity profile of the bacteria to determine their interactions with the channel floor. This velocity profile will show interactions between the bacteria and the channel, leading to lower velocities when bacteria are interacting. The amount of total biofilm will also be measured using a crystal violet assay after the channel analyses are finished.

Once it has been shown that the channel works as expected, the flow rate will be changed for the same *S. aureus* biofilm analysis. The flow rates that will be used for these experiments are 1, 10, and 100mL/h. The flow rate will influence the interactions between the bacteria and the floor of the channel. Flow rate also affects the density of biofilms. The impedance is expected to increase with flow rate due to the positive relationship between flow rate and biofilm density. This process of measuring the effect of flow rate should also be applied to the future experiments with drug testing and different surfaces.

Novel drugs can be tested in the MOBIC device. There has been research into different compounds that have potential to treat biofilm infections. Imidazole derivates, indole derivatives, different plant-derived compounds, marine-derived compounds, N-acyl homoserine lactone-based inhibitors, peptides, and more have had their antibiofilm properties examined and should be tested in the MOBIC channel [42]. These drugs can be tested by pumping them into the channel after bacteria have attached to the surface, causing an increase in impedance, and measuring if the impedance then decreases. If there is a significant decrease in impedance, it would mean that the drug was at least partially able to clear the biofilm or the attached bacteria. These drugs should also be tested at the three different flow rates described previously to see if their effectiveness is variable depending on the flow rate. They could also be tested for their effectiveness clearing biofilms on different types of surfaces.

These experiments should be done using various materials as the channel floor so that the rates of biofilm attachment in the channel can be compared between different

surfaces. This would include stainless-steel, titanium, carbon, latex, silicon, and more to cover the main types of medical devices. Surface properties should be altered to experiment with the attachment of bacteria to surfaces with variable composition, charge, hydrophobicity, porosity, and roughness. These changes can be accomplished by mixing components into the polymer or applying them to the surface [43]. Surfaces that show resistance to biofilm adhesion would have lower rates of attachment and lower impedance. The additional information provided by the fluorescence microscopy will give velocity information that will show interactions between the surface and the bacteria. The goal of this set of experiments would be to find if there is a surface type that is resistant to the initial attachment of bacteria and thus, resistant to biofilm formation in a flow channel. These experiments should also use the changes in flow rate and could use the drug applications as well.

Once preliminary data is recorded for these experiments using *S. aureus,* a heterogeneous mixture of many bacteria should be substituted for the pure *S. aureus* culture in the channel. This is because most biofilms are not homogeneous. *Pseudomonas aeruginosa* should be included in the heterogeneous mixture because it is known for form cocultures with *S. aureus,* and they are both found in chronic biofilm-related infections [44]. Finding a specific surface that is resistant to a range of bacterial attachment or a drug that could clear biofilm on medical implants could be the first step in preventing medical implant-related infections from the source.

Anticipated Problems and Alternative Approaches

Like all scientific experiments, there is room for problems in the use of the MOBIC device. One problem that may arise is that bacterial attachment may be observed by fluorescence microscopy, but the impedance may not show a measurable change until hours into the experiment. In this case, the frequency transmitted by the Potentiostat can be lowered for the next trial so that the small changes are able to be detected. In the opposite case, if there are measurable changes in impedance without clear bacterial attachment, it could be due to protein attachment which can be analyzed. In this case, the surface is able to be preconditioned by flowing media over it for an hour before seeding the bacteria into the channel. Another potential problem is contamination. If the bacterial culture becomes contaminated with other bacteria or microorganisms, the data will be skewed depending on the effects of the other organisms. This can be avoided by use of aseptic or sterile techniques and the use of a fume hood if the problem is persistent. If the three flow rates that were chosen do not show any difference in impedance and bacterial attachment, a larger range of flow rates can be chosen. This could include 0.1mL/h and 1000mL/h to add a ten-fold higher and lower rate. If the bacteria do not attach to the PDMS floor, the ratio of elastomer to hardener can be adjusted to see if a harder or softer polymer will allow for attachment.

Applications

Examining the initial steps in biofilm formation in a MOBIC device will be used to test the attachment of bacteria to different types of surfaces, effects of different drugs on biofilm formation, and the effects of flow rate of biofilm formation. The applications in biomedical sciences for the prevention of infections associated with medically implanted devices has been discussed. There are other applications of the MOBIC channel design. One use of the MOBIC device is applying a potential to the channel surface to measure if the bacteria are electrostatically repelled without the potential being cytotoxic. The flow channel can be used to examine the growth of different organisms in environments that experience flow, like rivers and other bodies of water.

