Interaction of Bacteria with Hydrophobic and Hydrophilic Interfaces

by

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Abstract

This thesis compares the thermodynamic approach used to predict initial bacterial adhesion to real-time confocal images of bacteria attaching to self-assembled monolayer substrates. Bacterial adhesion is believed to be governed by physical-chemical laws in the initial stages of attachment. After longer periods of time, biological processes work to strengthen and establish the attachment through changes in gene expression and metabolic functions. In this experiment, the free energy of adhesion for a *Pseudomonas* putida bacterium interacting with either a mercaptoundecanol or dodecanethiol self-assembled monolayer is calculated using the Young-Dupré equation. This equation relates experimentally measured contact angles of liquids on bacterial and substrate surfaces to the work of adhesion.

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Introduction

The interaction of biological cells with surfaces has many important applications in both industry and medicine. It has been shown that the initial adhesion process is governed by physical interactions between the cell and the substrate while biological processes, like growth and phenotype adaptation, involve larger time scales[1]. The initial stages of this interaction appear to be a thermodynamically driven passive process.

Initial bacterial adhesion is of special interest due to the biological processes that are a consequence of this interaction. For example, living cells experience a large number of signaling cues from changes in the chemistry of the attachment surface. Understanding the effect substrate conditions have on cellular development is important in cancer research. Biofilm development is another consequence of bacterial adhesion. Biofilms are defined as a layer of prokaryotic and eukaryotic cells anchored to a substratum surface and embedded in an organic matrix of biological origin[2]. They exhibit a high level of organization and their structure is influenced by the chemical nature of the substrate. Many current technological issues require a better understanding of the factors controlling biofilm formation or, more specifically, the initial attachment process. For example, there is a desire within many industries to create a 'non-stick' surface to prevent microbial fouling. The problem of microbial fouling is especially prevalent in heat exchangers, pasteurization and drinking water pipes, and ship hulls. The US Navy has estimated that a biofilm several hundred micrometres thick causes a 20% increase in fuel consumption[2]. This is equivalent to approximately $400 USD/hour at a speed of 48 km/h. Pathogenic biofilms also form on medical implants causing infections in patients. On the other hand, it can be advantageous to improve biofilm formation in order to optimize the degradation of environmental contaminants. Regardless of the ultimate goal, it is of utmost importance to

understand and model the initial bacterial adhesion process before any advancement can be made.

In this project, the work of adhesion is calculated based on contact angle measurements. These calculations will be used to model the initial attachment of bacteria to a surface. As a test to the feasibility of this theoretical approach, the interactions between the bacteria and the SAM substrates are monitored using real-time laser scanning confocal microscopy (LSCM).

Since bacterial adhesion is influenced by the characteristics of the substrate, a highly characterized surface is desirable when studying the interaction process. Self-assembled monolayers (SAMs) offer a highly ordered, smooth, compact monolayer with consistent chemical properties ideal for this type of investigation. Self-assembly can be defined as the formation of oriented monolayer films on a surface by the spontaneous adsorption of molecules from solution[3]. In this experiment, SAMs of dodecanethiol and mercaptoundecanol were used to make hydrophobic and hydrophilic surfaces respectively.

$CH_3(CH_2)_{10}CH_2S$
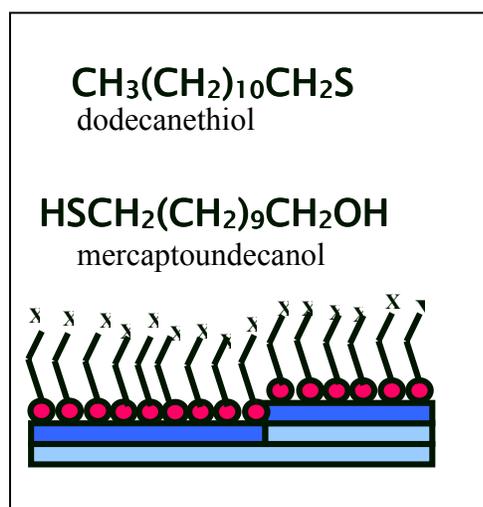dodecanethiol

$HSCH_2(CH_2)_9CH_2OH$
mercaptoundecanol

Figure 1: SAM of either dodecanethiol or mercaptoundecanol (the circles represent the thiol group). Notice how the hydrocarbon chains are oriented so that the functional group X (either a methyl or a hydroxide group in this case) is presented at the surface.

