Fundamental Investigation of Biological Interactions for Applications in Infection Prevention and Biomaterial Development

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FUNDAMENTAL INVESTIGATION OF BIOLOGICAL INTERACTIONS FOR
APPLICATIONS IN INFECTION PREVENTION AND BIOMATERIAL DEVELOPMENT

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WORCESTER POLYTECHNIC INSTITUTE
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by

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Dedication and Acknowledgements

This dissertation is dedicated to my father, Liu, Wenping (刘文平) and my uncle, Liu, Wenfan (刘文范).

As I finally reach the acknowledgments in my dissertation, which I intentionally put at the end; I know it is the hardest part. We specialize in measuring the adhesion forces in the retraction curve, i.e. the forces needed to surmount the attachment. It is only at this moment that I can measure how strongly I have bonded to my Ph.D. lab and WPI.

My deepest appreciation and gratitude go to my advisor, Dr. Terri A. Camesano. I have been marvelously fortunate to have her as my advisor. She guided me through pathway of amazing biological interactions using systematic approaches. Next, she gave me the freedom to explore on my own and always directed me when I was stuck. Finally she taught me how to share our research with peer scientists by composing papers and attending international conferences in a timely fashion, to benefit the public by presenting the lab research in a fundamental way, and to provide practical applications by connecting with industry and patenting. I still keep all the drafts she corrected. Her guidance, trust, encouragement, patience, and support made the past five years unforgettable, fruitful, and enjoyable.

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I suppose that is everyone, right? Ok. Cheers and best wishes!
Well, maybe I should also mention my girlfriend, Shan Gao. You know I am saving the best for the last with a hope to last. As such a kind, lovely, selfless, and caring individual in my life, I feel so lucky to have you as a company even though New York City is 171 miles away from Worcester. Thanks for finding me so many papers and coming to Worcester from NYC to make me thousands of dumplings. She is always standing by my side and supporting me in whatever I do. I am so grateful to her and can’t wait to start the next phase with her.
Abstract

Bacterial infections persist as a public threat due to the ease by which bacteria adapt to commonly used antibiotics. In addition, bacteria on surfaces develop protective communities called biofilms that hinder the ability of antibiotics to completely eliminate the pathogens. The rapid development of bacterial resistance to antibiotics has made pharmaceutical companies reluctant to fund new antibiotics research. Hence, novel approaches to prevent and treat infections are needed.

The development of infections can be divided into three steps: adhesion, invasion and multiplication. Antibiotics target at the latter two step and are prone to bacterial resistance as passive strategies. Bacterial adhesion to host cells/implanted medical devices is the first step leading to following invasion and multiplication. However, fundamental understanding of bacterial adhesion process is still lacking.

The current studies are aimed to systematically investigate biological interactions between pathogenic bacteria and host cell, proteins and biomaterials with both macro and micro scale approaches. The macro scale methods include bacterial adhesion assay, viability studies, and thermodynamic modeling. The micro scale methods include direct adhesion force measurements, ultra surface visualization via atomic force microscopy (AFM) and surface structure modeling.

Our work combines experiments and modeling aimed at understanding the initial steps of the bacterial adhesion process, focusing on two case studies: 1) Mechanisms by which cranberry can prevent urinary tract infections through interfering with bacterial adhesion; and 2) Design of anti-adhesive and antimicrobial coatings for biomaterials. We make direct adhesion force
measurements between bacteria and substrates with an atomic force microscope (AFM), and combine such experiments with thermodynamic calculations, in order to develop a set of tools that allows for the prediction of whether bacteria will attach to a given surface.

These fundamental investigations of the bacterial adhesion process help elucidate the underlying mechanisms behind bacterial adhesion, thus leading to improved clinical outcomes for a number of biomedical applications.
Authorship

The majority contents of this dissertation are a representation of the work done by the Ph.D. candidate, Yatao Liu. Contributions to this dissertation research also include Amparo M. Gallardo-Moreno, a visiting professor from Universidad de Extremadura, Spain; Paola A. Pinzon-Arango, a Ph.D. candidate in the department of chemical engineering at Worcester Polytechnic Institute; Joshua Strauss, a Master student in the department of chemical engineering at Worcester Polytechnic Institute; and Matthew J. Black, a former undergraduate student in the department of chemical engineering at Worcester Polytechnic Institute.

Amparo helped me to develop the technique to coat bacteria onto AFM tip and the thermodynamic modeling. Paola did *E. coli* adhesion assay on uroepithelial cells. Josh did *S. epidermidis* adhesion assay. Matt did AFM experiments between bare AFM silicon nitride tip and *E. coli* HB101.
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Chapter 1: Overview

Fundamentals of Bacterial Adhesion Applied Towards Infection Prevention: Focus on Two Case Studies

1. Bacterial adhesion is the first step in infection development

   As one of the earliest life forms, bacteria have evolved into many thousands of species and can survive in a wide range of environments. According to National Institutes of Health (NIH), fewer than 1% of bacterial species can cause disease, with most bacteria being harmless or even beneficial to humans, such as bacteria residing in human intestines that help digest food (1), or cultures that contribute to the fermentation processes of yogurts and cheeses. Despite the fact that most bacteria are not pathogens, infectious diseases claim 1500 deaths per hour worldwide (2). Bacterial infections that lead to pneumonia, tuberculosis, and severe diarrheal diseases, along with infectious agents of malaria, measles and HIV/AIDS, account for half of all premature deaths worldwide, especially affecting children and young adults (2). Due to antibiotic resistance, some infections cannot be cured by conventionally prescribed antibiotics. For example, nearly 19,000 people died in the United States in 2005 after being infected with methicillin-resistant Staphylococcus aureus strains that have spread rampantly through hospitals and long term care facilities (3).

   The increasing public health crisis caused by bacterial resistance necessitates alternative approaches to preventing and curing infections. The initiation of a bacterial infection requires that bacteria first attach to host tissue. The attachment of bacteria to a surface is typically described as occurring through two stages: long range, non-specific forces help the bacterium make a close contact with host cells or a substratum, where stronger specific forces can become operative. Once attached, bacteria grow, secrete extracellular material, and can develop a biofilm, which is a dense and protective community of microorganisms.
The initial adhesion process is considered to be governed by specific and non-specific interaction forces between bacteria and substrata. Non-specific interactions typically refer to Lifshitz-van der Waals (LW) forces that are almost always attractive and operate between any two bodies; electrostatic interactions, which are often repulsive because bacteria and many surfaces each possess negative charges; and the so-called electron-donor/electron-acceptor or Lewis acid/base (AB) forces, which include hydrogen bonding. Specific forces, which are much stronger, refer to bonds between ligands and receptors of two biological samples. We discuss our approach to modeling and measuring the forces involved in the initial bacterial adhesion process.

2. Methods in studying bacterial adhesion

Bacterial adhesion can be studied at various scales, from macroscale studies that show the adhesion behavior of a population of bacteria, to nanoscale studies that probe individual cells or molecules associated with bacteria. Although macroscale studies are phenomena-oriented, they cannot provide information needed to disclose the underlying mechanisms. A combination of studies at different length scales can provide a more detailed picture.

2.1 Direct force measurements.

Interaction forces between bacteria and host cells or implanted medical devices directly determine whether bacteria will adhere. Although the quantification of adhesion forces between bacteria and a substrate represents the most accurate and straightforward way of gaining information on bacterial adhesion, in practice, there are two crucial issues that need addressing. (a) Tiny forces. The interaction forces between bacteria and a substrate are very small, with values typically at the pico-Newton (pN) to nano-Newton (nN) scales, i.e. \( (7-70) \times 10^{-12} \text{ lb}\cdot\text{ft/s}^2 \). Currently only two techniques can be used to directly detect such forces. One is optical tweezers (4), and the other is atomic force microscopy (AFM) (5-7). AFM provides larger measurement
range and more sophisticated controls such as the loading rate, besides providing simultaneous high resolution imaging (Figure 1). There are additional indirect techniques used to estimate the interaction forces. These techniques include total internal reflection microscopy (TIRM), total internal reflection aqueous fluorescence (TIRAF) microscopy, surface forces apparatus (SFA), and quartz crystal microbalance with energy dissipation (QCM-D), etc. Interested readers are encouraged to refer to a comprehensive review paper on the use of these techniques in bacterial adhesion studies (8).

