

Study of the Effect of Mechanical Stiffness Substrata, Assembled with Polyelectrolyte Multilayer Thin Films, on Biofilm Forming Staphylococcus Epidermidis' Initial Adhesion Mechanism

By

Maricela Delgadillo

Submitted to the Department of Materials Science and Engineering  
in Partial Fulfillment of the Requirements for the Degree of

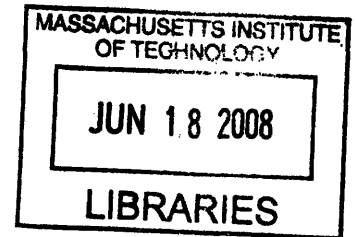
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# Study of the Effect of Mechanical Stiffness Substrata, Assembled with Polyelectrolyte Multilayer Thin Films, on Biofilm Forming *Staphylococcus Epidermidis*' Initial Adhesion Mechanism

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## ABSTRACT

**Polyelectrolyte multilayer thin films are polymer films assembled through a layer-by-layer sequential addition of oppositely charged polymers. The layer-by-layer film assembly technique allows for properties such as film thickness, chemical functionality, and elastic moduli to be easily altered by changing the pH in solution, or the number of bilayers added. This thesis examined the use of polyelectrolyte multilayer films, assembled with poly(allylamine hydrochloride) (PAH) and poly(acrylic acid) (PAA), to alter substrata mechanical stiffness, which was used to explore the response of biofilm forming *staphylococci epidermidis*.**

**The formation of biofilms on medical device surfaces is currently responsible for a significant amount of infections acquired in hospitals. Currently mechanisms responsible for the initial adhesion of bacteria are not completely understood. Previous work completed in the Rubner and Van Vliet labs at MIT suggests a mechanoselective adhesion mechanism in prokaryotes. The existence of a positive correlation between mechanical stiffness and bacterial adhesion, independent of surface roughness or charge density, has already been shown in a non-biofilm forming strain of bacteria. This thesis focused on exploring the role mechanical stiffness substrata has on biofilm forming bacterial adhesion by conducting bacterial assay experiments on polyelectrolyte multilayer films. The results showed no positive correlation between mechanical stiffness and cell adhesion with biofilm forming *staphylococcus epidermidis*. Also, even under an applied shear force the amount of bacteria adhered on the surface was not affected. In all cases tested, the biofilm forming strain of bacteria was able to adhere and grow successfully.**

Thesis Supervisor: Professor Michael F. Rubner  
Title: TDK Professor of Materials Science and Engineering

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## 1 Introduction

Polyelectrolyte multilayer thin films were used to test the mechanoselective adhesion mechanism of a biofilm forming strain of *staphylococcus epidermidis*. Previous work completed in the Van Vliet and Rubner labs at MIT found that mechanical stiffness had a positive correlation with a non-biofilm forming strain of *staphylococcus epidermidis*. Here these results were further analyzed by using the same substrata functionalized with polyelectrolyte multilayers, and a biofilm forming strain of bacteria.

Substrates were functionalized with polyelectrolyte multilayers through the sequential addition of oppositely charged polymers in a layer-by-layer process. Two systems were analyzed: those assembled at pH values of 2.0, which have a mechanical stiffness of about 1MPa, and those assembled at pH values of 6.5, with a mechanical stiffness of 100 Mpa. Previous work demonstrated that in these systems, the only significant difference was the mechanical stiffness.

Finally, a protocol a bacterial attachment assay was adapted and modified from Stepanovic et al. [7]. After the bacteria were incubated on the surfaces for 2 or 24 hours, the initial adhesion mechanism was studied through microscopy, and atomic force microscope imaging. The goal of my thesis was to prove that bacterial adhesion of a biofilm forming strain of bacteria correlates positively with mechanical stiffness. However, the bacteria were able to overcome any mechanoselective obstacles.

## 2 Biofilms Background and Research Motivation

Currently, biofilm contamination of surfaces is a major cause of concern for a wide array of industries. Biofilms are a complex system of microorganisms that form irreversible attachments to both living and non-living surfaces [11]. In industrial materials and equipment, biofilms lead to fluid flow resistance, reduced heat transfer efficiency, product contamination, and bio-corrosion [10]. Biofilms are of special interest in the medical field because they have the ability to kill. According to the Center for Disease Control and Prevention report in 2007, biofilms account for 1.7 million infections and 99,000 deaths each year, 22 % of which are from surgical site infections [10]. In the United States alone, 60-70% of hospital acquired infections arise from implanted medical devices [10], such as prosthetic hip implants, venous catheters, prosthetic heart valves, cardiac pacemakers, vascular prostheses, and contact lenses. [10,11]. Surgical site infections usually lead to implant removal, placing the patient in danger of more surgery, added medical costs, or even death.

The biomedical devices industry is a \$180 billion dollar per year and growing industry. Because all medical devices are vulnerable to biofilm formation, research on addressing biofilm attachment and growth is of large interest. The initial attachment mechanism of bacteria onto a surface is currently not well understood, but whether or not bacteria attach to a surface is a crucial step in the development of biofilm. Others have looked at the effects of surface charge, hydrophobicity, surface roughness, and surface conditioning [9] to the initial attachment mechanism, however, the results are still inconclusive, and may include a combination of characteristics [9]. Previous work in the Rubner and Van Vliet labs at MIT has resulted in promising results showing the positive correlation of polymeric substrata stiffness to adhesion of non-biofilm forming type of *staphylococcus epidermidis* [1]. In order to further explore this

mechanism, a biofilm forming type of bacteria was explored. This thesis addresses the question of what effects surface mechanical stiffness has on the initial attachment of a biofilm forming type of *staphylococcus epidermidis*, the most common organism found in hospital acquired infections [10].

## **2.1 Biofilm Development and Growth**

Forming a biofilm has several steps, which include physical, biological, and chemical processes [10]. Confocal scanning laser microscopy technology in 1992 made it possible to observe images of the complicated bacterial networks [8]. John Lawrence introduced the first confocal images of a biofilm where it was possible to see bacterial cells enclosed within a viscous transparent material. This revelation opened the door to understanding the complexity of biofilms that is still not clear today [8].