The formation of strong covalent gold-sulfur bonds drives the spontaneous assembly. The hydrocarbon backbones of these molecules align and stabilize the monolayer through van der Waals interactions[4].

The interaction between the bacteria and the substrate can be treated as an thermodynamic process[5] and the interacting surfaces are assumed to physically contact one another without mixing. This thermodynamic approach is often used. It has been postulated that the initial adhesion process is reversible and only becomes irreversible over time[2]. As mentioned earlier, the initial attachment process is governed by physical interactions whereas biological interactions that contribute to the strengthening of the attachment occur over larger time periods. Thus, initial attachment is a spontaneous change that results in a decrease in the free energy of the system where

$$\Delta G_{adhesion} = - W_{adhesion} \qquad (I)$$

It is possible to measure the work of adhesion using Young's equation and experimentally measured contact angles. Contact angle measurements are widely used for the evaluation of surface wettability, hydrophobicity, adhesion and surface tension[6]. In this experiment, interfacial free energies between interacting bacterium and substratum surfaces are compared with the ultimate goal of obtaining the free energy of adhesion for a bacterium attaching to a SAM substrate. As mentioned earlier, two SAMs with differing wettablilities were tested. Since surface hydrophobicity is related to the work of adhesion[5], SAMs of different hydrophobicities were expected to have different effects on the bacterial attachment process. The bacteria chosen for this experiment was *Pseudomonas* putida. As a genus, pseudomonads are extremely diverse; they are omnivorous and have an innate ability to adapt to unfavourable environments. This makes them especially proficient at degrading environmental contaminants as well as resisting

antibiotics. *Pseudomonas* putida is a soil bacterium that lives in a mutualistic association with plants; it obtains nutrients from plant roots and provides protection against many plant pathogens. It has a versatile metabolism and, like many of its genus, can degrade toxic substances. Since they are the focus of many novel industrial applications, many current studies use pseudomonads as model organisms[7]. They also readily form biofilms in a laboratory environment. Of particular interest to this investigation is its biofilm development pattern, which starts with a single cell attaching to the substrate.

Theory:

The work done when a bacterium attaches to a substrate can be estimated using experimentally measured contact angles of water on the bacterial and substrate surfaces. To begin, it is necessary to define a few thermodynamic quantities for clarity.

In general, the interaction energy between two molecules, 1 and 2, at any given separation distance is the product of some property, A, of molecule 1 multiplied by some property, B, of molecule 2[8]. As a specific example, consider the binding energy of two particles under gravity.

$$U_{AB} \approx -G\frac{m_A m_B}{r^2}$$

$$U_{AB} \approx -\sqrt{G}\frac{m_A}{r}\sqrt{G}\frac{m_B}{r}$$

$$U_{AB} \approx -AB$$

In general, the binding energy of molecule 1 in contact with molecule 2 can be expressed as,

$$U_{AA} = -A^2 \text{ or } U_{BB} = -B^2 \qquad \text{(for like molecules)}$$

or $\qquad U_{AB} = -AB \qquad\qquad\qquad$ (for unlike molecules)

(i.e. $U_{AB} = \sqrt{|U_{AA}||U_{BB}|}$).

The work done by the system is the work done to bring two bodies into contact from infinity (i.e. the negative of the change in potential energy). Thus,

$$W = -\Delta U$$

So now

$$W_{AA} = A^2 \qquad\qquad\qquad \text{(II)}$$

and $\qquad W_{AB} = \sqrt{W_{AA}W_{BB}} \qquad\qquad \text{(III)}$

for this simple example.

Note, in the literature it is conventional to use capitol letters even when referring to quantities in terms of 'per unit area'. Since the quantities being considered in this experiment are all in terms of unit area, this convention will be followed throughout this paper.