(b) **Obtaining correct orientations of biological molecules.**

In order for bacterial ligands to correctly bind with receptors, the molecules on bacterial surfaces, including fimbriae (pili), and lipopolysaccharides, must expose the appropriate orientation. Experimentally, it is challenging to maintain correct orientation when biological cells are trapped in optical tweezers or immobilized on an AFM tip. In our lab, we invented a novel coating method that can be used to attach bacteria to an AFM tip (Figure 2), such that they possess the correct orientation for direct force measurements (9).

Some technical issues also need to be resolved in force measurements such as the timescale and loading rate. The timescale needed to build a ligand-receptor bond can be difficult to determine. Further, the loading rate needed to make AFM force measurements has to be specified for each experiment. These parameters should be appropriately determined to obtain correct force measurements.

2.2 Thermodynamic modeling of bacterial-surface interactions.

Classical and extended Derjaguin-Landau-Verwey-Overbeek (DLVO) theory has been utilized to explain and predict the adhesion behavior of bacteria in aqueous media. The DLVO model takes into account van der Waals interactions, electrostatic interactions, and often includes
electron donor/electron acceptor interactions when the extended DLVO model is applied. Parameters to include in the thermodynamic models need to be estimated for each bacterium, substrate, and solution. For example, zeta potential measurements on bacteria in a suspension are used for the modeling of electrostatic interactions.

Parameters for the thermodynamic calculations are taken from contact angle measurements on bacterial lawns and on the substrates of interest, using probe liquids with varying polarities. Individual surface tensions can be calculated from the measured contact angles by using the Young-Dupré equation (10). The Gibbs free energy change due to adhesion is calculated from the interfacial tensions for bacteria/substrate, bacteria/water, and substrate/water. If bacteria can attach to a substrate, then the newly formed interface (bacteria-substrate) must be more stable than the two old interfaces (substrate-liquid and bacteria-liquid). The Gibbs free energy change during the process must be negative to favor the new interface, which represents bacteria attached to the substrata. If the Gibbs free energy change is positive, bacteria prefer to not attach to the surface, but to remain in the aqueous media. One advantage for using thermodynamic modeling is that the method is reliable for many kinds of substrates, especially when at least one non-biological surface is applied. In addition, this method has a strong and well-defined theoretical foundation, which helps to fundamentally explain bacterial adhesion and offer a theoretical guide for biomaterial development or infection-prevention strategy. However, the thermodynamic modeling only accounts for non-specific interactions. If both surfaces are biological samples, ligand-receptor interactions may be present. Then the interaction forces calculated from the thermodynamic model will be greatly underestimated, as we reported earlier (11). A detailed explanation on the use of these models for bacterial adhesion calculations was reported in our previous studies (11).
2.3 Macroscale studies of bacterial attachment

One of the simplest ways to quantify bacterial attachment to a surface is via a retention assay. Bacteria are incubated with host cells or the biomaterial of interest; either statically or under flow conditions. After a pre-determined time, host cells or the substrata are removed and washed to remove the loosely attached bacteria. The percentage of attached bacteria that are viable can be quantified using a dual DNA staining kit, in which green and red fluorochromes can be used to discern the number of viable cells (Figure 3).

Although a bacterial retention assay is a quick way to screen various surfaces or treatments, it does not provide mechanistic information on why bacteria attach. In addition, it can be difficult to conduct the experiments reproducibly, particularly if bacteria aggregate, making it difficult to get accurate cell counts. Numerous trials may be required to obtain statistically meaningful data. However, this simple assay may be used as a reference method to compare with other methods of quantifying bacterial adhesion.

3. Case studies

While there are numerous types of bacterial infections with varying degrees of clinical severity, we focus on two examples that our lab has studied extensively.

3.1 Case I: Cranberry as a preventive measure for urinary tract infections (UTIs)

3.1.1 UTIs and antibiotic resistance

Urinary tract infections are defined as infections of the kidneys, ureters, bladder, or urethra, and are the second most common type of infection in the U.S. Symptoms generally include a frequent urge to urinate, and pain and burning in the area of the bladder or urethra during urination, and sometimes may be accompanied by fever, fatigue, and trembling. Women, infants, and elders are more prone to UTIs. Approximately one third of women will have at least one UTI
in their lifetime (12). The annual rate of infection among women in the United States is 11.3 million symptomatic cases (13) and over 10 million asymptomatic cases (14). The estimated annual medical expenditures are more than $1.6 billion (15). The Gram-negative bacterium *Escherichia coli* is the main culprit, responsible for 85-95% of cystitis cases (bladder infection) and 90% of acute pyelonephritis cases (a serious kidney infection) (16).

Although most bacterial infections are treatable with antibiotics, bacterial resistance to currently available antibiotics has become an increasing threat to public health, largely due to inappropriate dosing and administration of antibiotics, as well as the rapid ability of bacteria to exchange genetic information that confers resistance. Cotrimoxazole (trimethoprim/sulfamethoxazole) is the current first-line treatment for uncomplicated UTIs in the U.S. and many other countries, but cotrimoxazole resistance exceeds 15% and can be as high as 25% in Canada and the U.S. (17).

3.1.2 Cranberries and UTIs

Native American used cranberries as a food source, and for many years cranberries have been experientially recognized for their benefits of maintaining urinary tract health. Preliminary clinical studies of cranberry’s benefits began in the early 1920s (18, 19). In 1994, Avorn et al. were the first to successfully demonstrate that consumption of cranberry juice reduces the frequency of recurrent urinary tract infections in a population of elderly women. Although very early studies hypothesized that increased acidity produced in the urine by eating cranberries was the reason for the beneficial effect (18), more recent work has shown that the pH of urine after cranberry juice cocktail consumption only changes slightly (20) and is transient (21).

In 1984, Sotoba et al. found that preincubation of *E. coli* and uroepithelial cells in cranberry juice decreased bacterial adhesion (22), leading to a paradigm shift in the understanding of the
action of cranberry on bacterial adhesion. Since that time, researchers have focused their efforts on gaining a detailed molecular-scale understanding of the mechanisms behind this action.

3.1.3 Molecular mechanisms of cranberries preventing UTIs

Compounds in cranberries affect molecules on the surface of Gram-negative bacteria. For example, fimbriae are proteinaceous structures that extend from *E. coli*, and contain a specific adhesin molecule (PapG) that helps the bacteria bind to a receptor on uroepithelial cells, known as the α-Gal(1→4)β-Gal oligosaccharide receptor. *E. coli* that possess P type fimbriae can cause more serious types of UTIs, such as acute kidney infection (pyelonephritis), in addition to the less severe cystitis (bladder infection). We review some of our recent work, focusing on P-fimbriated *E. coli* and a non-fimbriated mutant strain, which allowed us to better understand the role of cranberry compounds on P fimbriae.

(a) Bacterial retention assay

Building upon the available clinical studies, we performed *in vitro* bacterial adhesion assays that were designed to help understand the mechanisms behind cranberry’s action on the *E. coli*-uroepithelial cell interaction. Using neutralized cranberry juice so that the effects of pH on bacterial adhesion could be eliminated, we found that the number of attached *E. coli* per uroepithelial cells decreased from 50.2 ± 22.9 bacteria/uroepithelial cell without cranberry juice treatment, to 13.6 ± 5.7, 9.3 ± 4.1, and 2.9 ± 1.5 bacteria/uroepithelial cell, corresponding to 5, 10, and 27 wt.% cranberry juice treatment, respectively (23). These *in vitro* attachment results confirmed that cranberry juice cocktail can reduce bacterial attachment to host tissue, and that lower pH is not the underlying mechanism that makes cranberry juice an effective agent for preventing UTIs.
(b) P-fimbriae morphology characterization

Through atomic force microscopy (AFM) measurements, together with steric modeling, we found that the average P-fimbriae length on *E. coli* HB101pDC1 was 147 nm (around 600 × 10⁻⁸ inch) without cranberry juice treatment, but decreased to 50 nm (around 200 × 10⁻⁸ inch) when bacteria were exposed to cranberry juice (Figure 4) (24). Thus, we directly demonstrated that although P fimbriae are not removed by exposure to cranberry juice, the proteins become compressed significantly after cranberry juice treatment, which may account for their decreased ability to adhere to uroepithelial cells.

