Adhesion of Pseudomonas aeruginosa to surface modified and unmodified polystyrene with and without a conditioning layer
by Jenny Kathleen Thompson

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University

Abstract:
The composition of a surface is known to affect the adhesion of bacteria. The goal of this experiment was to determine how a defined chemistry change would affect the adhesion of Pseudomonas aeruginosa to polystyrene and surface modified polystyrene with and without a conditioning layer.

A polystyrene coupon was surface modified via a molecular beam apparatus. This added oxygen in the form of such functional groups as carboxylic acids, epoxides, carbonyls, and hydroxyls into the surface of the polystyrene. Analysis of the surfaces was done through XPS to determine if the surface chemistry alteration had gone as planned. The coupon was then placed into a parallel-plate flowcell, and P. aeruginosa diluted to 3xl07 cell/ml from a chemostat were flowed across the surface of the coupon for six hours. Images of the adhering cells were taken with a digital camera and computer, which were attached to the microscope. Images were taken every 5 minutes for the first two hours, then every 10 minutes for the next two hours, and finally every 20 minutes for the last two hours. The images were then counted to obtain the number of cells adhered as well as their x-y positions. This data was analyzed to determine if the four treatments affected adhesion to the surface.

Surface modification through the addition of oxygen to polystyrene increases the adhesion of P. aeruginosa to polystyrene by 55% at six hours. The addition of a conditioning layer increased adhesion on both the control and the modified polystyrene, resulting in increases of 25% and 95% respectively from the control polystyrene at 6 hours. P. aeruginosa attached to polystyrene and surface modified polystyrene in a random fashion.

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APPROVAL

of a thesis submitted by

Jenny Kathleen Thompson

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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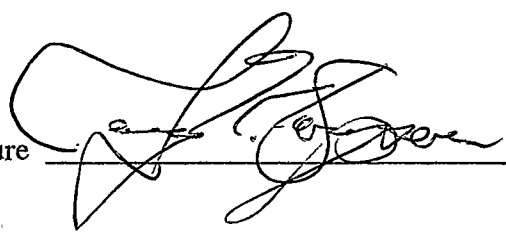
Signature 

Date April 28, 1999

VITA

Jenny Kathleen Smith was born June 28, 1973 in Great Falls, Montana, to Luella Jean Smith and Ronald Hugh Smith. Jenny and her parents moved to Billings, Montana when she was 9 months old. Her brother, Kevin Michael Smith, was born there on March 30, 1975. Her other brother, David Robert Smith, was also born in Billings on May 10, 1976. The family moved to Gillette, Wyoming, for two years in 1977, after which they moved back to Billings. Jenny grew up in Billings, where she attended Meadowlark Elementary school grades K-6, Will James Jr. High School for grades 7-8, and Billings West High School for grades 9-12.

Jenny K. Smith attended Eastern Montana College (now Montana State University–Billings) from August 1991 through July 1993, where she majored in Chemistry and Music, maintaining a GPA of 3.76. During this time, she met and became engaged to her future husband, Terry L. Thompson of Great Falls (b. January 16, 1965). After this education, she transferred to Montana State University — Bozeman, where she went into Chemical Engineering.

Jenny K. Smith was married to Terry L. Thompson on June 25, 1994. She graduated with honors from Montana State University–Bozeman with a BS in Chemical Engineering on May 11, 1996, with a GPA of 3.36.

Jenny K. Thompson entered graduate school at Montana State University–Bozeman in August of 1996. Jenny and Terry Thompson had a baby girl on November 12, 1996. She was named Kathleen Renée Thompson.

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ABSTRACT

The composition of a surface is known to affect the adhesion of bacteria. The goal of this experiment was to determine how a defined chemistry change would affect the adhesion of *Pseudomonas aeruginosa* to polystyrene and surface modified polystyrene with and without a conditioning layer.