### **2.1.1 Structural Composition of a Biofilm**

Biofilms are highly organized complex structures of microorganisms. The microorganism community is composed of bacterial cells enclosed within a viscous transparent exopolysaccharide matrix. Within the matrix there is a network of open water channels which serve to carry nutrients and dispose of waste. The channels do not have cells and act as a centralized circulatory system [8]. The physiological need to carry nutrients and waste to and from the cells creates a “lumpy” architecture, which is made up of tower and mushroom microcolonies. Mushroom microcolonies are used to establish a well-organized diffusion path [8]. On the other hand, well-fed biofilms form “flat” conformations [8]. The abundance of nutrients makes a tower or mushroom architecture unnecessary for these types of biofilms to survive. The bacteria used in this thesis will most likely form a “flat” conformation, since

nutrients are provided at every step of the experimental procedure and they are grown under static conditions.

### 2.1.2 Cell Organization and Communication

Once bacteria are attached to the surface, which will be discussed later, there appears to be communication among the cells termed “quorum sensing.” The existence of “quorum sensing” is a plausible hypothesis because it is observed that individual bacterial cells are organized within biofilms at a distance of about 3 to 5  $\mu\text{m}$  [8]. The cellular distribution also affects whether or not the mushroom configuration is adapted. If the hypothesis is true and organization is not random, a network of pili resembling that of the cytoskeleton of eukaryotic cells may control the organization mechanism [8]. The pili have a contracting mechanism that controls cell distribution [8]. More evidence of the existence of communication between cells lies in the fact that gene transfer between biofilm cells happens at a rate 1000 times faster than in cells suspended in fluids. Furthermore, recently Gory et al. 2006, has added evidence that biofilm sections are connected by linear protein structures or “nanowires” [8]. In addition, Paul Stoodley has indicated that individual microcolonies act like viscoelastic solids that can be deformed by high shear forces leading to oscillations forming waves that detach biofilm from the matrix [8]. Thus, it is also a possibility that the tower and water channels are produced from shear forces and not by direct morphological development. This idea, however, indicates that the presence of structure is random, and based on whether or not shear forces are present, which is difficult to accept. It is highly unlikely that such complicated networks can be attributed to random events. Whether or not these mechanisms are the explanation for the structure within cells, it is undeniable that cell-cell communication within biofilms exists.

### **2.1.3 Matrix Composition**

Once a cell is successfully attached onto a surface, it secretes an exopolysaccharide matrix. The exact composition the matrix is still not fully understood. However, there are several components that exist in every matrix [8]. These are acid polysaccharides, polymers of sugar molecules (usually uronic acids), and recently, Lawrence et al. 2003, has identified large blob-like structures of exopolysaccharides that do not enclose bacterial cells [8]. More surprisingly, DNA has been identified as a large part of some biofilms' matrix. This poses the question of whether DNA is simply a type of polysaccharide chain composed of polymer deoxyribose, or if it is present to carry information. There has been evidence that identifies a large amount of DNA in specific parts of biofilm such as the cap formation of the mushroom structures, which may indicate its presence for the sake of information (Tolker-Nielsen, 2006) [8]. This leads to the possibility that there exist cells that sacrifice themselves and release their DNA into the matrix for structural order. Whatever the case, it is certain that DNA is part of the matrix.

## **2.2 Initial Bacterial Attachment Mechanisms**

The initial attachment of bacterial cells onto a substrate is a crucial step in biofilm formation. The initial attachment governs whether or not a biofilm will develop and grow. The question of what affects whether or not bacteria irreversibly attach to a surface is still not agreed upon. Research has focused on factors such as surface conditioning, surface charge, surface roughness, and hydrophobicity [9]. Some researchers have found that conditioning the surface with proteins will compete for bacterial binding sites; others disagree and report that the presence of proteins on a surface could serve as nutrients for bacteria [9]. Surface charge experiments also produce inconclusive results. Because bacterial walls have a slight negative charge, there

should be repulsion from a negatively charged substrata such as stainless steel. Gilbert et al. 1991, proved just the opposite by showing a decreased attachment in *E.coli*, but no effect on *S. aureus* [9]. Thus, they demonstrated that surface charge is not consistently affecting the attachment mechanism. Surface roughness also yields competing results. Cracks and crevices in substrata may provide a large area protected from cleaning and shear forces. Consistent with this theory, several research groups report a positive correlation between bacterial adhesion with surface roughness, and others show no such correlations [9]. With all these factors positively correlating in some cases, it is possible that the overall mechanism is a combination of all of them, or it could depend on what factor is most influential in each particular case.

When bacteria approach a surface, the surface will see pili or flagella composed of proteins 2-6 nm wide, exopolysaccharide and lipopolysaccharide fibers, and some outer membrane exposing the hydrophobic parts of lipopolysaccharides [8]. All of these surface structures protect the cell and further support the hypothesis that the initial attachment is probably not controlled by a single surface characteristic. Also, bacteria are dynamic creatures with the ability to easily adapt; therefore, a single surface characteristic may delay the attachment mechanism, but may not completely overcome it. This thesis will look independently at the mechanical stiffness effect on biofilm forming *staphylococcus epidermidis*. Previous work, which will be discussed in chapter 2.3, has already demonstrated that other factors do not play a significant role in the experiments that were conducted.

### 2.2.1 Current Research on Preventing Adhesion

Currently, research has focused on preventing bacterial colonization by either functionalizing the surface to kill bacteria, or preventing the initial adhesion of bacteria. Surfaces that are functionalized to kill bacteria include: microbicidal agents, the inclusion of slow

releasing biocides such as gold or silver [11], antibiotics, or the addition of specific antimicrobial peptides and polymers [1]. Neither of these techniques has been successful at permanently preventing the adhesion and growth of bacteria. Killing bacteria does not completely solve the problem of bacterial attachment because eventually the functionalized surfaces will degrade and run out of whatever addition the surface had. Once the surface functionality degrades, bacteria are free to attach and grow on top of the dead bacteria surface. In addition, as was discussed in chapter 2.2, the mechanisms through which bacteria adhere are not clear, and may vary depending on the environment and the type of bacteria analyzed. Thereby, making it difficult to prevent adhesion with only surface characteristics. Previous work, discussed in chapter 2.3, has proven a mechenoselective adhesion mechanism in *staphylococcus epidermidis*, which was further explored in this thesis.

### **2.3 Previous work done on mechanical stiffness correlation to adhesion**

In work previously completed in the Van Vliet and Rubner labs at MIT, we identified a positive correlation between polymer substrata mechanical stiffness and initial bacterial adhesion, independent of surface interaction energy, surface roughness, and charge density [1]. Table 1 below shows the exact physiochemical and mechanical characteristics of the polymeric substrata used in order to conduct the bacterial experiments [1]. The difference between every characteristic, except elastic modulus and surface roughness, was statistically identical. Surface roughness showed no correlation with bacterial attachment, which left elastic modulus as the only important surface characteristic for our experiments. The assembly of the polymeric substrata was done with polyelectrolyte multilayers. The elastic moduli of the final polymeric substrata depended on the assembly process and will be further discussed in chapter 3.