(c) Direct force measurements

In addition, AFM was used to show that the adhesion force between *E. coli* and a uroepithelial cell was ~10 nN (7.233 × 10⁻⁸ lb•ft/s²) when no cranberry juice cocktail was present, but decreased to ~0.50 nN (0.362 × 10⁻⁸ lb•ft/s²) after cells were exposed to 27% cranberry juice cocktail (25). The specific adhesion forces between PapG adhesin and receptors on uroepithelial cells were significantly decreased after cranberry juice treatment. This was the first study to directly demonstrate that cranberry juice treatment reduces the nanoscale adhesion forces between bacteria and uroepithelial cells.

(d) Thermodynamic modeling

Through thermodynamic modeling, we showed that the Gibbs free energy change (ΔG_{adh}) between *E. coli* and uroepithelial cells in the absence of cranberry juice treatment was -20 mJ/m² (around -150 ft-lbs/ft²), where the negative value implies that bacterial adhesion is favorable. With increasing concentrations of cranberry juice treatment, ΔG_{adh} increased and became positive when the bacteria and uroepithelial cells were exposed to at least 20 wt.% cranberry juice cocktail, suggesting that at or above this concentration, bacterial adhesion is unfavorable.
(26). These results imply that cranberry juice can impair non-specific interactions between bacteria and uroepithelial cells and hence prevent bacterial adhesion.

Therefore, cranberry can provide protection at three different levels:

a. Cranberry juice exposure compresses P fimbriae of *E. coli*, thus preventing adhesion between the bacterium and the uroepithelial cell.

b. Cranberry juice increases the repulsive energy barrier to adhesion, over a range of hundreds of nanometers ($400 \times 10^{-8}$ inch), thus preventing the bacteria from coming into contact with the uroepithelial cells.

c. Even if bacteria are able to penetrate the repulsive energy barrier, the action of cranberry juice on the bacteria decreases the ability of the bacteria to attach to uroepithelial cells, as demonstrated through direct force measurements.

3.1.4 Future Directions for Cranberry Research

Although progress has been made in understanding cranberry’s actions against *E. coli* towards the protection of urinary tract health, there are a number of key research issues that remain to be addressed. For example, a large body of research is devoted to identifying the critical compounds in cranberry that cause the anti-adhesive benefits, and in elucidating the needed dose and duration of exposure to such compounds. Due to the acidity of cranberry juice, commercially available cranberry juice cocktails are sweetened with fructose, water, and vitamin C, yielding 25-27 wt% cranberry juice. Therefore, there are more than 120 different compounds in cranberry juice (27). Most research has focused on isolating and identifying the class of A-type proanthocyanidins (PACs) or non-dialyzable materials, which have shown decreases in bacterial adhesion *in vitro* (28-33).
However, it is not easy to translate the dose required to impart an anti-adhesion effect in an \textit{in vitro} study to the dose needed for clinical relevance. Our \textit{in vitro} studies showed that 5.0 wt.% cranberry juice was sufficient to prevent bacterial adhesion (24, 25) from the molecular scale perspective for the first time, although similar results were observed in prior \textit{in vitro} bacterial adhesion assay experiments (30, 34). Although it is not yet known how these \textit{in vitro} thresholds will translate to \textit{in vivo} conditions, researchers are actively engaged in trying to extend laboratory-scale mechanistic studies towards clinical trials. Increased understanding of the molecular action of cranberry juice on \textit{E. coli} and uroepithelial cells can lead to better estimation of needed cranberry juice dose and duration.

3.2 Case II: Infections of implanted medical devices

3.2.1 Infections on biomaterials

Modern medicine is highly dependent on implanted medical devices, such as catheters, cerebrospinal fluid shunts, prosthetic heart valves and prosthetic joints, vascular grafts, cardiac pacemakers, and intraocular lenses, etc., which have significantly improved quality of treatments for patients. However, any time a foreign material is introduced into the body; this surface becomes a likely site of bacterial infection. For example, 4.3 % of 2.6 million orthopedic implants and 7.4 % of cardiovascular implants become infected per year (35, 36). Bacterial infections occur in over 2 million surgical cases each year in the U.S. alone, which burdens patients both physically and financially (37). Annually in the U.S., there are over 250,000 catheter related bloodstream infections (CRBSIs) (38). The Gram-positive bacterium \textit{Staphylococcus epidermidis} has evolved as a leading cause of nosocomial sepsis, and is the most frequently isolated causal organism for infections of numerous types of catheters, shunts, and
other implanted medical devices (39-41) For example, *S. epidermidis* and other coagulase-negative *Staphylococci* were the causal agents in ~50% of CRBSIs (42).

Once bacteria attach to implanted medical devices, they can easily form a protective biofilm because the biofilm community is encased in a matrix of polysaccharides and proteins, which presents a diffusion barrier for antimicrobial agents’ penetration. Further, the reduced metabolic rate of the bacteria in the biofilm causes a slow rate of uptake of antimicrobial agents. The biofilm also shields bacteria from environmental stresses (43). Often the only effective treatment of an infected implanted medical device is surgical excision (44). In addition to increasing the patient’s morbidity, mortality, and recovery time, the economic expenditure on bacterial-infected medical devices exceeds $3 billion per year in the U.S. alone (35).

### 3.2.2 Strategies toward preventing implanted medical device related infections

Current research is focused on designing materials that resist bacterial adhesion or that inactivate attached bacteria. One strategy has been to coat antimicrobial agents directly onto the implanted materials to kill bacteria upon initial adhesion or as they begin to grow. A variety of antibiotics such as vancomycin, gentamicin, clindamycin, fusidic acid, ciprofloxacin, cefuroxime, cefotaxime, and chlorhexidine have been tested *in vitro* and in animal models (45-47). However, only limited success has been obtained. The main challenge is that it is difficult to maintain a steady release of drug from the biomaterial. The focus of several research groups, including ours, is to develop materials that resist the adhesion of bacteria to surfaces. Coatings such as self-assembled monolayers (SAMs) and polymers have demonstrated the ability to prevent bacterial adhesion by modifying surface properties such as hydrophobicity, roughness, and surface charge (7, 48, 49). However, bacterial adhesion results do not show consistent trends in terms of the physicochemical properties of the surfaces. For example, we showed that surface wettability and
roughness were insufficient properties to correlate with bacterial adhesion (7). A better ability to characterize the properties of biomaterials at the molecular level may lead to better design of antibacterial biomaterials.

3.2.3 Use of self-assembled monolayers (SAMs) to create anti-adhesive coatings

SAMs possess a layer of molecules with the same terminal group and uniform orientation, properties that facilitate the study of bacterial adhesion since bacteria are always exposed to the same chemical groups. In our laboratory, we developed a series of SAMs with varying terminal groups that were designed to resist bacterial adhesion and/or inactivate bacteria. The two most promising candidates we identified were dodecanethiol-based SAMs (terminating in isophthalic acid or isophthalic acid with silver). The silver-containing SAMs were of interest because the antibacterial properties of silver have been demonstrated, and bacteria are unable to develop a resistance to silver’s antimicrobial abilities (50). In addition, silver has been shown to be nontoxic to mammalian cells at similar concentrations (51).

When evaluating the potential of an antibacterial coating for a particular biomaterial, it is also important to consider how serum and plasma proteins, such as fibronectin, laminin, fibrin, and albumin, will adsorb to the biomaterial. In our work, we also tested the adsorption of model proteins (fetal bovine serum and fibronectin) to the SAM-coated materials.

We found that the attachment of *S. epidermidis* to the protein-coated material depended on the particular protein present. Fetal bovine serum adsorption reduced the attachment of *S. epidermidis* to the material, while fibronectin coating promoted *S. epidermidis* attachment (52). SAMs terminating in isophthalic acid (IPA) and isophthalic acid with silver (IAG) resulted in lower non-specific adhesion forces with *S. epidermidis* compared to bare surfaces, as supported by thermodynamic modeling. When serum proteins were adsorbed on the SAMs, non-specific
interactions between the bacteria and substrate decreased (Figure 5). While the LW forces were unchanged, AB forces were found to dominate the overall interaction, and showed more variability in terms of the type of SAM and protein put on the substrate. Since AB forces mainly reflect hydrogen bonds, we suggest that a fruitful approach to enhanced development of antimicrobial biomaterials would be to select materials that prevent or limit the formation of hydrogen bonds (11).