A polystyrene coupon was surface modified via a molecular beam apparatus. This added oxygen in the form of such functional groups as carboxylic acids, epoxides, carbonyls, and hydroxyls into the surface of the polystyrene. Analysis of the surfaces was done through XPS to determine if the surface chemistry alteration had gone as planned. The coupon was then placed into a parallel-plate flowcell, and *P. aeruginosa* diluted to $3x10^{7}$ $^{cells}/_{ml}$ from a chemostat were flowed across the surface of the coupon for six hours. Images of the adhering cells were taken with a digital camera and computer, which were attached to the microscope. Images were taken every 5 minutes for the first two hours, then every 10 minutes for the next two hours, and finally every 20 minutes for the last two hours. The images were then counted to obtain the number of cells adhered as well as their x-y positions. This data was analyzed to determine if the four treatments affected adhesion to the surface.

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CHAPTER 1

INTRODUCTION

Biofilms

The adhesion of bacteria to a surface is the starting point of all biofilms. Adhesion begins once a cell has come into close contact with the surface, usually through diffusion, settling, or active locomotion. This process is known as transport. Once a microorganism has reached the substratum, it either simply transports away, or becomes loosely associated with the surface. However, this initial association is by no means permanent. Once on the surface, a cell often remains for only a short period before spontaneously detaching back into the flow. If the cell stays, sometimes it moves around on the surface before reaching the final stage of attachment, permanent adhesion. Once a microorganism has reached this stage, it rarely detaches again unless subjected to some form of stress. These permanently attached bacteria begin to grow and subsequently form a biofilm.[1, 2, 3]

A biofilm consists of a layer of bacteria contained within an exopolysaccharide matrix (EPS), colloquially known as slime. Biofilms do not consist of a homogenous layer of uniform thickness. Instead, they contain layers of different bacteria, channels through the biofilm, "mushrooms," "streamers," and an assortment of other odd formations.[4] These formations affect the availability of nutrients available to the

microorganisms. Whereas in a true slab the bacteria at the base of a biofilm can be nutrient limited, if there are channels through the structure of the biofilm, then cells at the base may have plenty of nutrients available.

Bacteria within a biofilm are also proving to be different from the same species of bacteria existing planktonically. Phenotypic changes have been observed, and attached cells express different genes than free-swimming cells.[1, 4] One important ramification of this difference is that they are often far more difficult to kill, whether through reactions within the cells or diffusion limitations.[4, 5] This has serious implications for industries that need to kill biofilms that exist in their systems. Heat exchangers, which often have chemicals within their fluids designed to kill microorganisms, still become fouled, necessitating tubing replacement. In medicine, biofilms that form on catheters, shunts, or implants can lead to large systemic infections, which can be lethal.[6] In addition, water distribution systems have many problems with microbially induced corrosion, where microbes within the biofilm actually cause disintegration of the pipes.[5] Biofilms within water distribution systems can also lead to blooms of coliforms, as these indicator organisms occasionally detach from the walls of the pipes.

Observation of Biofilms

In recent years, many methods of studying biofilms have been devised. Coupons have been inserted into pipes, which can later be removed for analysis. Annular reactors, drip plates, and a variety of other configurations have been designed in an attempt to mimic the variety of systems that exist in both nature and the man-made world. This is in order to understand how to use and control biofilms to our benefit.

The parallel-plate flowcell has recently been used extensively.[7, 8, 9, 10, 11, 12, 13] It allows for real-time observation of the bacteria *in situ* as an experiment proceeds. This system utilizes a small sample of the substratum of interest, known as a coupon. When a coupon is removed from other types of systems, an air-liquid interface is introduced that can affect a force of around 10^{-2} dynes (which is far greater than the forces introduced by shear in most flowcell systems) .[14] These forces can induce detachment of cells, as well as rearrangement of adhesion patterns.[8] In a flowcell, if stains or other fixatives are needed, they can be introduced into the flow without ever exposing the coupon to these forces. In some cases, the need for stains can even be eliminated, since direct observation of the microorganisms is possible.[8]

Attachment Studies

Although it is unknown if initial events have an effect on the final shape of a biofilm, knowledge of how a biofilm begins may be used to retard biofilm formation. Interactions between the attached bacteria and the surface to which it is bound may become important in attempts to induce spontaneous detachment. Although it is extremely unlikely that a surface will ever be found that is totally resistant to bacterial adhesion, hopefully an understanding of how a biofilm forms can help find ways to make biofilms controllable.