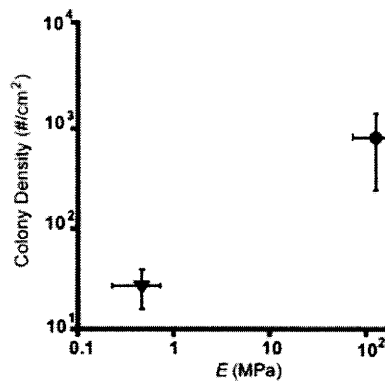
<b>Assembly pH (PAA/PAH)</b>	<b><math>\Delta</math>GMWP (microbe- water-PEM)  (mJ/m<sup>2</sup>)</b>	<b>[RMS] (root Mean Square)  (nm)</b>	<b>Q (surface charge density)</b>	<b>E (MPa)</b>
<b>2.0/2.0</b>	<b>29.0 ± 7.5</b>	<b>30.2 ± 29.5</b>	<b>-2.29 ± 0.1 mC/m<sup>2</sup></b>	<b>0.75 ± 0.05</b>
<b>6.5/6.5</b>	<b>27.2 ± 8.95</b>	<b>2.7 ± 1.6</b>	<b>-3.18 ± 1.4 mC/m<sup>2</sup></b>	<b>80.4 ± 38.0</b>

**Table 1:** This table shows the values of the physiochemical and mechanical properties affecting initial bacterial attachment of the polyelectrolyte multilayers used. There was only a significant difference in mechanical stiffness (E); while surface charge density (Q), and total interaction energy of the microbe-water-polymer system ( $\Delta$ GMWP) have statistically identical values. Although root mean square (RMS) surface roughness is different, no correlation with bacterial attachment was observed [1].

Once the substrata characterization demonstrated only a difference in mechanical stiffness, we were able to confirm that substrata mechanical stiffness promotes cell adhesion in a non-biofilm forming strain of *staphylococcus epidermidis*. As Figure 1 shows below, the density of colonies on the stiffer polyelectrolyte multilayer surface assembled at a pH of 6.5 was higher than the more compliant surface assembled at a pH of 2.0.

These results were explored by following the same procedure of substrata assembly and attempting to repeat these results with a biofilm forming strain of *staphylococcus epidermidis*.





**Figure 1:** Plot of colony density versus modulus for different polyelectrolyte multilayers. For all cell concentrations the density of colonies that attached on the polyelectrolyte multilayer substrata assembled at pH 6.5 (•) was greater than the substrata assembled at a pH 2.0 (τ) [1].

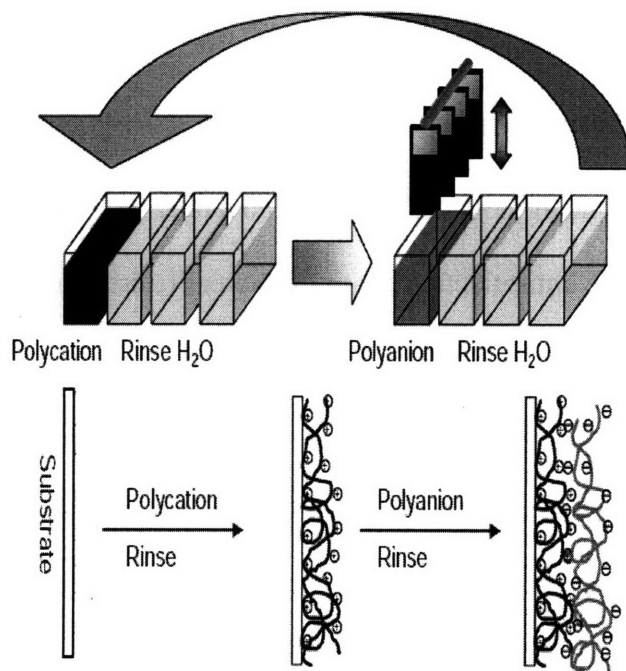
### 3 Polyelectrolyte Multilayer Film Background

Polymer thin films are one of the most important advances in the field of Materials Science. Their applications range from antireflection coatings to wear resistant coatings against chemical or thermal effects. They allow for the modification of a surface without altering the properties of the bulk material [3]. Polyelectrolyte multilayer film applications are especially important because they are easy to process, inexpensive, easily conform to any surface, and can be biocompatible [3]. This thesis emphasized the utility of polyelectrolyte multilayer films as biomaterials used to prevent bacterial adhesion by altering the mechanical stiffness of the substrata used in the bacteria experiments.

#### 3.1 Layer-By-Layer Assembly and Polyelectrolytes

Polyelectrolyte multilayer films are assembled through the sequential addition of oppositely charged polymers. This layer-by-layer approach allows the assembly parameters including pH and the number of bilayers to be easily altered to create the desired surface characteristics. During the layer-by-layer process, the substrate is first dipped into a

polyelectrolyte solution until the polymers have enough time to absorb onto the surface. The next step washes off the excess loosely bound polymer from the surface by sequentially dipping it in three different water baths. Next, the substrate is immersed in a polyelectrolyte solution of the opposite charge. The second polymer solution is electrostatically attracted to the substrate and switches the surface charge. Again, this is followed by three water rinsing steps. One of these cycles creates a bilayer, and the number of bilayers can easily be altered. Figure 2 below shows the process here described.



**Figure 2:** Layer-by-layer assembly process of polyelectrolyte multilayers courtesy of Michael Berg [2].

The layer-by-layer technique is not only effective for polyelectrolytes assembled via electrostatic interactions; they have also been successfully assembled with hydrogen bonding interactions. Furthermore, other materials such as proteins, DNA, nanoparticles, cells, and light emitting polymers have also been included into polyelectrolyte multilayers [2].

The weak polyelectrolytes used in multilayer assembly are polycation poly(allylamine hydrochloride) (PAH) and polyanion poly(acrylic acid) (PAA), shown in Figure 3 below. Polyelectrolytes are polymer molecules that have water-ionizable group along their backbone, and can be labeled strong or weak depending on the extent of ionization. The extent of ionization is measured relative to their pKa, that is, the pH value where half of the ionizable groups are dissociated [3].



**Figure 3:** Chemical Structure of PAH and PAA courtesy of Michael Berg [2].

### 3.1.1 pH effects on Polyelectrolyte Multilayers

Poly(acrylic acid) and Poly(allylamine hydrochloride) are weak polyelectrolytes; thus, their charge density varies depending on the pH of the solution. Poly(acrylic acid) is a weak polyanion, whose charge density increases as pH increases, and has a solution pKa of about 6.5 [2]. On the other hand, poly(allylamine hydrochloride), a polycation, has a solution pKa of about 8.8, and its charge density decreases as the pH of the solution increases [2].