The thermodynamic modeling was supported by direct AFM force measurements between an S. epidermidis-coated AFM tip and the various SAMs or protein-coated surfaces. Stronger adhesion forces were observed between S. epidermidis and fibronectin than between the bacteria and fetal bovine serum, due to the formation of strong ligand-receptor bonds that can only occur with fibronectin (53). Since protein coatings can mask the underlying surface properties, it is important to consider the competition between S. epidermis and serum protein for adsorption to the biomaterial.

In our study, the IPA-terminating SAM showed the best activity in terms of preventing bacterial adhesion and inactivating bacteria. IAG showed strong anti-bacterial adhesion properties similar to IPA. In addition, IAG killed around 60% attached S. epidermidis (Figure 6) (11). IAG coated with a protein layer of more than 100 nm (3.94 × 10^{-6} inch) still was able to present antibacterial activity, since we assume some silver ions could diffuse through the protein layer. However, when the protein coating was thicker than 250 nm (9.85× 10^{-6} inch), the ability of the SAM to inactivated bacteria decreased significantly (52). These results emphasize again that biomaterial development studies need to consider the interactions of materials with in vivo proteins, as well as with bacterial pathogens.
Conclusions

As a crucial step leading to infection development, the creation of new tools to experimentally measure and model bacterial adhesion can lead to health benefits. In particular, we discussed how atomic force microscopy and thermodynamic modeling could be used to study the fundamental adhesion processes related to urinary tract infections and bacterial infections on biomaterials.

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Figure List

Fig 1 Representative AFM amplitude imaging of the deposition of *E. coli* culture solution after washed three times with phosphate buffer saline. Then the solution was deposited onto a mica surface. They formed a tree-like crystallization.

Fig 2 Representative SEM imaging of *S. epidermidis* coated AFM tip.

Fig 3 Representative image of *S. epidermidis* stained with live/dead kit for an adhesion assay.

Fig 4 The average equilibrium length of P fimbriae on *E. coli* surface derived from steric modelling based on AFM surface characterizations. Adapted with permission from Liu et al., *Biotechnology and Bioengineering*, 2006, 93, 301. Copyright (2005) Wiley Periodicals, Inc.

Fig 5 Correlation between Gibbs free energy change and *S. epidermidis* retention. Adapted with permission from Liu et al., *Langmuir*, 2007, 23, 7138. Copyright (2007) American Chemical Society

Fig 6 Correlation between Gibbs free energy change and *S. epidermidis* viability. Adapted with permission from Liu et al., *Langmuir*, 2007, 23, 7138. Copyright (2007) American Chemical Society
Fig 1 Representative AFM imaging of the deposition of *E. coli* culture solution
Fig 2 Representative SEM imaging of *S. epidermidis* coated AFM tip
Fig 3 Representative image of live/dead kit stained *S. epidermidis* adhesion assay
Fig 4 The average equilibrium length of P fimbriae on *E. coli* surface derived from steric modelling based on AFM surface characterizations. Adapted with permission from Liu *et al.*, *Biotechnology and Bioengineering*, 2006, 93, 301. Copyright (2005) Wiley Periodicals, Inc.
Fig 5 Correlation between interfacial free energy and *S. epidermidis* retention results.
Fig 6 Correlation between interfacial free energy and retention cellular viability
References

Part I: Tools in Studying Biological Interaction Forces
Chapter 2: Measuring Bacterial Adhesion at Environmental Interfaces With Single-Cell and Single-Molecule Techniques

Abstract

A synopsis is provided of techniques currently used to quantify the interactions between bacterial cells and surfaces. Focus is placed on techniques which allow for direct probing of nano, pico, or femto-scale interaction forces between bacteria and surfaces of relevance for environmental science and engineering. We focus on bacterial adhesion measurements and surface characterizations via techniques that measure forces on individual bacterial cells or cellular macromolecules, particularly atomic force microscopy (AFM) and related force spectroscopy. However, we also include overviews of other techniques useful for evaluating cellular forces, such as optical tweezers, evanescent wave scattering-based techniques (i.e. total internal reflection microscopy; TIRM and total internal reflection aqueous fluorescence microscopy; TIRAF), and the quartz crystal microbalance (QCM). These latter techniques, while not providing direct measurements of forces of adhesion, can be used to explain adhesion and interaction forces in bacterial systems. The operating principles, advantages and limitations of each technique is reviewed, and key bacterial adhesion studies from each area are presented. Qualitative and quantitative methodologies for relating force measurements to bacterial attachment, particularly to bacterial retention in porous media, are discussed.
I. Introduction

Environmental systems by their nature are comprised of interfaces that provide chemical and mechanical stimuli for microorganisms. Often, attachment to a surface is the way in which microbes respond to environmental stimuli, and this attachment affects numerous environmental problems. Potentially-adherent bacteria enter the environment through such varied sources as domestic, agricultural, medical and industrial activities. There is a great need to be able to accurately predict bacterial attachment and transport in geologic media. Much research in this area is concerned with the potential for pathogenic microorganisms to contaminate drinking water, which remains a significant problem [1]. In addition, the significance of bacterial attachment to soil has been noted recently in several other processes, including biostimulation [2], bioaugmentation [3], biobarrier technologies for containment of pollutants [4, 5], and microbial-facilitated transport of contaminants [6, 7]. The ability to measure nanoscale interactions between microbes and surfaces in the environment provides crucial information needed to formulate an understanding of microbial adhesion in aquifers.

Classical cell adhesion assays used for eukaryotic cells are typically described in terms of the type of force being applied, such as normal or hydrodynamic. For example, centrifugal assays are useful for measuring detachment forces of cells, as was done for basophilic leukemia cells coated with immunoglobulin E, to substrates coated with a ligand, namely dinitrophenol [8]. Detachment forces in the 2-4 microdyne range (2-4 x 10^{-5} N) were detected. Shear devices based on spinning disks [9] or microfluidics-based devices with parallel-plate constructions [10] have also been useful for these applications. Information on the shear stress needed to detach a cell from a substrate is combined with geometry information on the cell and (where applicable) the density of receptors on the substrate, to construct a profile of the average adhesive strength.
between the cell and the substrate. Experiments using a radial stagnation point flow chamber have been used recently to quantify bacterial attachment and detachment kinetics [11].

Micropipette aspiration represents a direct force probing technique, classically used for mammalian cells [12]. In this approach, a single cell is brought into contact with a planar substrate, such as a vesicle or membrane bilayer. At a given time point, a pipette holding the cell (perpendicular to the bilayer) is retracted, and the aspiration pressure is used to calculate the adhesion force between the two. In a later advancement of this technique, [13], the biomembrane force probe (BFP) was developed. This probe was used as a way of carefully controlling the loading force applied between ligand-receptor pairs [14]. We are not aware of this technique being used in any microbial studies.

The surface forces apparatus (SFA) is also a powerful force tool that has been used for proteins and other biomolecular interactions. While the force resolution is somewhat low (± 10 nN), distances can be measured with much greater resolution (± 0.1 nm) [15]. While the technique has been very useful for probing molecular interactions (i.e. protein behavior), we are not aware of its use in any bacterial studies.

Characterization of microbial/bacterial adhesion has evolved significantly in recent years, and developments have been most significant in the single-cell/single molecule detection of forces. While past research relied upon population-based techniques and indirect methods, today we can measure interaction forces between single cells and surfaces of interest [16], and we can even characterize microbial properties that vary across locations on a single cell [17, 18]. In addition, we can better probe the surface-associated and extracellular [19-21] macromolecules associated with bacteria, which are often providing the first contact between a bacterium and a substrate surface.
In this article, we concentrate on a review of current best methods for measuring bacterial adhesion, focusing on techniques in which single cells can be probed. Many of the topics and techniques discussed are applicable to other types of microorganisms, although we mainly concentrate this review on bacteria. Examples from fungal or yeast adhesion studies, as well as eukaryotic cells, are cited where relevant. For a more detailed review on interaction forces in other microbial systems, or on particular biomolecules (i.e. proteins, polysaccharides), the reader is referred to other articles [15, 16, 22, 23]. We also explore the relationship between microbial adhesion measurements and the transport of microbes in porous media. Finally, we discuss research needs in this field and likely areas for future research.