It is known that different surfaces affect how bacteria adhere. What is not known is why. There are contradictory studies where certain characteristics of a surface, such as hydrophobicity, have been correlated to rates of adhesion. In some cases, there was little or no correlation.[15, 16] However, other studies found that there was a strong connection

between hydrophobicity and adhesion.[9, 17, 18] Other studies have looked at the presence of variables such as electrolyte concentration, detergents, fluid velocity, and the pH of the media.[1, 19, 20] Although some of these variables were correlatable within a species, these could not be extrapolated to the general bacterial population.[21]

Adhesion studies in the past have found that bacteria (as well as other adhering organisms) adhere in greater numbers to rougher surfaces. Studies that looked at the roughness of a surface and how it affects adhesion almost always found a relation between increasing roughness and an increase in the number of attached microorganisms.[5, 9]

Most studies on stainless steel (as well as other surfaces) did not analyze actual bacterial patterns of adhesion. More recent studies found that bacteria adhered along the grain boundaries of the stainless steel, and even concentrated the effects of corrosion along those boundaries.[10, 11, 12] However, grain boundaries not only have a change in topography, but also a change in the actual chemistry of the substratum.[22, 23, 24] Although it was believed that the topography was the main influence affecting where the bacteria were adhering, no studies had been done which tried to separate the two variables of chemistry and topography in relation to adhesion patterns.

Topography Study

A study done by T. Rush Scheuerman used three organisms in order to determine how a defined change in topography with known effects on the flowstreams would affect the adhesion of the bacteria to the surface. *Pseudomonas aeruginosa*, *P. fluorescens* mot

(+), and *P. fluorescens* mot (–) were used. Rush Scheuerman looked at attachment with a confocal scanning laser microscope, which enabled her to observe bacteria both along the surface of the coupon and in the bottom of the grooves. She found that the cells adhered to the downstream edge of the groove, and that there was not a significant increase in adhesion in the bottom of the grooves. This was not the expected result—the hypothesis was that the cells would adhere in the bottom of the grooves.[19]

Conditioning Layers

Another variable in the realm of bacterial adhesion is the presence of a conditioning layer, or film. Any surface that is exposed to a natural environment, and especially the environment of the human body, develops a conditioning layer almost instantaneously. The conditioning layer consists of a variety of organic molecules, and alters the surface properties of the substratum.

Composition The true composition of a conditioning layer still remains largely unknown, as Stephen Klotz pointed out when he wrote that "...it seems to this author that the compositions of the coating substances on plastics when exposed in vivo are poorly characterized or unknown in most circumstances." He further states that until reliable models that consistently predict bacterial interactions with plastics *in vivo* are made, "...no firm conclusions about the role of hydrophobicity or any other virulence factor can be made; only correlations will be possible."[21]

A conditioning layer on a surface will affect its surface properties, since the surface is no longer the same. Although actual alteration of the physical surface is unlikely to occur

except in unusual circumstances, what an organism 'sees' is filtered by the conditioning layer.[25] The characteristics of the original surface affect the original composition of the conditioning layer[21].

The extent to which the conditioning film affects what the microbe senses of the underlying surface chemistry is unknown.[1] Different surfaces affect how proteins in the conditioning layer adhere to their surface.[26, 27, 28, 29] One study found that different surfaces even affected the structure of the film: Rudee and Price found that in some circumstances, a uniform conditioning film was not even formed, instead forming a "networked structure" on the surface.[30] Whether this incomplete coverage of the surface affects adhesion is unknown.

Adhesion Studies It has generally been found that a conditioning layer interferes with adhesion, reducing the total number of cells that initially adhere to the surface.[1,25,31,32,33] However, this was not universally true.[13, 34] One study even found both results, depending on the surface underneath the albumin conditioning film. Taylor et al. found that at low levels of albumin concentrations, the presence of a conditioning layer significantly reduced bacterial adhesion to hydrogel contact lenses. As the concentration of the albumin increased, however, adhesion levels increased on one type of lens but dropped on the other. These effects were most noticeable with *P. aeruginosa* .