Acknowledgments

I would like to thank Tom Partington for his amazing work in the machine shop, Professor Nikolaos Gatsonis for his contribution of the computational fluid dynamics analysis, Chelsea Barr for her diagram of a mature biofilm, and Professor Christopher Lambert for advising this project.

References

[1] Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. Bacterial biofilms: from the Natural environment to infectious diseases. *Nature News* (2004) **2** 95-108.
[2] Costerton, J. Introduction to biofilm. *International Journal of Antimicrobial Agents* (1999) **11**(3-4) 217–221.
[3] Donlan, R. M. Biofilms: microbial life on surfaces. *Emerging infectious diseases* (2002) **8**(9) 881-890.
[4] Van Houdt, R., & Michiels, C. Biofilm formation and the food industry, a focus on the bacterial outer surface. *Journal of Applied Microbiology* (2010) **109**(4) 1117-1131.
[5] Pandit, A., Adholeya, A., Cahill, D., Brau, L., & Kochar, M. Microbial biofilms in nature: Unlocking their potential for agricultural applications. *Journal of Applied Microbiology* (2020) **129**(2) 199-211.
[6] Kesaano, M., & Sims, R. Algal biofilm based technology for wastewater treatment. *Algal Research* (2014) **5** 231-240.
[7] Singh, R., Paul, D., & Jain, R. Biofilms: Implications in bioremediation. *Trends in Microbiology* (2006) **14**(9) 386-397.
[8] Stewart, P., & Costerton, J. Antibiotic resistance of bacteria in biofilms. *The Lancet* (2001) **358**(9276) 135-138.
[9] Rabin, N., Zheng, Y., Opoku-Temeng, C., Du, Y., Bonsu, E., & Sintim, H. O. Biofilm formation mechanisms and targets for developing antibiofilm agents. *Future Medicinal Chemistry* (2015) **7**(4) 493-512.
[10] Agarwal, A., Singh, K. P., & Jain, A. Medical significance and management of staphylococcal biofilm. *FEMS Immunology & Medical Microbiology* (2010) **58**(2) 147–160.
[11] Fernandes, A., & Dias, M. The microbiological profiles of Infected prosthetic implants with an emphasis on the organisms which form biofilms. *Journal of Clinical & Diagnostic Research* (2013) **7**(2) 219-223.
[12] Thorpe, K. E., Joski, P., & Johnston, K. J. Antibiotic-Resistant infection treatment costs have doubled since 2002, now Exceeding $2 billion annually. *Health Affairs* (2018) **37**(4), 662-669.
[13] Dufour, D., Leung, V., & Lévesque, C. M. Bacterial biofilm: Structure, function, and antimicrobial resistance. *Endodontic Topics* (2010) **22**(1) 2-16
[14] Archer, N., Mazaitis, M., Costerton, J., Leid, J., Powers, M., & Shirtliff, M. (2011). Staphylococcus aureus biofilms: Properties, regulation, and roles in human disease. *Virulence* (2011) **2**(5) 445-459.
[15] CDC biofilm reactor - Biosurface Technologies Corporation. (2021).
[16] Rabin, N., Zheng, Y., Opoku-Temeng, C., Du, Y., Bonsu, E., & Sintim, H. O. Biofilm formation mechanisms and targets for developing antibiofilm agents. *Future Medicinal Chemistry* (2015) **7**(4) 493-512.