The ability to characterize bacterial surfaces and interactions at an increased resolution, and to probe these entities at the nanoscale, has significantly enhanced our understanding of microbial interfaces [24]. Environmental engineering and science fields are in the midst of enjoying the benefits of recently available surface characterization techniques. While it was previously necessary to examine whole populations of bacteria and measure averaged responses, we are now routinely able to measure properties at the level of a single cell, and sometimes our resolution can be further increased to probe properties that vary within or across a single cell. Thus, we are no longer relegated to viewing a bacterium as a “homogenous solution of proteins” [25]. For example, we can trace the swimming path of a single motile bacterium as it comes in the proximity of a surface [26, 27], probe the polysaccharides on the surface of a single microbial cell [18, 28], or identify nanometer-sized structures on a cellular surface, such as the morphologies of proteins that distinguish germinating from dormant fungal spores [29]. With careful imaging and preparation protocols, bacterial structures such as flagella can be observed (Figure 1).
II. Direct Techniques for Measuring Single-Cell Adhesion: Advantages and Limitations

In this review, we concentrate on methods to probe individual cells, to allow for the quantitative measurement of microbial adhesion forces. Various indirect or non-quantitative methods, such as by counting cells attached to a substrate, or separation of cells using flow chambers, spinning disks, columns, etc., are also available to measure cellular adhesion, as mentioned briefly in section I. We choose to focus the major portion of this review on direct and indirect methods for probing single cells or cellular biomolecules. While each technique will be described in detail, an overview of the properties of these methods is provided in Tables 1 and 2.

One of the most important direct methods for measuring bacterial adhesion forces is atomic force microscopy (AFM), which has emerged as an extremely potent tool for environmental interface research. We also describe another technique that allows for the direct and quantitative probing of interaction and adhesion forces, optical tweezers, and indirect techniques based on total internal reflectance and the quartz crystal microbalance.

A. AFM and Related Techniques for Direct Force Measurements

1. Overview of AFM for Bacterial Interface Research

Although not even 20 years old, the AFM has spread widely into many scientific and engineering fields for its use as an imaging tool and for the measurement of interfacial phenomena. In the AFM technique (Figure 2), a very sharp tip (attached to a cantilever arm) is used to “probe” the interfacial region between the sample and tip, providing either a map of surface topography or a measure of interaction forces. By aligning a laser on the cantilever, the deflections of the cantilever, due to sample-tip interactions or topography, are detected using a split-detector photodiode. The deflection signal is digitally processed to reconstruct a topographic image or an interaction force profile of the sample. Before force microscopy was a common
technique, the AFM was widely used for the imaging of cellular molecules, such as the polysaccharide acetan [30], and biofilms [31-33]. An example of a microbial biofilm (Aureobasidium pullulans) captured in an AFM tapping mode image, under water, is shown in Figure 3.

The behavior of single cells can be investigated to study such properties as the relationship between macromolecule expression (and heterogeneity wherein) with interaction forces or adhesion affinity [18], the relationship between cell swimming behavior or orientation with attachment [17], and the ability of single cells to experience reactions when in proximity to a surface [34].

The individual macromolecules on a bacterial surface can be probed with nanometer-scale resolution. For example, the AFM was used to determine the thickness of extracted and isolated membrane vesicles and lipopolysaccharides (LPS) on Pseudomonas aeruginosa [35]. By using force spectroscopy in combination with high resolution AFM imaging, Stoica et al. could obtain the spring constants of molecules that make up the membrane vesicles of P. aeruginosa. The elastic response of the membrane is believed to play a role in the virulence and ability to incite infection for this bacterium.

AFM has been used to measure the sizes of molecules on bacterial surfaces, even without their removal. For example, Amro et al. [36] imaged the surfaces of E. coli strain JM109(pSF815A) and determined the sizes of LPS and proteins in the outer membrane. Through careful operation of the AFM, they achieved image resolutions of 50 and 5 Å, in the lateral and vertical directions, respectively.

Functionalized AFM probes are useful for imaging bacterial surfaces with heterogeneous compositions. For example, the contrast of hydrophilic molecules on a fungal cell surface could
be enhanced by imaging the cell with a hydrophobic probe, such as one with an –OH or –CH₃ terminal group [37]. AFM tips were coated with self-assembled monolayers (SAMs) bearing –OH and –CH₃ terminal groups, and force maps were created over areas of the fungus *Phenerochaete chrysosporium*. In this case, the fungal cell surface was shown to be hydrophilic, since no adhesion forces with either type of hydrophobically-functionalized tip were observed with the AFM.

Some studies have utilized a combination of biochemical assays or reactions along with single-cell probing in order to increase the amount of information that can be obtained. In one such study, the enzyme pullulanase was applied to cells of the microfungus *Aureobasidium pullulans*, and the adhesion forces between the microbe and a silicon nitride tip were quantified [38]. Contrary to expectations, pullulan was not found to be the dominant macromolecule in controlling the adhesion of *A. pullulans*. In fact, uronic acid-based polymers that were also present on the cell surface were seen to control the adhesion forces to silicon nitride, and were also correlated with attachment of the cells to quartz. A technique that could distinguish between populations of macromolecules was necessary in order to differentiate the roles of multiple biopolymers on the microbial surface.

In terms of quantifying microbial adhesion via direct force measurements, the AFM can be used in several conformations. The microbe of interest can be immobilized to a flat substrate by techniques including physisorption onto an oppositely charged filter [39], or coated glass slide [40]; chemical attachment to glass [41]; and entrapment in the pores of a membrane [42] or a gel [43]. The interacting probe can be a clean smooth surface, such as silicon nitride or silica, or it may be a functionalized probe, such as an organic-bearing or polymer-coated probe [44]. This conformation of the AFM allows for extremely good resolution, due to the sharp probe
interacting with the microbial surface. However, a limitation is that the technique is limited to studying the interactions between microbes and relatively small molecules that can be attached to the probe.

Alternately, the microbe itself can be the probe if it can be attached to a cantilever. Yeast cells [45], bacterial lawns [20], and individual bacterial cells [46] have all been used as probes. In this latter arrangement, a wider variety of substrates can be examined. However, the disadvantage is that the greater surface area of the probe decreases the resolution of the images and averages the force measurement over a larger area. Also, information on the exact probe radius and the orientation of the cell (or cellular macromolecules) with respect to the substrate can be more difficult to obtain.

When collecting images or force data with the AFM, these representations depend on the balance between attractive and repulsive forces governing tip-sample interactions. van der Waals forces are always present, which are relatively long-range (i.e. few to 10 nanometers), weakly attractive forces that are not very sensitive to solution ionic strength. The repulsive forces between the two surfaces arise from a combination of atomic-level repulsion, caused by the overlapping of electron orbitals, as well as solvation and hydration forces. Longer range electrostatic double layer (EDL) forces are also present, and are very sensitive to the ionic composition of the liquid solution in which measurements are performed. EDL interactions for many microbial systems of environmental relevance are repulsive. Bacteria can be represented as negatively charged colloids, and therefore, their interaction with soil, glass, negatively charged polymeric organic compounds, clay particles, etc. is repulsive. EDL interactions may be attractive if microbes are interacting with positively-charged minerals, for example, mineral surfaces with iron or other metal functional groups present. In systems in which polymers are
present, such as microbial surface or extracellular macromolecules, steric repulsion also must be considered.

2. Applications of AFM for Studying Bacterial Adhesion at Environmental Interfaces
a. AFM to Measure Bacterial Interactions with Trace Metals from Mineral Surfaces

AFM has been used to probe geochemical and biogeochemical reactions that occur in the subsurface aqueous environment. In 1996 and 1997, formative studies in this area laid the groundwork for future explorations of microbe-mineral interactions in biogeochemical applications [47-49]. For example, Maurice et al. used tapping mode AFM imaging to probe the dissolution of Fe(III)-(hydr)oxide from hematite and goethite mineral surfaces by a *Pseudomonas* sp. bacterium [49]. They concluded that the dissolution was heterogeneous, and controlled by a surface process, either due to microbial attachment to the rough mineral surface or to a surface-controlled dissolution reaction. Since this was an early study on the subject, the authors also pointed out the need for appropriate control experiments and cautioned on the difficulties involved in the preparation of samples (purity, etc.), as well as the need for multiple images to extract meaningful results based on the complexity of the system.