It is worthwhile to note that in this experiment pressure and temperature are held constant. The substances we are considering are incompressible so there is no change in volume either. Thus

$$\Delta U = T\Delta S - W$$

so then $F = U - TS$

$$\Delta F = -W$$

Also since

$$G = F + PV$$

$$\Delta G = \Delta F = -W \qquad \text{(IV)}$$

Surface energy, γ, is defined as the Helmholtz free energy change that occurs when the surface area of a medium is increased by unit area. The process of creating one unit area is equivalent to separating two half-unit areas from contact. Thus,

$$\gamma_A = \tfrac{1}{2}\, W_{AA} \qquad \text{(V)}$$

Similarly, the interfacial free energy, γ_{AB}, is defined as the free energy change that occurs when expanding an interface by unit area. To understand this process, it is convenient to describe it as occurring in two steps; in the first step unit areas of A and B are created, and in the second step they are brought into contact[9]. Thus,

$$\gamma_{AB} = \gamma_A + \gamma_B - W_{AB} \qquad \text{(VI)}$$

or $W_{AB} = \gamma_A + \gamma_B - \gamma_{AB}$

where W_{AB} is the work of adhesion and γ_{AB} is the energy of the interface. This equation is known as the Young-Dupré equation and is valid for both solid and liquid interfaces[6]. It gives the free energy change associated with bringing unit areas of surface A into contact with surface B.

The above argument can be applied to the case of a particle interacting with a surface in a third medium as in Figure 2.
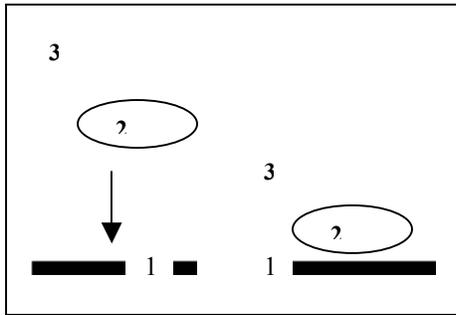

Figure 2: Simple case of a particle interacting with a surface in a third medium

In this case,

$$W_{123} = -\gamma_{12} + \gamma_{23} + \gamma_{13} \qquad \text{(VII)}$$

or $\quad \Delta G = \gamma_{12} - \gamma_{23} - \gamma_{13}$

describes the creation of a unit 1-2 interface with the removal of 2-3 and 1-3 interfaces. Since surface and interfacial free energies determine how macroscopic liquid droplets behave when they adhere to a surface, the above equations for ΔG and γ make it possible to determine interfacial free energies based on contact angle measurements. The following figure describes a liquid droplet in contact with a solid substrate in air.
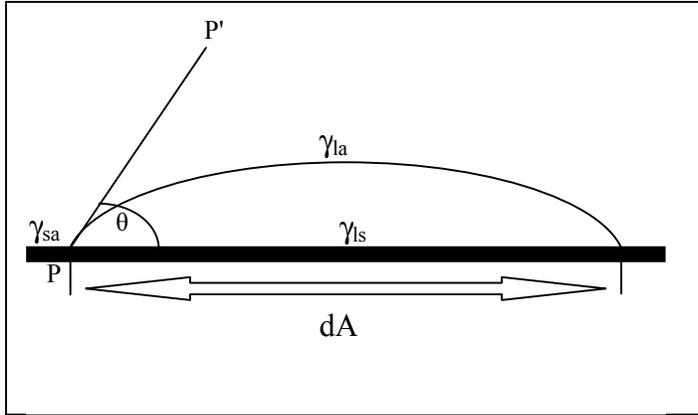
Figure 3: Geometry of a water drop on a surface

The line PP′ is the line tangent to the interface where all three media meet. The infinitesimal change in the liquid-solid interfacial area is represented by dA, hence, the change in the solid-air interfacial area is −dA. Finally, the change in the liquid-air interfacial area is dAcosθ. Assuming this to be an equilibrium process, the change in free energy per unit area of solid substrate is equal to zero.

$$dG_{sla} = (\gamma_{ls} - \gamma_{sa} + \gamma_{la}cos\theta)dA = 0 \quad \text{(in equilibrium)}$$

or $\gamma_{sl} = \gamma_{sa} - \gamma_{la}cos\theta$ (VIII)