Adhesion to Chemically Modified Surfaces

No studies were found that addressed the issue of bacterial adhesion to a chemically altered surface with actual changes in the chemical structure of the surface. However, a

study has been done with batch yeast cells that compares rate of adhesion to control polystyrene, plasma-modified polystyrene, and oxygen molecular beam modified polystyrene.[8]

Previous work has been done on surface modification in order to determine the changes made in the surface chemistry by two methods—oxygen plasma modification and oxygen molecular beam modification.[35] This study characterized these surfaces through XPS and derivatization in order to determine the functional group changes that were made to the surfaces by both procedures. It was found that both the plasma modified surface and the 100-pulse oxygen molecular beam modified surface significantly reduced the adhesion of the yeast cells, and that they did so to a similar extent.[8]

Suzuki and Sakai have proposed that the "surface charge polarity rather than surface hydrophobicity (including the adsorbed proteins) could be a dominant factor in determining the initial attachment rates."[33] Others have also posed this opinion.[3, 18, 21, 36, 37] It should be noted that with the incorporation of oxygen into the polystyrene surface, the polarity of the surfaces would be radically changed.

Current Work

The goal of this work is to determine the effect of a defined surface chemistry change on bacterial adhesion. Polystyrene surfaces were chosen because of their significance in tissue culture research, medical research, and widespread use in general research.[1, 8, 18, 20, 25, 26, 30, 36, 37, 38, 39, 40, 41, 42, 43] Previous studies of adhesion to polystyrene and tissue culture polystyrene did not show the specific chemical changes that were made to the polystyrene, and the modifications were simply chosen for their ability to affect the hydrophobicity of the polystyrene.

This study will look at how a defined chemical change affects adhesion. New studies by Suzuki and Sakai as well as others have suggested that it is not the hydrophobicity of the surface that is the strongest influence on adhesion, but surface charge and polar groups.[3, 18, 21, 33, 36, 37] If this theory is correct, then the addition of oxygen into the polystyrene backbone would be the major factor influencing any observed adhesion changes for this study.

CHAPTER 2

MATERIALS AND METHODS

Designing the System

The design of this experiment was based on the system used by T. Rush Scheuerman in her work[44]. Modifications to the system were based on the need for rigorous cleaning that comes with doing surface chemistry experiments. Figure 1 shows the overall layout of the experiment.

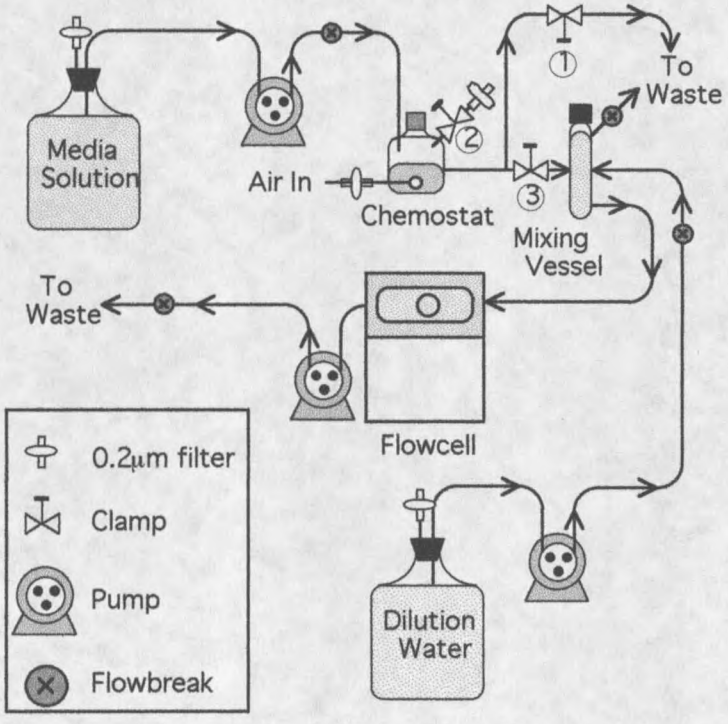

Figure 1. General Experimental Layout

The basic change in design was to use Teflon® tubing where possible, and Viton® rubber tubing otherwise in order to withstand the corrosive cleaning environment. Any fittings used were of Teflon®, polypropylene, glass, or stainless steel.