Microbial dissolution by another soil microorganism that is a facultative, Gram-negative anaerobe, *Shewanella putrefaciens* 200, was also probed via AFM measurements, and the authors could “observe” redox reactions occurring at the microbe-mineral interface. Attached bacteria from nutrient-poor media corroded the surface iron coating on ferric iron hydroxydioxide-coated silica, releasing as much as 10% of the iron in the coatings, while leaving bacterial shaped depressions in the mineral surface [48]. In a subsequent study with this same organism, the authors compared microbial dissolution of iron and aluminum oxyhydroxide mineral coatings under aerobic and anaerobic conditions [47]. Under aerobic conditions, the
authors found localized pitting on the Fe$^{3+}$ oxyhydroxide-coated quartz within 72 hrs of incubation with *S. putrefaciens* 200. The slow but steady release of iron into solution was attributed to a redox reaction at the bacterial-mineral interface, followed by reoxidation of Fe$^{2+}$ on the surface. This localized pitting (and the redox reaction) were not observed on Al$^{3+}$ oxydyroxide-coated surfaces. The iron loss under anaerobic conditions was non-localized and the weak adhesion between the bacteria and the mineral surface suggested that the cells remained passively mobile on the surface.

Interactions between individual, viable and metabolically active microbial cells and mineral surfaces have been probed for several systems, and this research is being applied to increasingly sophisticated systems. For example, the adhesive interactions between *Shewanella oneidensis* and goethite were probed via AFM [50]. The energy values calculated from AFM (force) profiles suggested that, under anaerobic conditions, electrons were being transferred from the bacteria to the mineral surfaces. A putative iron reductase protein on the bacterium was speculated to have been mobilized in the bacterial outer membrane, and it is this protein that appears to interact with the mineral to facilitate electron transfer. Adhesion forces were much lower under aerobic conditions, where such electron transfer would not be expected.

An AFM imaging study combined with Electrochemical Impedance Spectroscopy (EIS) measured the extent to which *Acidithiobacillus ferrooxidans* is able to oxidize chalcopyrite (CuFeS$_2$), one of the major constituents of ore materials [51]. *Acidithiobacillus ferrooxidans*, (formerly known as *Thiobacillus ferrooxidans*), is an acidophilic chemolithoautotrophic bacterium that primarily oxidizes metal sulfides in order to survive. AFM and EIS were used to determine open-circuit potential and electrochemical impedance of chalcopyrite, and the surface roughness of adhered *Acidithiobacillus ferrooxidans*. Within 30-60 minutes of exposure,
adhesion of the bacterial strain was detected on chalcopyrite, and after 71 hours, a complete biofilm was detected. The biofilm, including cells, biomolecules, and sulfur, served as a capacitor that allowed diffusion of molecules to and from the chalcopyrite surface.

In a study of mineral dissolution using a Gram-positive bacterium, AFM images of calcite surfaces showed dissolution pits with different morphologies, depending on the concentration of cell wall material from *Bacillus subtilis* (strain 168) that was present [52]. Dissolution pits were allowed to develop after cell walls were contacted with the mineral for relatively long time periods, from 5 min to 1 hr. Cell walls were released from *B. subtilis* by exposure to strong acid. When the concentration of equivalent functional groups from the cell wall was low (0.004 mM), and in distilled water, the dissolution pits were anisotropic. However, isotropic dissolution pits were observed when the cell wall functional group concentration was increased to 4.0 mM or with the same concentration of EDTA. An interesting point to note is that since only cell walls rather than intact cells were used here, dissolution could be linked to purely physicochemical mechanisms, rather than being a combination of physicochemical binding and microbiological effects.

The interactions between bacteria and mineral surfaces will certainly be strain specific. A study on the interactions between eight strains of Gram-negative bacteria and one Gram-positive strain revealed differences in the way each interacted with metal oxide surfaces [53]. AFM in addition to contact angle measurements and bioadhesion tests were used to characterize these bacteria. Hydrophobicity of a mineral surface had a greater effect on bacterial adhesion than did surface charge. Glass surfaces produced generally less adhesion than metal-oxide surfaces (coated with Fe). Some correlation was seen between the total free energy (taking into
account bacterium and substrate properties) and the observed number of attached bacteria to the surfaces, but low energy surfaces produced a weak correlation coefficient ($R^2=0.68$).

b. AFM to Probe Bacterial Polymers and their Relationship to Adhesion in Environmental Systems

Microbial polymers have long been recognized for their role in bacterial attachment to mineral surfaces and transport in the subsurface environment [1, 54-59]. With recent advancements in the application of AFM to microbial systems, we can now quantify polymer-surface interaction forces and characterize the elastic and mechanical properties of microbial surface polymers. The retraction portion of a typical force cycle and quantification of adhesion interactions for a bacterial cell is shown in Figure 4.

i. Polysaccharides on Bacterial Surfaces

Interactions between individual cells of either Burkholderia cepacia G4 or Pseudomonas putida KT2442, and silicon nitride cantilevers were probed in buffer [41]. Bacterial interaction forces were described in terms of an electrosteric model, due to polyelectrolyte polymers being present on the bacterial surfaces. When EPS was removed from the surface of $P. \text{putida}$ KT2442, repulsive interactions between the bacterium and the AFM tip markedly decreased.

A follow-up study further elucidated that cellulose (or related molecules) were the major polymers influencing the adhesion and interaction forces for $P. \text{putida}$ KT2442 [60]. Some samples of $P. \text{putida}$ were treated with the enzyme cellulase, to degrade cellulose molecules on the bacterial surfaces. Decreased cell-tip adhesion forces were measured for bacteria treated with the enzyme, due to the loss of cellulose and cellulose-like molecules.

Colanic acid is an extracellular polymer produced as a capsule by some pathogenic strains of $E. \text{coli}$, and is believed to allow the cells to form biofilms [61]. One study looked at mutant bacteria that over-express colanic acid by 1,000-fold (strain $E. \text{coli}$ CPS$^+$), along with
bacteria that do not express colanic acid (strain CPS\textsuperscript{−}), in comparison with the wild type (strain CPS). The interactions of these \textit{E. coli} cells (as a confluent layer attached to an AFM probe) to hydrophilic glass, hydrophobic glass, and two forms of hydrophobic silicone were quantified via AFM approach curves [62]. The CPS\textsuperscript{+} strain had less repulsion with either hydrophilic or hydrophobic glass than the wild type or CPS\textsuperscript{−} strain. The CPS\textsuperscript{+} strain showed repulsion for hydrophobic silicone, while the CPS\textsuperscript{−} strain showed attraction. An unexpected result was that the wild type also showed repulsion to the hydrophobic silicone. The AFM could be used to describe the interaction forces between these bacteria with different colanic acid productions and various surfaces.

Extracellular polymers have also been studied with respect to the bonds they can form between bacterial cells and AFM probes. Bond aging was considered for its importance in understanding the bacterial adhesion process [63]. Force measurements were made on \textit{Streptococcus thermophilus} and the time-dependence of bond aging between the bacterium and the silicon nitride tip of an AFM was measured. The bond strength increased after contact had been maintained for 100 seconds, and this phenomena was attributed to specific interactions caused by EPS on the surface of \textit{S. thermophilus}.

ii. Lipopolysaccharides (LPS) on Bacterial Surfaces

LPS on bacterial surfaces, especially for \textit{E. coli}, have been probed with AFM to reveal complex results. An early study on this topic examined the interaction forces between monolayers of \textit{E. coli} cells and the AFM tip [64]. By using a series of isogenic mutants, the authors aimed to elucidate the role of LPS length on bacterial interaction forces. Wild-type cells showed an attraction with the silicon nitride tip, while mutants with truncated LPS showed only repulsion in the approach cycles. The authors extended this work and later applied the cell probe
technique to study the interactions between *E. coli* and glass, hydrophobicized glass, polystyrene, and Teflon [65]. Bacterial adhesion could be partially explained by hydrophobic and electrostatic interactions, and steric interactions also played a role in systems with more complex LPS layers. For example, the more hydrophobic and more charged strain D21f2 (missing some of its carbohydrate moiety) was more repelled by the hydrophilic and negatively charged surfaces (mica and glass). Changing the ionic strength of the solution confirmed that the effect was in part electrostatic. An attractive force from AFM approach cycles was observed between D21f2 and the more hydrophobic surfaces (polystyrene and Teflon). The *E. coli* strain with a complete LPS layer (D21), that was also more hydrophilic and less negatively charged, showed attraction to glass and mica in AFM approach curves. These latter interactions were not affected by ionic strength, suggesting that the interaction is not electrostatic in nature.