The above equation is known as Young's equation and is widely used to determine the interfacial free energy between liquid and solid surfaces. It is possible to obtain the work of adhesion between two surfaces using Young's equation. Assuming that the adsorption at the solid-air or liquid-air interface is negligible (i.e. $\gamma_{sa} = \gamma_s$ and $\gamma_{la} = \gamma_l$)[6], Young's equation becomes,

$$\gamma_{sl} = \gamma_s - \gamma_l cos\theta \quad \text{(IX)}$$

Substituting this into Dupré's equation (equation VI) yields the Young-Dupré equation for the reversible work of adhesion;

$$W_{sl} = \gamma_l(1 + cos\,\theta) \quad \text{(X)}$$

The above derived equations provide the necessary framework with which to examine the thermodynamic behaviour of a bacterium interacting with a solid substrate. Consider the specific case of a *Pseudomonas* putida bacterium in a liquid suspension interacting with a SAM substrate, as in Figure 4.
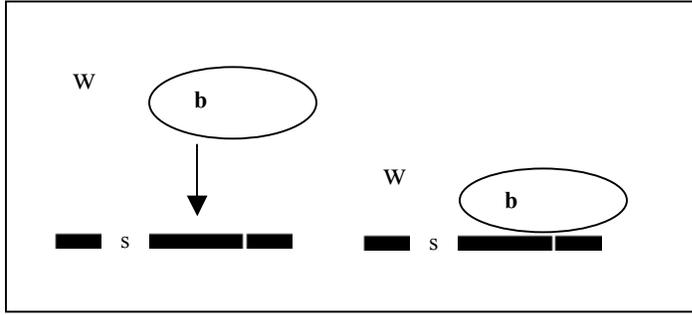

Figure 4: Simple diagram of a bacteria interacting with a SAM substrate

The change in free energy in going from a free swimming (planktonic) to an attached (sessile) microorganism can be approximated using the following equilibrium equation. From equation VII,

$$\Delta G = -W_{sbw}$$

$$-W_{sbw} = \gamma_{sb} - \gamma_{sw} - \gamma_{bw}$$

Using equation VI, this become

$$-W_{sbw} = -2\gamma_w - W_{sb} + W_{bw} + W_{sw} \qquad (XI)$$

where w represents water, b represents the bacterium and s represents the substrate. The equation can be solved if W_{bw}, W_{sb} and W_{sw} are known. These values can be obtained through contact angle measurements and the Young-Dupré equation (i.e. equation X) as

$$W_{bw} = \gamma_w(1 + \cos\theta_b) \qquad\qquad (XII)$$

and $\quad W_{sw} = \gamma_w(1 + \cos\theta_s) \qquad\qquad (XIII)$

where θ_b represents the contact angle measured from the bacteria-air-water interface, and θ_s represents the contact angle measured from the SAM-water-air interface. Also, for dispersion forces (see equation III),

$$W_{bw} = \sqrt{|W_{bb}||W_{ww}|}$$
$$W_{bw} = 2\sqrt{\gamma_b \gamma_w} \qquad \text{(XIV)}$$

So, equating equation XVI and equation XII yields

$$\gamma_b = \frac{\gamma_w}{4}(1+\cos\theta_b)^2 \qquad \text{(XV)}$$

Similarly

$$\gamma_s = \frac{\gamma_w}{4}(1+\cos\theta_s)^2 \qquad \text{(XVI)}$$

Using equations XV and XVI

$$W_{sbw} = -2\left[-\gamma_w - \frac{\gamma_w}{4}(1+\cos\theta_b)(1+\cos\theta_s) + \frac{\gamma_w}{2}(1+\cos\theta_s) + \frac{\gamma_w}{2}(1+\cos\theta_b)\right] \qquad \text{(XVII)}$$
$$W_{sbw} = \frac{1}{2}\gamma_w(1-\cos\theta_s)(1-\cos\theta_b)$$

According to published tables[2] $\gamma_w = 72.8\text{mJ/m}^2$. Thus, the work of adhesion for a SAM-bacteria interface in a water medium can be approximately determined using experimentally measured contact angles.