The thickness of polyelectrolyte multilayers assembled with poly(acrylic acid) and poly(allylamine hydrochloride) ranges between 5 and 80 Å over a small window of pH values [4]. As the charge density of weak polyelectrolytes changes, so does the adsorbed thickness, which can greatly affect overall bulk surface characteristics [2,4]. At low pH extremes of about 2.0, the degree of ionization of the poly(acrylic acid) chains in solution is only about 10% [4]. When polymers are adsorbed at this pH, they have a loopy film conformation because

poly(acrylic acid) is not highly charged and does not lie flat on the poly(allylamine hydrochloride) surface [2]. Also, a small amount of poly(allylamine hydrochloride) is attracted to the film surface since poly(acrylic acid) is highly protonated [2]. On the other hand, as the pH value increases to about 6.5, both polymers are highly ionized resulting in thin adsorbed layers that are highly interpenetrated, lie flat within the multilayer, and are about 3 Å thick [4].

## **4 Experimental Procedures**

The experimental procedure included several steps. First the substrata, in this case six-well bacterial culture plates or aminoalkylsilane coated glass slides, were functionalized through the assembly of polyelectrolyte multilayers, described in chapter 4.1. After the six-well plates were prepared to have coatings with the desired mechanical stiffness, they were ready to be included in the bacterial assays part of the experiment.

In the second part of the experimental procedure, bacterial solutions (consisting of  $10^8$  cells/mL) and glucose solutions were prepared, added to the sterilized six-well plates, and incubated overnight under at a temperature of 37° C. Following the incubation for either 2 or 24 hours, the six-well plates were washed and the cells were fixed onto the surface. The final step consisted of staining and imaging the plates in order to visualize the density and distribution of the attached cells.

### **4.1 Substrata preparation: PEM assembly procedure**

The substrata used in the experiments were six-well bacteria culture plates from Becton Dickinson labware, or aminoalkylsilane coated glass slides from Sigma-Aldrich. The surfaces were functionalized with polyelectrolyte multilayers assembled via the layer-by-layer sequential addition of oppositely charged polymers described in Chapter 3. The solutions were composed

of  $10^{-2}$  M concentration of poly(acrylic acid) (molecular Weight =200 000 g/mol; 25% aqueous solution; Polysciences), and poly(allylamine hydrochloride), (molecular weight of 70 000 g/mol; Polysciences). The solutions were prepared in 18M $\Omega$  Milli-Q water, and the pH values of the solutions were adjusted by adding one molar hydrochloric acid and sodium hydroxide.

The first layer deposited on the plain six-well plates, by HMS programmable slide stainer from Zeiss, Inc. was the positively charged poly(allylamine hydrochloride) for 10 minutes followed by three rinse baths of 18M $\Omega$  Milli-Q water, with dipping times of 2 minutes, 1 minute, and 1 minute. After the three wash steps, the six well plates were dipped into poly(acrylic acid) for ten minutes, and followed the same rinse cycle before re-entering the poly(allylamine hydrochloride) solution. The six-well plates assembled at a pH 2.0 were prepared with 10 bilayers, and the six-well plates prepared at a pH of 6.5 were built up to 50 bilayers. On the aminoalkylsilane treated slides, the first layer deposited was the polyanion, poly(acrylic acid), due to the positively charged surface of the slide. In this system the slides assembled at a pH value of 2.0 had 9.5 bilayers, and the slides assembled with a pH value of 6.5 had 49.5 bilayers. In both pH systems, poly(acrylic acid) was deposited as the top layer in order to keep the surface characteristics the same as the work done previously.

#### **4.2 Bacterial attachment assays**

The experimental procedure for the bacteria attachment assays was adapted and modified from Stepanovic et al. [7]. These experiments take place over the course of several days. Each step of the experimental procedure must be carried out carefully because the slightest error, such as touching the solution with any un-sterilized surface, may contaminate the bacterial solution, and give false results.

#### 4.2.1 Bacterial Inoculation

During the first day of the experiment, Trypticase Soy Broth (BD) was inoculated with a biofilm forming monoclonal strain of *staphylococcus epidermidis* (S. epidermidis, ATCC # 35894). The first step was to make sure that the lab space, the pipettes used, the Trypticase Soy Broth container, and researchers hands were sterilized by spraying them with a 70% Ethanol solution (Pharmoco-Aper). Once everything was sterilized, the bacteria was inoculated by taking a frozen vial of bacteria, touching a sterile pipette tip to it, and inoculating the Trypticase Soy Broth. The Trypticase Soy Broth was then be loosely covered, and incubated for  $18 \pm 1$  hour at  $37^{\circ}\text{C}$  with shaking agitation.

#### 4.2.2 Preparation of Bacterial Solution and Incubation on Substrata Surface

In order to make sure that there was no contamination of any other strains of bacteria, the polyelectrolyte multilayer functionalized substrata that were used to grow the biofilm were sterilized by spraying them down with 70% ethanol solution and left to air dry. Ensuring that all pipettes used and lab space were kept completely sterile was a crucial step in making sure this part of the experiment was successful.

After the bacteria culture was grown overnight, two 20 mL aliquots of the primary culture were centrifuged at 2700 RPM for ten minutes at  $4^{\circ}\text{C}$  (Centrifuge 5804 R). The Trypticase Soy Broth was then decanted into a 10% Bleach solution, and the bacteria pellet was re-suspended into two 15 mL aliquots of Trypticase Soy Broth. This solution was then centrifuged at 2700 RPM for 5 minutes at  $4^{\circ}\text{C}$ . Again, the Trypticase Soy Broth was decanted into the 10% Bleach solution, and the bacteria pellet was finally re-suspended into 10 mL of Trypticase Soy Broth. The following step was to dilute the bacterial solution until it reaches an optical density of one at a wavelength of 540 nm on a uv-vis spectrophotometer, which

corresponds to a cell density of  $10^9$  cells/mL. The final step was to dilute 10 mL of bacterial solution into 90 mL of Trypticase Soy Broth corresponding to a final bacterial solution with a density of  $10^8$  cells/mL.

In order to ensure the bacteria have enough nutrients to attach and grow a biofilm, a 2% glucose solution was added to the bacteria solution before it was placed on the substrate. Adding a glucose solution was an important step because it is necessary for biofilm growth. The solution was prepared by with Glucose Monohydrate (Biochemika), 50 grams was diluted into 500 mL of 18M $\Omega$  Milli-Q water, and autoclaved for 15 minutes.