Compared to measurements on the confluent layers of *E. coli*, different results were seen when force measurements were performed on isolated *E. coli* cells, interacting with a silicon nitride AFM tip. AFM approach curves on the center of *E. coli* D21, D21f2, and JM109 were identical and insensitive to ionic strength, suggesting that neither electrostatics nor steric interactions were important in distinguishing interaction forces for these bacteria [66].

In another investigation of *E. coli* JM109, removal of some portion of the LPS through treatment of cells with EDTA decreased the steric repulsion observed from AFM approach curves, and also decreased adhesion forces between the bacteria and silicon nitride from AFM retraction curves [19]. These effects were also sensitive to ionic strength, with LPS removal showing the same effect on adhesion and interaction forces (namely, decreased adhesion forces and reduced repulsion forces) in either water or buffer.
iii. Modeling Conformational Properties of Macromolecules on Bacterial Surfaces

One approach to provide more detailed information on bacterial polymers is to use force spectroscopy in combination with models from the polymer literature. Mechanical properties of bacterial polymers can be quantified by applying entropic based, statistical mechanical models to AFM retraction curves. The five models used to quantify polymer conformation are the Gaussian chain (GC), freely-jointed chain (FJC), wormlike chain (WLC), extensible freely jointed chain (FJC+), and extensible wormlike chain (WLC+) models, as reviewed in [67]. Van der Aa et al. [68] were the first to apply these models to the macromolecules on a microbial cell. The FJC model was used to characterize the Kuhn length and segment elasticity of macromolecules on the surface of *Aspergillus oryzae*. The values they estimated from fitting the FJC model to the force-extension curves were in agreement with literature values for the elastic deformation of dextran and amylase. Therefore, the authors concluded that stretching of cell surface polysaccharides explained the measured elongation forces.

Application of polymer models to stretching events on bacterial cells was also performed. The FJC model was used to estimate the elasticities of biopolymers on the surface of *P. putida* KT2442, as a function of solvent chemistry [69]. The range of conformations of the biopolymers as a function of salt concentration in solution could be examined. A transition in the flexibility of the biopolymers (as estimated by the segment length values) was observed as the salt concentration increased from that of ultrapure water to 0.01 M KCl [70]. A range of segment lengths was predicted, even for the macromolecules on bacteria from the same culture, reflecting the heterogeneity of these extracellular polysaccharides [18]. The conformation of the biopolymers was then related to their adhesion forces with a model surface [23]. For *P. putida* KT2442 in low salt solution, the polysaccharides were rigid and highly extended from the bacterial cell, resulting in high steric repulsion in AFM approach curves and low adhesion with
silicon nitride. When the bacterial cells were in a higher salt solution, the macromolecules on the surface became more flexible and coiled on the surface. Under the latter conditions, repulsive steric interactions with silicon nitride were diminished in AFM approach curves, and higher adhesion forces (with silicon nitride) were measured from the retraction portions of AFM force cycles.

c. AFM as a Detachment-Force Technique

Measurement of the force required to detach an attached bacterium from a surface represents a novel application of the AFM, and has been used in some cases [71]. The initial events of adhesion of *Entrococcus faecalis* to commonly used biomaterials, polyurethane (PU), polyamide (PA), and poly(tetrafluoroethylene) (PTFE), were probed [72]. A silicon nitride cantilever was used to measure bacterium-substrate interactions. A special method was applied, in which bacterial detachment was initiated by increasing the force of the AFM tip. The force required to detach *Entrococcus faecalis* varied for each polymer, with average detachment forces ranging from 19±4 nN for PU, 6±4 nN for PA, and 0.7±0.3 nN for PFTE. In addition, the authors observed that bacteria adhered in clusters on PU, but as individual cells on PA. Adhesion to PFTE was minute, and easily detachable. This technique has not yet been applied to a bacterial detachment study for a mineral grain surface, but perhaps this will be a useful approach for future researchers to pursue.

3. Limitations in the Application of AFM to Measure Microbial Adhesion at Interfaces
   
a. Determination of the Cantilever Spring Constant

Although the AFM is an exceptionally useful tool for the study of microbial interfaces, some cautions must be applied in the data interpretation. One of the main limitations in force microscopy is that it can be difficult to accurately measure the spring constant of an AFM tip.
Since force magnitude is the cantilever spring constant times the deflection, all quantitative force measurements require an accurate estimation of the cantilever’s spring constant.

One of the easiest techniques to measure the spring constant for routine AFM users is that of Cleveland et al. [73], in which the cantilever’s length and width are either measured or estimated by the manufacturer, and used in combination with a user-made measurement of the cantilever’s resonant frequency. However, recent studies suggest that agreement between manufacturer’s values and measured spring constants is poor, and reproducibility is also problematic with these cantilevers and geometric methods, especially for silicon nitride cantilevers [74]. All cantilevers suffer from some uncertainties in force calibration, as noted in a recent review by Burnham et al. [74].

An improved method for calibrating cantilevers has been proposed by Burnham et al. [74] that is both more precise and accurate. This method relies on acquiring a thermal power spectrum of a cantilever, which can be obtained by collecting thermal fluctuations as a function of time on a cantilever that is hanging in free space. The fluctuations are Fourier-transformed into a power spectrum and the data are fit to a simple harmonic oscillator model. This method has been very successful with silicon cantilevers over a wide range of spring constants, and silicon cantilevers can be used to probe bacterial interactions in both air and liquids. Unfortunately, this method is more difficult to apply for the silicon nitride cantilevers that are commonly used in biological AFM research, due to their lower quality factors. Hopefully, more efforts in this area will lead to better methods for spring constant determination for those cantilevers, as well.

b. Immobilization of Bacteria for AFM Measurements
Researchers have used a variety of methods for immobilizing bacteria for AFM studies. Unfortunately, few studies have systematically addressed the way in which the cell preparation method affected the interaction force profiles.

In an AFM imaging study, immobilization of bacteria on gelatin-coated mica and poly-L-lysine coated mica were compared [43]. Doktycz et al. examined *Staphylococcus aureus*, *E. coli*, and *Rhodopseudomonas palustris* CGA009 in air and under liquid using a silicon cantilever. Poly-L-lysine coatings of varying molecular weights were tested, and they resulted in the bacteria adsorbing poorly to mica. The highest molecular weight studied (300 kDa) was able to adsorb bacteria from minimal media, but did not hold bacteria to the mica well when they were adsorbed from a distilled water solution. Although good immobilization could be obtained with gelatin, products from different manufacturers produced widely varying results.

The use of glutaraldehyde in the immobilization of *E. coli* for AFM measurements was tested to some degree. A subgroup of bacteria was treated with the fixative, and all were physisorbed to polyethyleneimine-coated glass before probing with silicon nitride tips. The cantilever deflection observed from measurements on *E. coli* D21 was dependent on the presence of glutaraldehyde [75]. Adhesion forces (from retraction curves) were not reported in this study, but may also be expected to vary when cells are treated with glutaraldehyde. Although not specifically tested, glutaraldehyde treatment would be expected to impair bacterial viability, since, for example, this chemical is a commonly used disinfectant in the dental and biomedical device industries [76, 77].