Chemostat

A new chemostat was designed that could withstand the pressure build-up that occurred with this experimental design. The old design, with a rubber stopper on the top of a beaker, was ineffective. A 1000-ml Pyrex® bottle with a screw-on cap was used instead.

The working volume of the chemostat was determined by filling the chemostat (Figure 2) to overflowing, turning on the stir bar and air pump, letting the reactor stabilize for 5 minutes, and then measuring the remaining volume of water. The working volume of the chemostat was 480 ml. To attain the desired 5-hour residence time to match conditions used by Rush Scheuerman, a flow rate of 1.6 $^{ml}/_{min}$ was used.

Flowcell

The design of the flowcell is based on previous designs used throughout the Center for Biofilm Engineering, with modifications to withstand the cleaning needed to investigate the influence of surface chemistry attachment. This flowcell was successfully used in yeast adhesion studies[8] The base and fittings of the flowcell were constructed from Teflon®, and the gasket was Viton® rubber. An exploded view of the flowcell is shown in Figure 3.

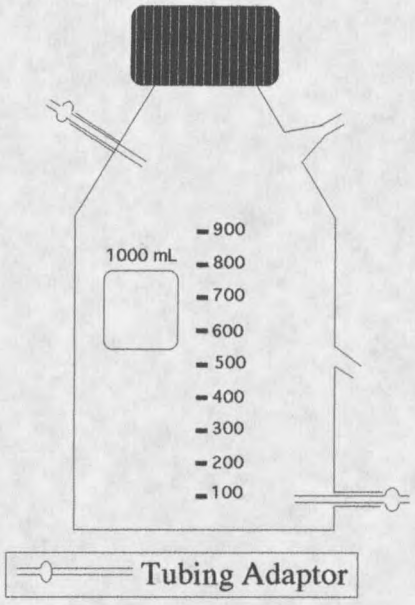

Figure 2. Adapted Chemostat Design

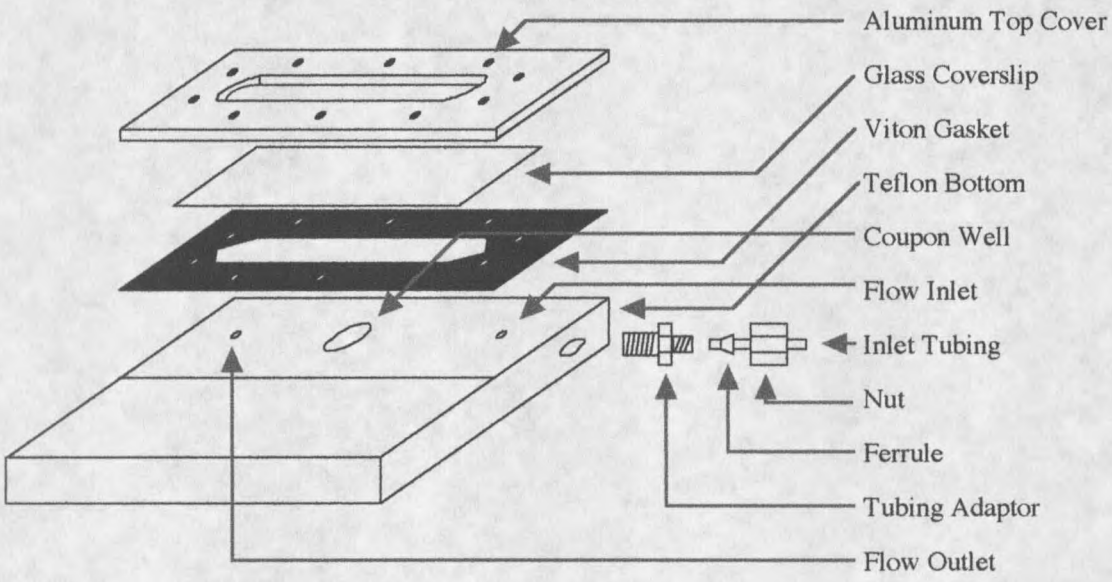

Figure 3. Exploded View of Flowcell Design

The desired Reynolds number in the flowcell was 5.5, in order to match the conditions used in the previous study[44]. Since the width of the channel in the flowcell used was determined by the size of the polystyrene coupon, the flow rate was the variable parameter. Through the equation[45]:

$$N_{\mathrm{Re}} = \frac{D_{eff} \cdot \overline{V} \cdot \rho_{water}}{\mu_{water}} \quad (1)$$

where N_{Re} is the Reynolds number, D_{eff} is the effective diameter of the flowcell channel, ρ_{water} is the density of water at 70° F, and μ_{water} is the viscosity of water at 70° F. The effective diameter is 4 times the hydraulic radius, which is found by the equation[45]:

$$R_H = \frac{l \cdot w}{2 \cdot l + 2 \cdot w} \quad (2)$$

where l is the width of the channel and w is the height of the channel. Inserting the dimensions of the flowcell channel, it was determined that a flow rate of 3 $^{ml}/_{min}$ would generate a Reynolds number of 5.5.

Cleaning Procedures

Since this was a surface chemistry experiment, all components were cleaned in either base bath or acid bath for a period of at least an hour.

Base Bath

Base bath was made in a chemical hood as follows: Nanopure® water was first saturated with NaOH over the course of at least three days. Once the solution was

saturated with the NaOH, ethanol was added until the color of the solution became dark brown to black. This step is very exothermic, and done over at least three days.