Experimental Techniques

Contact Angle Measurements

Contact angle measurements are widely used in the determination of free energy of adhesion via the Young-Dupré equation as well as in determining the wettability of a surface. They are easier to measure than surface biochemistry and represent an average of the total surface properties[5]. Contact angles between the bacteria/water/air interface and the SAM/water/air interface were used in this experiment to calculate the free energy of adhesion for bacteria attaching to a SAM substrate (see equation XVII). They were also used to verify SAM formation on the gold film. Contact angles were measured on horizontal profiles of sessile drops of water using a 5X eyepiece constructed in the lab. Water drops were 10μL and were deposited using a micropipette. The image was taken using a Sony DSC-P30/P50 digital camera 10-15 seconds after the drop was deposited. Angles were measured using an ImageJ "measure" plugin.
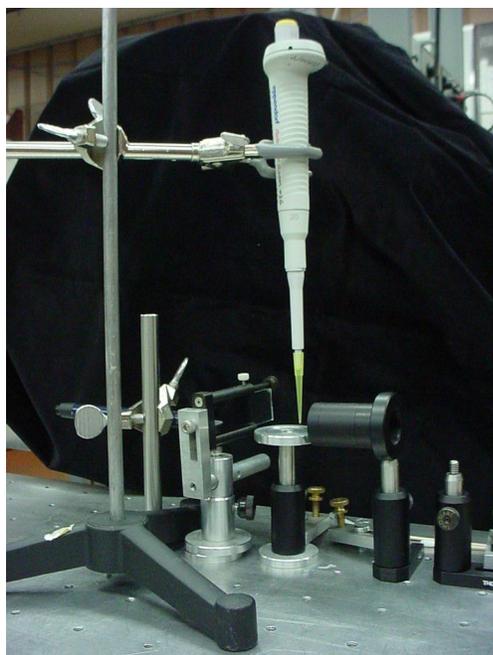

Figure 5: Contact angle apparatus

Centrifugation

In previous studies contact angles were made on bacterial lawns[5] (i.e. biofilms that have developed for some period of time). Since biofilms include both the bacteria and the organic matrix that they are embedded in, the contact angle that is measured on the bacterial lawn includes information about the extracellular polysaccharides (EPS) that the bacteria produce. In this experiment, however, the contact angle must represent the actual bacterial surface, not including the EPS. Thus, the bacteria must be harvested in a way that does not encourage biofilm growth. In order to obtain the contact angle for the bacteria/water/air interface the bacteria were harvested on an 18mm diameter slide by centrifugation. A device was made to hold the slide in a 50mm centrifuge tube that allowed the slide to be removed without disturbing the bacteria collected on the surface. A Thermo IEC Centra CL3R centrifuge was used in this experiment. The bacteria were spun at 4000g for 10 minutes. The layer of bacteria deposited on the slide was homogenous in its distribution and thickness as confirmed by confocal microscopy. The average thickness of the bacterial layer collected on the slide was 22μm. The slide was then allowed to dry under sterile conditions for 1h before contact angle measurements were taken. In addition, the bacteria were centrifuged onto each SAM and the water contact angles on the bacteria were measured and compared to those taken for the bacteria centrifuged onto the glass slides.

Scanning Laser Confocal Microscopy

The confocal microscope has several advantages over a traditional microscope. The use of a pinhole, or confocal aperture, allows only light from the focal plane to reach the detector. Since out of focus light is eliminated, the resulting image is highly detailed. In addition, the confocal aperture acts as a spatial filter, thus allowing for non-invasive optical sectioning of a

thick sample. The thickness of each section can be controlled by varying both the pinhole size and by choosing the right objective; choosing a high numerical aperture objective allows for thinner optical sections.
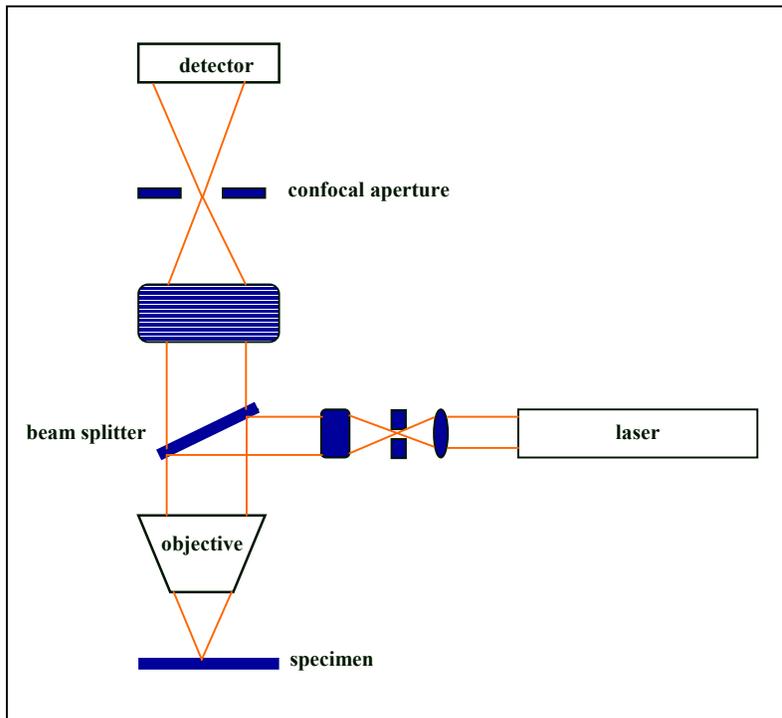

Figure 6: Simple schematic of a laser confocal microscope

Zooming is also possible by reducing the size of the pinhole, thus eliminating the need to change objectives and disturb the sample.