[17] Moormeier, D. E., & Bayles, K. W. Staphylococcus aureus biofilm: A complex developmental organism. *Molecular Microbiology* (2017) **104**(3) 365-376.
[18] Fanning, S., & Mitchell, A. Fungal biofilms. *PLoS Pathogens* (2012) **8**(4)
[19] Böhme, A., Risse-Buhl, U., & Küsel, K. Protists with different feeding modes change biofilm morphology. *FEMS Microbiology Ecology* (2009) **69**(2) 158-169
[20] Costerton, J. Introduction to biofilm. *International Journal of Antimicrobial Agents* (1999) **11** 217-221.
[21] Taylor, T., & Unakal C. G. Staphylococcus aureus. *StatPearls [Internet].* (2020). https://www.ncbi.nlm.nih.gov/books/NBK441868/
[22] Mueller, M., & Tainter C. M. Escherichia coli. *StatPearls [Internet].* (2020). https://www.ncbi.nlm.nih.gov/books/NBK564298/
[23] Iglewski, B. Chapt. 27 “Pseudomonas.” *Medical Microbiology* (1996) 4th edition.
[24] Maurice, N., Bedi, B., & Sadikot, R. (2018, April). Pseudomonas aeruginosa Biofilms: Host response and clinical implications in lung infections. *Am J Respir Cell Mol Biol* (2018) **58**(4) 428-439
[25] Davin-Regli, A., & Pagès, J. (2015, May 18). Enterobacter aerogenes and Enterobacter cloacae; versatile bacterial Pathogens confronting antibiotic treatment. *Front Microbiol* (2015) **6** 392
[26] Giannella, R. Chapt. 21 “Salmonella.” *Medical Microbiology* (1996) 4th edition.
[27] Metwalli, K., Khan, S., Krom, B., & Jabra-Rizk, M. Streptococcus mutans, candida albicans, and the human mouth: A sticky situation. *PLoS Pathogens* (2013) **9**(10)
[28] Rodriguez, J. O., & Kahwaji, C. I. Vibrio cholerae. *StatPearls [Internet].* (2020). https://www.ncbi.nlm.nih.gov/books/NBK526099/
[29] Wilson, C., Lukowicz, R., Merchant, S., Valquier-Flynn, H., Caballero, J., Sandoval, J., . . . Holmes, A. Quantitative and qualitative assessment methods for Biofilm growth: A mini-review. *Res Rev J Eng Technol* (2017) **6**(4)
[30] Kurmoo, Y., Hook, A. L., Harvey, D., Dubern, J., Williams, P., Morgan, S. P., . . . Alexander, M. R. Real time monitoring of biofilm formation on coated medical devices for the reduction and interception of bacterial infections. *Biomater Sci* (2020) **8** 1464-1477
[31] Johnson, L. (2020, December 28). Fluid dynamics (overview): BASICS, terminology & equations.
[32] Zhou, J.-H., Sui, Z.-J., Li, P., Chen, D., Dai, Y.-C., & Yuan, W.-K. Structural characterization of carbon nanofibers formed from different carbon-containing gases. *Carbon* (2006) **44**(15) 3255-3262.
[33] Grossi, M., & Riccò, B. Electrical impedance spectroscopy (eis) for Biological analysis and food characterization: A review. *J. Sens. Sens. Syst.* (2017) **6** 303-325.
[34] Basics of Electrochemical impedance spectroscopy. *Gamry Instruments* (n.d.).

[35] Ben-Yoav, H., Freeman, A., Sternheim, M., & Shacham-Diamand, Y. An electrochemical impedance model for integrated bacterial biofilms. *Electrochimica Acta* (2010) **56**(23) 7780-7786.
[36] Lichtman, J., & Conchello, J. Fluorescence microscopy. *Nature Methods* (2005) **2** 910-919.
[37] Fischer, A., Srenz, L. and Schrenzel, J. From the Laboratory of the Geneva University Hospitals (HUG). CH-1211 Geneva 4, Switzerland.
[38] Lopez, D., Vlamakis, H., & Kolter, R. Biofilms. *CSH Perspectives* (2010) **2**(7).
[39] Ribeiro, M., Monteiro, F., & Ferraz, M. Infection of orthopedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial-material interactions. *Biomatter* (2012) **2**(4) 176-194.
[40] Estrada-Leypon, O., Moya, A., Guimera, A., Gabriel, G., Agut, M., Sanchez, B., & Borros, S. Simultaneous monitoring of staphylococcus aureus growth in a multi-parametric microfluidic platform using microscopy and impedance spectroscopy. *Bioelectrochemistry* (2015) **105** 56-64.
[41] Hiroshi, K. A. (2020). *Characterization of Biofilms using EIS Spectroscopy* (Major Qualifying Project No. E-project-060920-002124). Retrieved from Worcester Polytechnic Institute Electronic Projects Collection: https://digital.wpi.edu/concern/student_works/73666695q?locale
[42] Rabin, N., Zheng, Y., Opoku-Temeng, C., Du, Y., Bonsu, E., & Sintim, H. O. Agents that inhibit bacterial biofilm formation. *Future Medicinal* Chemistry (2105) **7**(5) 647-671.
[43] Cattò, C., & Cappitelli, F. Testing anti-biofilm Polymeric Surfaces: Where to start? *Int J Mol Sci* (2019) **20**(15) 3794.
[44] Cendra, M., Blanco-Cabra, N., Pedraz, L., & Torrents, E. Optimal environmental and culture conditions allow the in vitro coexistence of pseudomonas aeruginosa and staphylococcus aureus in stable biofilms. *Scientific Reports* (2019) **9**.