The final step of this part of the experimental procedure was to culture the bacteria on the six-well plate substrata, or the aminoalkylsilane treated slides, and incubate at 37°C. Three sets of six-well plates were used (assembled at pH values of 2.0 and 6.0, as described in chapter 4.1) and a control substrata without no polyelectrolyte multilayers. Using a sterile pipette, 2 mL of bacterial solution with 2% glucose were added to wells 1, 2, 4 and, 5. Well 3 was used as a control and only broth and glucose were added. Nothing was added to well 6 in order to make sure the polyelectrolyte multilayer did not stain positively. Finally, the six well plates were covered, parafilm, and left to incubate for 2 or 24 hours. For the experiments with aminoalkylsilane slides, 5 slides (2 for the shear testing that will be discussed later) functionalized with polyelectrolyte multilayers were placed in Petri dishes (VWR) and inoculated with 15 mL of bacteria and glucose solution. Two slides were left as controls, and only broth was added, and one slide was used for staining. Furthermore, two aminoalkylsilane slides with no polyelectrolyte multilayers, and two glass slides were also inoculated with bacteria to serve as controls. The inoculation time for the first two bacterial experiments was 24 hours in order to make sure that biofilm was successfully growing on the polyelectrolyte substrata. After

confirming the existence of biofilm with the first two experiments, the next three were run at incubation times of 2 hours to observe the initial attachment of bacteria on the substrate. The short incubation time did not give the bacteria enough time to develop into a mature biofilm.

#### **4.2.3 Washing and Fixing Bacteria**

Following the proper incubation time, the next step was to rinse out the excess bacteria and fix the attached bacteria and biofilm formed onto the substrata. First, while making sure that there was no cross contamination between wells, the excess bacterial solution was drained out carefully using a pipette. Next, the wells were carefully rinsed with Phosphate Buffered Saline (PBS, from VWR) twice. It was especially important during the rinsing steps to ensure that the pipetting was done very gently so as not to disrupt the biofilm or the cells attached on the surface. The experiment with aminoalkylsilane treated slides followed the same rinsing procedure, however, one set of slides were rinsed more harshly in order to test the strength of the attachment of the biofilm on the polyelectrolyte multilayers. Next, the attached bacteria were fixed onto the surface by placing 2 mL of Methanol (Mallinckrodt Chemicals) into each of the wells, or 15 mL of Methanol into the Petri dishes, and letting them sit at room temperature for 20 minutes. Finally, the excess methanol was drained and the six-well plates were left to dry overnight.

#### **4.2.4 Staining and Imaging Bacteria**

The final part of the experimental procedure was to stain all of the wells in the six-well plates, or aminoalkylsilane treated slides, to observe the existence and attachment of bacteria on the surface. The staining, using a 2% solution of Gram Crystal Violet (BD) for 20 minutes, was done with 5 mL for each of the wells or 20 mL for the Petri dishes. The stain was then rinsed out



carefully using 18M $\Omega$  Milli-Q water. Once the plates dried, images were taken of each well at magnifications of 20X and 50X with AxioVision and a Axiovert 200 Zeiss microscope. Atomic Force Microscope (Nano Scope Control, Digital Instruments) images were only taken of the last experiment on the slides.

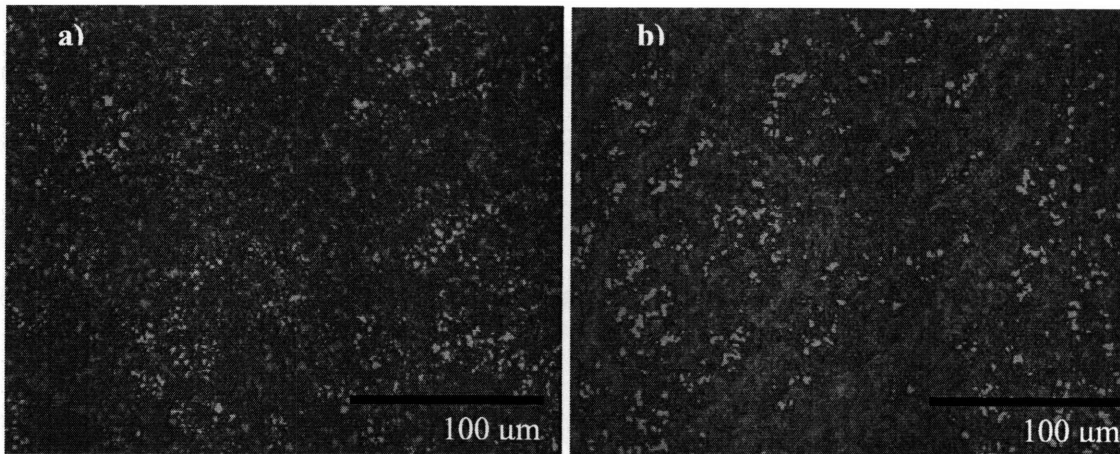
## **5 Results and Discussion**

After the development of the biofilm growth protocol, the next major accomplishment of my thesis was being able to positively identify the formation of biofilm under static conditions. The first two experiments with 24 hours of incubation time yielded positive biofilm results. The next step was to study the initial attachment mechanism. In order to do so, the next two experiments on six-well plate substrata were incubated for two hours. Both of these experiments did not show a significant difference in the amount of bacteria attached on the polyelectrolyte multilayers; therefore, the next experiment was conducted on aminoalkylsilane treated slides in order to ensure there was no cross contamination and the polyelectrolyte multilayers were evenly distributed on the substrata surface. In addition, the last experiment included a shear force component to test the stability of the attachment of the bacteria on the polyelectrolyte substrata. The hypothesis was that even if the amount of bacteria attached onto the surface did not change depending on the mechanical stiffness of the substrata, the compliant polyelectrolyte multilayers might require less shear force to remove bacteria as compared to the stiffer substrate.

### **5.1 24 Hour Incubation Results**

Positively identifying the existence of biofilm was a major step in the research for this thesis. Once the positive identification of biofilm on the surface was established, the initial adhesion mechanism could be further explored. Although there was hope to see a lower density

of biofilm on the substrata assembled at pH values of 2.0, there was none. Thus, indicating that the biofilm forming *staphylococcus epidermidis* overcame any effects the mechanical stiffness of the substrata had on the initial adhesion step. Both of the substrata functionalized with polyelectrolyte multilayers appeared to have the same amount of biofilm growth, as is observed in Figure 4 below.