Modern confocal microscopes operate in reflection mode. This means that the objective collects both the incident and the reflected light. Usually a beam splitter is used to separate the excitation from the emitted light. This makes the confocal microscope particularly conducive to fluorescence imaging. Typically, fluorophores are introduced into the sample by means of various labeling techniques, however, autofluorescence is usually exploited whenever possible. The strain of *Pseudomonas* putida used in this experiment was genetically engineered to express

green fluorescent protein (GFP) and will autofluoresce with an emission maximum at 510nm using UV excitation.

Laser scanning confocal microscopy (LSCM) avoids subjecting large portions of the sample to the excitation energy reducing damage to the sample and photobleaching. This is especially beneficial when the sample is being observed live and in situ for longer periods of time as in this experiment. Using a laser beam, illumination is confined to a single diffraction limited spot and the beam is scanned across the sample.   A diffraction limited spot results in rapid intensity fall-off above and below the focal plane which improves the final image quality.

An Olympus FLUOVIEW FV300 LSCM was used to image the bacterial attachment process. An argon laser (488 nm) was used to excite the GFP expressed in *Pseudomonas* putida. A 40X UPlanFl Olympus objective (NA 0.75, WD 0.51mm) was used to image the bacteria. In this experiment, initial adhesion was monitored using time-lapsed microscopy for 1h, with an image being produced every 60s.

Sample Preparation

SAM Preparation

SAM substrates used in this experiment were prepared by immersing flame annealed gold films (Au (111) surfaces) in dilute thiol solutions according to the method outlined by Allan MacDairmid[4].

Gold films were made by evaporating gold onto cleaved mica under vacuum at a pressure of 10^{-6} Torr. Prior to evaporation, the mica was heated by running a current of 2.6 A across a filament in contact with the mica to expel water trapped in the mica layers. The gold was evaporated from a tungsten basket at 10V supplied by a variac AC power supply. The mica was coated with gold for five minutes, after which the film was allowed to cool under vacuum overnight. Flame annealing was carried out using a natural gas flame.

The gold films were immersed in 1mM solutions of either mercaptoundecanol or dodecanethiol for 24h at room temperature to produce hydrophilic or hydrophobic SAMs respectively. The thiol solutions were prepared using ethanol as the solvent. Contact angle measurements were used to confirm the presence of the monolayer.

Bacteria Culture

Pseudomonas putida was maintained on tryptic soy agar (TSA) plates. Cultures were grown one day in advance in tryptic soy broth (TSB) at 30ºC with shaking. Cells were harvested by centrifugation and washed twice before each adhesion experiment. They were re-suspended in phosphate buffered saline (PBS) and used immediately.

Adhesion Experiment

      The SAM was attached to the bottom of a Grace Biolabs 1mm x 18mm silicon isolator (a gasket type device used to confine liquid suspensions of bacteria to small areas) using paraffin wax. 400μL of *P*. putida suspended in PBS was next added to the isolator which was then covered with a glass coverslip. Immediately after introducing the bacteria to the SAM substrate imaging began. This was accomplished by focusing the confocal microscope just above the SAM surface and taking images every 60s for 1h.

Results and Discussion

The water contact angles that were measured on various surfaces are reported in Table 1.