Acid Bath

Acid bath was prepared in a chemical hood by adding one packet NoChromix® to one liter of H_2SO_4.

Cleaning

Glassware, except for the media carboys, was soaked in a container of the acid cleaning solution. The carboys used for media and dilution water were initially cleaned with acid by placing approximately one liter of solution into the carboy, and letting it sit for thirty minutes. Then it was turned onto the side, and left for 30 minutes, then rotating so that another side was soaking for another 30 minutes, and so on until completely cleaned. After this initial cleaning, the carboys were merely rinsed after each run unless contamination occurred, whereupon they were again cleaned with acid.

In order to clean the tubing, the cleaning solution was pumped through the tubing. Acid was pumped through the chemostat and dilution vessel tubing, in order to clean them. It was necessary to use the base bath in the flowcell tubing if the stainless steel fittings were present.

While cleaning, as much of the tubing as possible was placed into a glass Anchor Hocking® casserole dish, as was the vessel containing the cleaning solution. This would contain any leaks that might occur. Since the pumps could not be placed into this dish,

paper towels were laid under the pumpheads, and the pumpheads were continuously monitored for leaking.

All components were then rinsed in Nanopure® water. Glassware was rinsed under running Nanopure® at least five times, and tubing was rinsed with at least ten times the inner tubing volume. The system was then assembled and autoclaved for 20 minutes under slow exhaust, to prevent the fittings from coming apart.

Polystyrene Coupon Preparation

A sample of commercially available polystyrene was obtained in 6x9 in sheets from Plaskolyte (Columbus, OH). These sheets had a thickness of 0.93 mm and an average molecular weight of 100,000 $^{g}/_{mol}$.

For this experiment, the sheets were cut into $^{1}/_{2}$-inch diameter circles with a punch and die set. After punching, the protective plastic film was removed from each side of the discs, and using tweezers the discs were swirled in hexane (HPLC Grade) for 10 seconds to remove any oxidative layer which may have formed. They were then sonicated in methanol (HPLC Grade) for at least five minutes, which was both a final cleaning and sterilization step. For the control polystyrene, this cleaning and sterilization was done within two hours of insertion into the flowcell. For the beam-modified samples, this was done immediately before placement into the molecular beam apparatus, and was not repeated before the experiment.