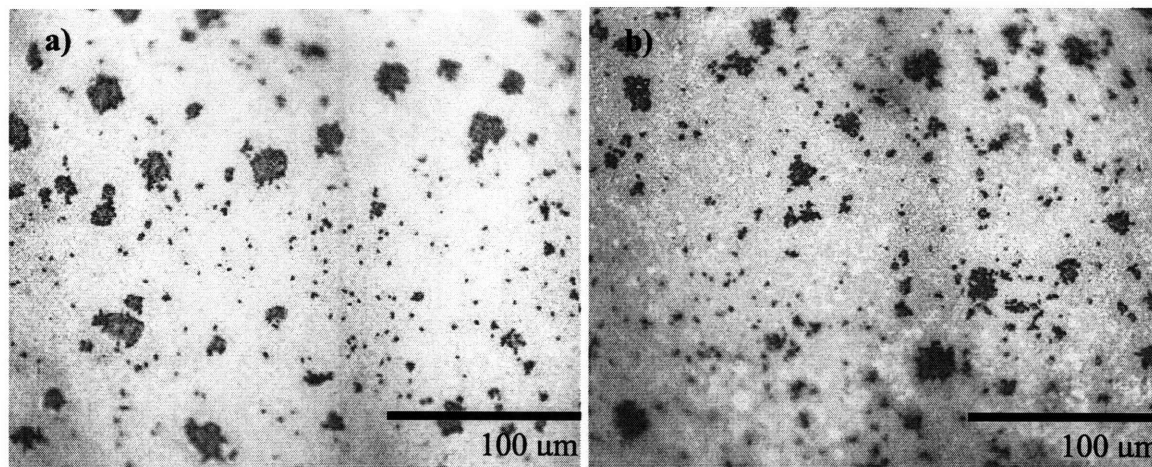
**Figure 4:** Images taken at a 50X magnification. Figure 5a is on substrata assembled at pH values of 2.0, while figure 5b is on substrata assembled at pH values of 6.5. There is not a visible difference, however, both do show biofilm formation.

Finally, with hopes of observing only the initial attachment mechanism of the bacteria, the next step was to repeat the experiment with an incubation time of only 2 hours. This allowed enough time for the initial adhesion mechanism to take place without enough time for the development of a mature biofilm.

## **5.2 2 Hour incubation results on six-well plates**

The next experiment was conducted at a two hour incubation time to observe the initial attachment mechanism of the biofilm growth. The results of the experiment showed no visible difference between the substrata assembled at the different pH values of 2.0 and 6.5. Figure 5

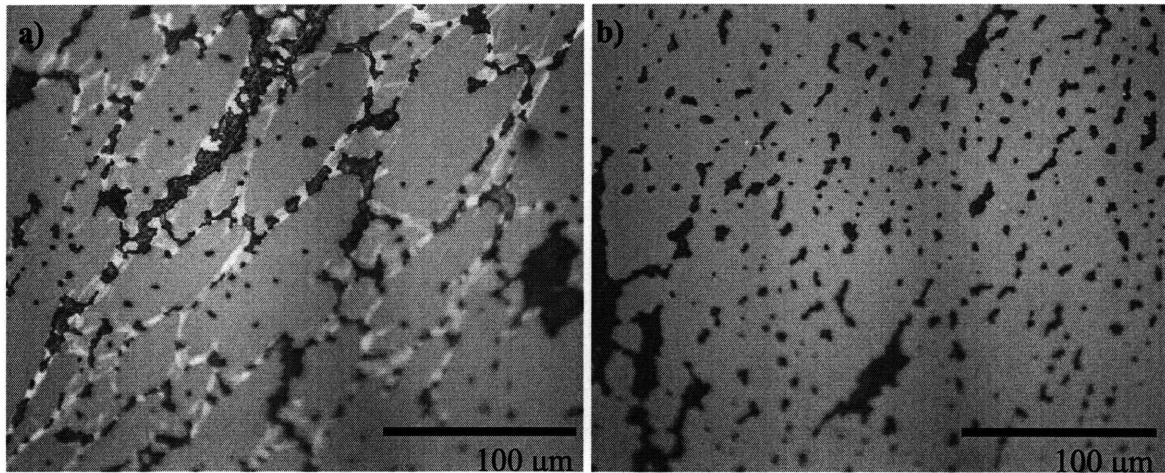
below demonstrates the minimal visible difference at a 50X magnification. Once the results of this experiment were analyzed, the next step was to repeat the experiment in order to make sure that experimental error, cross contamination, or uneven polyelectrolyte coatings were not the cause of the results.



**Figure 5:** Images taken at a 50X magnification for first experiment conducted with a 2 hour incubation time. Figure 6a is on substrata assembled at pH values of 2.0, while figure 6b is on substrata assembled at pH values of 6.5.

For the second experiment conducted with a two-hour incubation time on six-well plate substrata, the dipping was done with extra care to ensure that the polyelectrolyte multilayers were evenly coating the bottom of the wells. Also, the experimental procedure was carefully followed with the suspicion that the first experiment yielded false results due to cross contamination of the wells. However, after analyzing the images of the second the experiment, Figure 6 below, the results were the same. There still appeared to be no visible difference between the different substrata. The amount of bacteria attached on the surface of the wells was not significantly different. One consistency that was observed, however, was that the substrata with polyelectrolyte multilayers assembled at pH values of 2.0 had a darker staining than the substrata assembled at pH values of 6.5. The possibility that the polyelectrolyte multilayers were

also staining would have to be explored. Finally, if the bacteria did adhere on both surfaces equally, the question of the shear force required to remove bacteria from the compliant surface compared to that required for the stiffer surface, remained. The last experiment was conducted on individual slides in hopes of minimizing the window for error, and conducting staining, and shear experiments.



**Figure 6:** Images taken at a 50X magnification for the second experiment conducted with a two hour incubation time. Figure 7a is on substrata assembled at pH values of 2.0, while figure 7b is on substrata assembled at pH values of 6.5.