Average Contact Angles	
Substrate	**Average Contact Angle (degrees)**
SAM of mercaptoundecanol	37 (8)
SAM of dodecanethiol	81 (7)
Gold Film (Flame Annealed)	69 (6)
Glass Slide (Sterile)	47 (7)
Bacteria Centrifuged onto Glass Slide	49 (7)
Bacteria Centrifuged onto Mercaptoundecanol SAM	36 (6)
Bacteria Centrifuged onto Dodecanethiol SAM	41 (8)

Table 1: Water contact angles on various surfaces in air. Standard deviations of at least 12 different measurements are presented in parenthesis

The contact angle of water on a flame annealed gold film was compared to contact angles obtained after the gold films were immersed in the thiol solutions. This was done simply to verify SAM formation. SAMs that did not have appropriate contact angles were discarded. Rijnaarts et al. report a water contact angle of $40° \pm 4°$ for *Pseudomonas* putida. In their experiment they harvested the bacteria on porous membranes by suction. Although this method is very different from the method used in this project, they both give similar results. In Table 1, the water contact angles of bacteria centrifuged onto various surfaces are different. The contact angles for the bacteria centrifuged onto the SAMs give similar results; however, the bacteria centrifuged onto glass give a higher contact angle. This result seems anomalous since glass has an intermediate hydrophobicity between that of the two SAMs as is shown in Table 1. This apparent error could be caused by differences in the water content of the bacteria collected on the surface after centrifugation. Although all surfaces were allowed to dry for 1h, the

mercaptoundecanol surface may need more time since it is more hydrophilic than the other two surfaces. The presence of water around the bacteria will lower the measured contact angle.

In calculating the work of adhesion for the SAM/bacteria/water interface, the contact angle used for the bacteria/water interface (i.e. θ_b) was the contact angle measured for the bacteria centrifuged onto the matching SAM. In other words, to calculate the work of adhesion for the mercaptoundecanol SAM/bacteria/water interface, W_{mbw}, the contact angle between the water and mercaptoundecanol SAM and the contact angle between water and the bacteria centrifuged onto the mercaptoundecanol SAM were used. The same goes for the dodecanethiol SAM/bacteria/water work of adhesion. The results of these calculations are summarized in Table 2.

Work of Adhesion (mJ/m^2)
W_{mbw} = 1.41
W_{dbw} = 7.66

Table 2: The work of adhesion as calculated from equation XVII. The subscripts m, d, w, and b indicate mercaptoundecanol SAM, dodecanethiol SAM, bacteria, and water respectively.

From equation XVII, it can be seen that as a substrate surface becomes more hydrophilic (i.e. θ_s approaches zero) the work of adhesion, W_{sbw}, approaches zero. Since, $\Delta G_{adhesion} = -W_{sbw}$, the free energy of adhesion also approaches zero. Thus, an equilibrium condition results where bacteria neither preferentially attach nor detach.

According to the confocal data, more bacteria adhere to the hydrophobic SAM than to the hydrophilic SAM. This result is consistent with the thermodynamic theory. According to Fletcher[5], *Pseudomonas* putida have the ability to attach to both hydrophobic and hydrophilic surfaces, however, hydrophobic surfaces are colonized faster than hydrophilic ones. It was also

noticed that bacteria on the hydrophilic SAMs were more likely to detach and become free swimming than those that had attached on the hydrophobic SAMs. Similar results have been published[5,11]. However, Rijnarrts et al. have reported that they have found no obvious correlation between the water contact angles on the bacterial surface and the free energy of adhesion. They used the so-called Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, which includes electrostatic forces and Liftshitz-van der Waals interactions to calculate ΔG values. Although this model is less simplified than the thermodynamic approach, it predicts that hydrophobic adhesion is not possible. This is contradictory to the results of many groups including the results of this project. Due to this fact, Rijnarrts et al have reported that non-DLVO interactions, such as steric interactions, H-bonding, and ion-pair interactions, must control bacterial adhesion.

Summary

The thermodynamic theory has been used in many experiments over the years to try to predict bacterial adhesion to a given substrate. Contact angles are used to relate interfacial free energies between the interacting surfaces. The thermodynamic approach treats the bacteria/substrate interaction as an equilibrium process. In real systems, however, attachment may not be an equilibrium process and the results of this project indicate that the more hydrophobic a substrate is, the less of an equilibrium process the interaction becomes. The equation that we obtained for the free energy change that occurs when a bacterium attaches to a surface considers only dispersion forces.   Other forces, such as electrostatic forces, are not taken into account but probably contribute to the overall interaction process. Although the results of the thermodynamic approach presented in this paper agree with other published results, as well as those observed using time-lapsed confocal microscopy, it will be necessary to expand this simple model to include other interactions in order to accurately model initial bacterial adhesion.

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