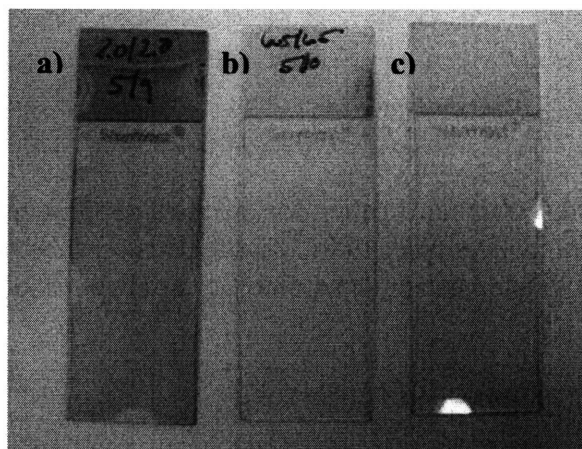
### **5.3 2 Hour incubation Results on aminoalkylsilane treated slides**

The last experiment on aminoalkylsilane treated slides was done to eliminate cross contamination, ensuring evenly coated polyelectrolyte multilayers, easily staining only the polyelectrolyte multilayers, and conducting a shear experiment to test the stability of the biofilm on both the slides assembled at pH values of 2.0 and 6.5.

#### **5.3.1 Staining Results**

The staining experiment was done by following the same staining procedure, described in chapter 4.2.4, on a plain aminoalkylsilane slide and aminoalkylsilane slides with polyelectrolyte multilayers. The results confirmed the suspicion that the polyelectrolyte multilayers were

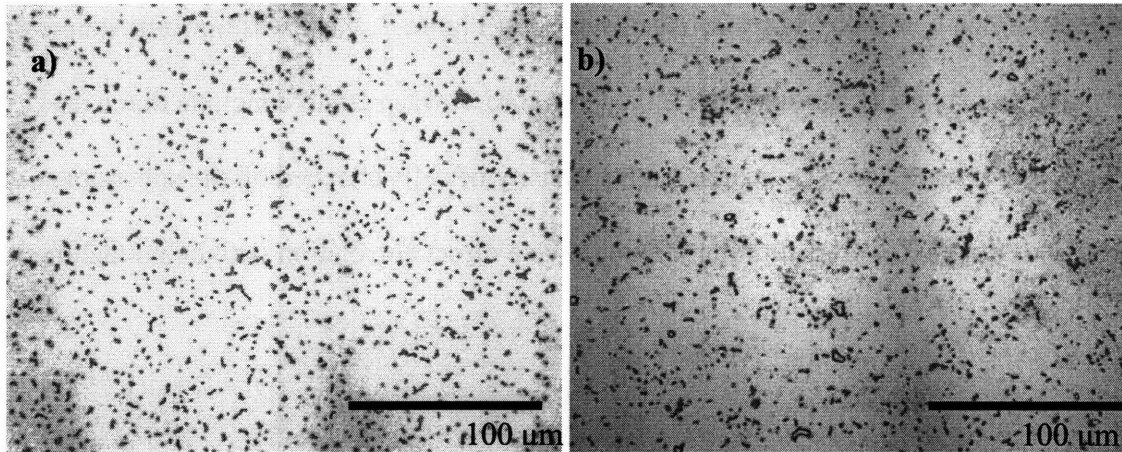
staining, as shown below in Figure 7. As observed in Figure 7, there was uniform staining only on the polyelectrolyte assembled at pH 2.0. Therefore, it was safe to conclude that a darker staining does not necessarily indicate a higher density of cells attached on the substrata surface. That is, the amount of overall staining did not have a direct correlation with cell attachment.



**Figure 7:** Image of the staining experiment results. 8a was assembled at a pH value of 2.0, 8b at a pH value of 6.5, and 8c is a plain aminoalkylsilane slide, only 8a demonstrates staining.

### 5.3.2 Microscopy Results

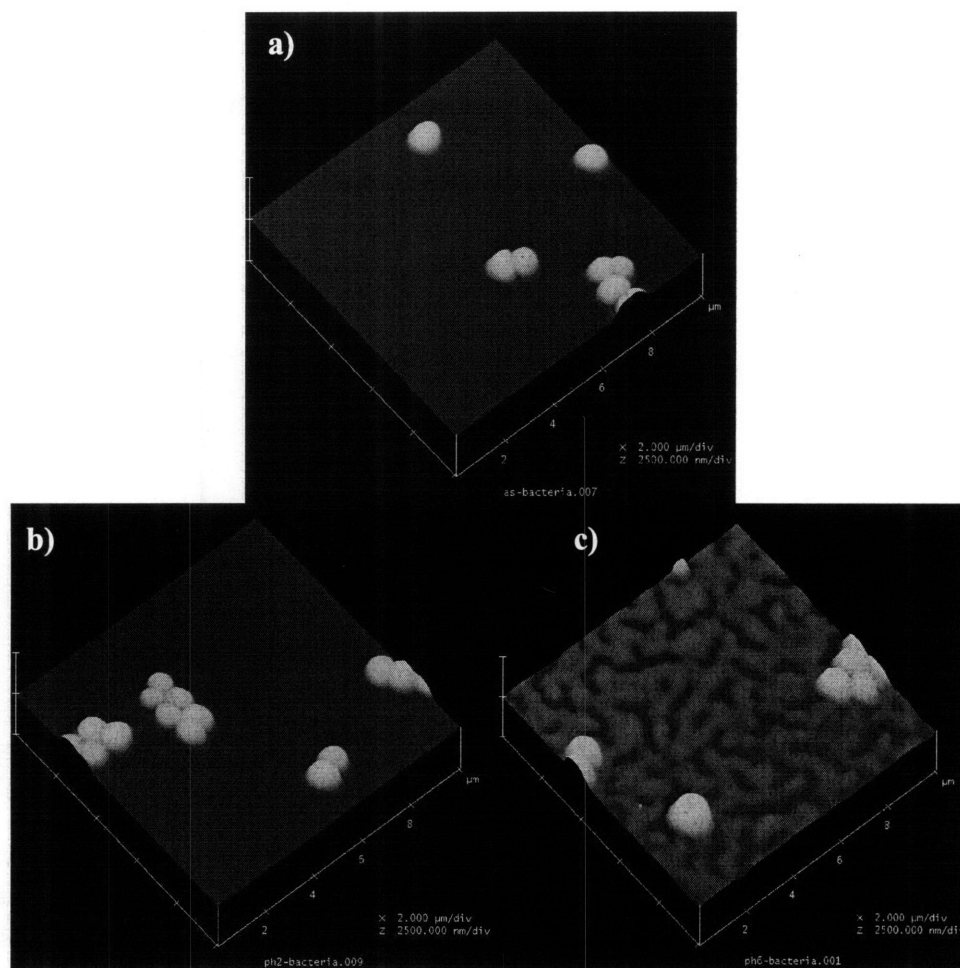
The result of the experiment on individual slides was consistent with the previous experiments on six-well plate substrata. The amount of bacteria adhered on the surface did not have a positive correlation with mechanical stiffness. As is shown in Figure 8 below, the amount of bacteria adhered was identical in both cases. The controls of the experiment, which only included the addition of broth without bacteria, did not show any growth; therefore, there was no cross-contamination during this experiment. With these images being consistent with the first three experiments, the conclusion that biofilm forming *staphylococcus epidermidis* does not show any positive correlation with mechanical stiffness, was more confidently accepted.



**Figure 8:** Images taken at a 50X magnification for the third experiment conducted with a two hour incubation time on aminoalkylsilane slides . Figure 9a is on substrata assembled at pH values of 2.0, while figure 9b is on substrata assembled at pH values of 6.5.

### 5.3.3 Atomic Force Microscope (AFM) Results

In order to further confirm the result that there is no quantifiable difference between the different substrata, AFM images were taken of the experiment conducted on slides. Again, the AFM images were consistent with the microscopy analysis of the previous section. The amount of bacteria adhered on the different substrata was not affected by mechanical stiffness. Analysis of the images, however, did serve to show how the bacterial cells clump together forming different networks on the substrata. Also, the difference between the polymer substrata assembled at pH values of 2.0 (Figure 9b), and pH values of 6.5 (Figure 9c), was observed, confirming that the different substrates had little to no effect on bacterial attachment.

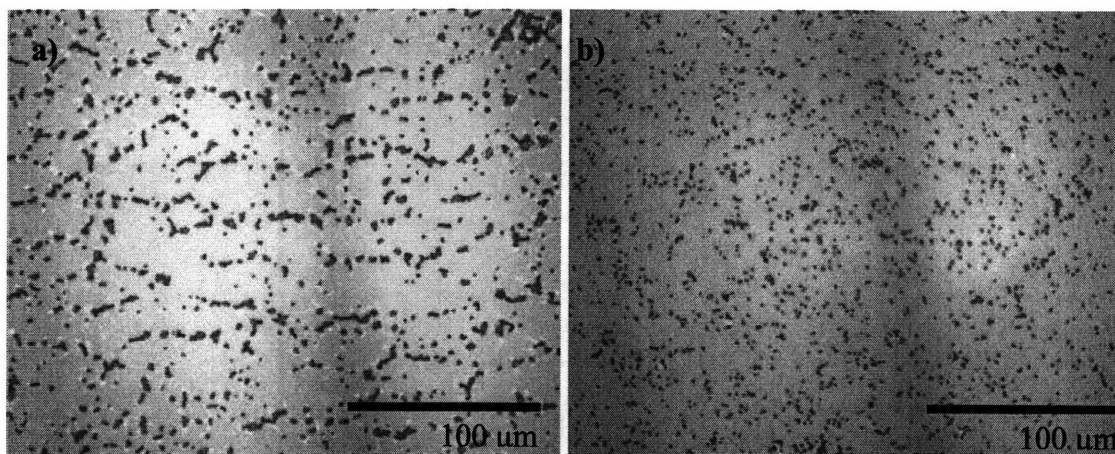


**Figure 9:** AFM Images for the second experiment conducted with a two-hour incubation time on aminoalkylsilane slides. Figure 10a is on plain a aminoalkylsilane treated slide, 10b corresponds to substrata assembled at pH values of 2.0, and figure 10c is on substrata assembled at pH values of 6.5. The amount of adhered bacteria was not affected by the underlying substrate.

### 5.3.4 Shear Experiment Results

The final experiment was conducted to test the stability of the attached bacteria on the different substrates under an applied shear force. Even if the amount of bacteria attached was not affected by the underlying substrate, the next hypothesis was that the bacteria on the more compliant surface could be removed under a lower applied shear force as compared to the stiffer surface. Again, the hypothesis was incorrect; microscopy analysis proved that even when placed under a shear force during the rinsing procedure, the amount of bacteria that remains attached to

the surface was the same in both cases. After shearing both samples with PBS three times for 5 seconds, the results showed that the amount of bacteria adhered on the substrate was not affected. The microscopy images were identical to the samples that were not sheared, as is seen in Figure 10 below.



**Figure 10:** Images taken at a 50X magnification for the third experiment conducted with a two hour incubation time on aminoalkylsilane slides. Figure 11a is on substrata assembled at pH of 2.0, while figure 11b is on substrata assembled at a pH of 6.5. Both samples were sheared during the rinsing procedure with Phosphate Buffered Saline.

## 6 Conclusions

The four experiments on six-well substrata and the experiment on individual slides yielded consistent results. There was no positive correlation between mechanical stiffness and cell adhesion with biofilm forming *staphylococcus epidermidis*. Although non-biofilm forming *staphylococcus epidermidis* did demonstrate a mechanoselective adhesion mechanism, no such mechanism was observed with the stronger biofilm forming strain. Also, even under an applied shear force the amount of bacteria adhered on the surface was not affected. In all cases tested in this thesis, the biofilm forming strain of bacteria was able to adhere and grow successfully.



## 7 Future Work

There remains a great amount of future work to be conducted to further explore the adhesion mechanism of *staphylococcus epidermidis* on polyelectrolyte multilayers. First off, the analysis in this thesis was not able to successfully quantify the amount of bacteria attached on the surface of the different substrata. Microscopy alone was not enough to quantify the amount of bacteria that was adhered. Further experiments should consider resolubizing the bacteria and measuring the optical density of the solution. Also the surfaces should be further explored with more extensive imaging on AFM or confocal microscopy.

Secondly, the last experiment conducted with aminoalkylsilane treated slides should be repeated at least twice to confirm all the results reported in chapter 5. In addition, because simply shearing the samples during the rinsing procedure caused no effect on the bacterial adhesion, a more harsh and quantifiable form of shearing should be executed. A possibility could be shearing by centrifugation. Also, a different kind of staining solution that does not stain the polyelectrolyte multilayers should be used, since the 2.0 films were positively staining.

This thesis analysis has shown no mechanoselective correlation with mechanical stiffness; however, a shorter incubation time should be further explored. Growing the bacteria under static conditions and may be facilitating the process of bacterial attachment and biofilm development. A shorter incubation time may be necessary to observe only the initial attachment of the bacteria before the formation of biofilm takes place.

If after all the suggestions above, there still appears to be no correlation, the difference in attachment may be affected by the amount of nutrients provided during the experiments. During the experiments conducted with non-biofilm forming *staphylococcus epidermidis*, the experimental methods testing the attachment of bacteria were performed in water, while here

they are performed with a Trypticase Soy Broth and glucose solution. Running the experiment following this protocol but using the non-biofilm form of bacteria, should demonstrate whether or not the amount of nutrients provided during the experiment affect the attachment mechanism. An alternative could be repeating this experiment in water, and comparing the results to those reported here.

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