A recent study examined three different bacterial immobilization techniques for a Gram-negative soil bacterium, *Klebsiella terrigena* ATCC 33527 [78], and both approach and retraction curves were considered. The three techniques included mechanically trapping bacteria
in a polycarbonate filter, adsorbing the negatively charged bacteria on a positively charged surface (coating with poly-L-lysine), and fixing the bacteria on the AFM tip using glutaraldehyde. The bacteria were cultured in nutrient broth and isolated using high force centrifugation (10,000 x g for 5 min.). A silicon nitride cantilever was used to determine force-distance cycles for the first two preparations, with the bacterial probe used to interact with a silicon nitride sheet for method three. The authors found differences in the force profiles for the three methods, with results suggesting that glutaraldehyde treatment decreased repulsive interaction distances due to cross-linking of proteins and amino acids in the peptidoglycan layer. This treatment imparts rigidity to the cell membrane that probably alters the way that surface macromolecules interact with the probe. Immobilization of cells on the tip gave rise to higher forces of adhesion from AFM retraction curves, which were attributed to the greater number of cells interacting with the substrate under those conditions. The authors also speculated that physical adsorption can cause higher adhesive forces in retraction curves due to the possible release of EPS into solution. These authors concluded that mechanical trapping was preferable, as it does not require chemical treatment or surface modifications.

While these studies are important steps in understanding how microbe preparation can affect the measured force profiles, much more work will be needed to fully resolve these issues. Other methods of cell immobilization have not been systematically compared. Some of these other methods include the technique of Lower et al., in which viable bacteria were immobilized onto an AFM tip and used as a bioprobe (without glutaraldehyde treatment) [79], chemical fixation of bacteria to a glass slide [21], or physical attachment of cells to other types of coated surfaces, such as self-assembled monolayers [40].
As a further note on methods to attach bacteria, some success has been observed with the use of tipless cantilevers to immobilize single bacterial cells. *Enterococcus faecalis* cells could be individually attached to the end of an aminosilane-coated (5% 3-aminopropyltriethoxysilane) AFM (tipless) cantilever by lowering the tip into solution until only 1 or 2 cells attached [46]. Field emission scanning electron microscopy was then used to verify the exact number and position of bacteria on the tip before and after AFM experiments. An earlier study immobilized single yeast cells, *Saccharomyces cerevisiae*, to a silica sphere that had been glued to a tipless cantilever [45, 80]. The yeast cell was attached to the silica sphere using a chemical glue, such as polylysine or cyanocrylate.

Different microbial strains may give rise to varied behavior depending on their surfaces characteristics, such that the “best” immobilization technique may be strain and experimentally-dependent.

B. Optical Tweezers

Optical tweezers represents a technique for making direct force measurements. An advantage of this technique is that very good force resolution can be achieved.

1. Overview of Optical Tweezers

The intensity of laser light can be used to move small particles, ranging from atoms and molecules to small dielectric spheres in the size range of tens of nanometers to tens of micrometers, and even to viruses, single living cells, and organelles within cells [81]. In 1986, Ashkin and colleagues developed a technique of one-beam gradient optical trapping and manipulation of small particles based on the forces of laser radiation pressure [82], which is known as optical tweezers (Figure 5).
Two theoretical models are commonly used. For particles much larger than the wavelength of the laser, besides reflecting part of the light, some light is refracted due to the difference in indices of refraction between the particle and its surrounding medium. The photons in the laser are redirected due to the acceleration or force exerted on them by the particle. According to the Newton’s third law of motion, equal and opposite reaction forces will exert on the particle which can be decomposed into a propulsive force in the direction of the light propagation (scattering force) and an attractive force along the intensity gradient perpendicular to the laser axis (gradient force) [83]. As a result, the particle is pushed to the higher flux of photons near the focus [84].

For particles much smaller than the wavelength of the laser, the particle will oscillate in the electric field, with the oscillations following a Gaussian distribution. Shortly after the induced electric dipole moment caused by the laser’s electric field forms, the polarized particle moves toward the location of highest intensity (the focus) driven by the laser’s electric field force [85]. By balancing the force of the photons with gravitational forces acting on the particle, the particle can be trapped and manipulated in a stable state.

The trapped particle’s geometry and the manipulation force (up to 150 picoNewtons [86]) given by the optical tweezers cover a range that is applicable for many biological processes, although the force may be lower than the maximum adhesion forces for certain applications. The reproduction of trapped E. coli and yeast cells was observed for the first time in 1987 [87], proving that optical tweezers can trap and manipulate living cells under damage-free conditions. This breakthrough can be considered a milestone marking the beginning of the application of optical tweezers in biology. Comprehensive reviews are provided elsewhere [81, 88].
2. Optical Tweezers for Measuring Adhesion Forces in Bacterial Systems

Besides trapping and manipulating the bacteria, optical tweezers also can be used to measure the adhesive forces between bacteria and surfaces. As a relatively new experimental approach, optical tweezers have only been applied in limited cases to study bacterial adhesion.

When the bacterium is stably trapped by the optical tweezers, the forces are in an equilibrium state. After introducing an adhesion event by moving the bacterium or substrate towards the other, a new force equilibrium forms. Next, an external force is applied to break the adhesive interaction between the bacterium and surface until detachment occurs, thereby quantifying the force required for detachment. The interested surface can be a bacterium or other surface of biological relevance, such as a substrate coated with proteins, ligands, or receptors.

The theoretical models described above do not work well when the laser wavelength and the particle size are similar. In practice, the strongest trapping of cells occurs when the laser wavelength is similar in size to the cell, and this is also the range where the potential for cell damage would be minimized. The two theoretical models in use are not yet able to provide good agreement with experiments in this intermediate regime of the particle size being similar to the wavelength [89].

An alternate approach based on empirical force calibration has been successful when the particle displacement due to the interaction is smaller than half the particle radius, since within the focal region where the particle is trapped, it may behave like a spring [90]. The displacement can be detected by an inverted microscope modified to merge the laser beam [91]. The “spring constant”, referred to as the trap stiffness, is determined by a force calibration prior to each force measurement. Two commonly utilized methods are based on either the viscous drag force or Brownian motion (thermal fluctuations).
In the first procedure, the viscous drag force is given by Stoke’s law, calculated from physical parameters of the system. Therefore the applied force can be calculated and related to the measured displacement. In the second approach, the trap stiffness can be modeled by measuring the frequency spectrum of positional fluctuations of the trapped particle due to random collisions with the fluid molecules [92]. Detailed discussion on the calibration procedures can be found elsewhere [91, 93].

Optical tweezers were used to quantify the adhesive force between a single bacterium (Staphylococcus epidermidis or Staphylococcus aureus) and the protein fibronectin (Fn), when the latter was coated onto a 10-μm diameter polystyrene bead [94, 95]. S. aureus strains were selected that had mutations leading to the expression of fibronectin-binding proteins with various degrees of modification. The optically trapped cell was brought into contact with the surface of an Fn-coated bead. After verifying the occurrence of the adhesion event, the trapped bacterium was either immediately moved back to the original trapped position or was held for 20 seconds before being moved, in order to study the time dependence of the adhesive interaction [94]. During the process, the displacement signals were recorded by the photodiode and then were converted to force using the stiffness constant from the calibration procedure. Due to the formation of multiple bonds, the force per adhesion event differed from measurement to measurement. However, the forces occurred as approximate integer multiples of an 18-pN base value for S. epidermidis [94]. The authors inferred that this represents the single-bond rupture force. Similarly, the forces ranged between 15 and 26 pN for the interaction of S. aureus with the protein-coated bead, depending on the extent of mutation in the fibronectin-binding protein [95].

In another bacterial adhesion study, Fallman et al. used the optical tweezers to measure the binding force between the E. coli P pili adhesin and its Galα1-4Galβ (galabiose) receptor
disaccharides, which are present on the surface of uroepithelial cells [91]. This was a pioneering study, since the direct characterization of the adhesive interactions between the P pili and the receptor had not been previously performed. In another study on the same system, the mechanical properties of *E. coli* P pili were probed [96]. Based on the force measurements, the authors learned that the detachment of a pilus is not dependent on its length, and that P pili can elongate up to 7 ± 2 times their unstretched length.

In brief, optical tweezers are a very new and useful tool in the study of bacterial adhesion, especially for binding force measurements. Since few systems have been probed, the potential exists to further exploit this technique for exploring specific and non-specific bacterial adhesion forces. This technique will likely become very important in future studies of bacterial adhesion in the environment. Some important limitations to keep in mind are that this technique works best for spherical particles within a narrow size range, so it may prove more difficult for elongated or rod-shaped bacteria. Also, light has to be able to pass through the trapped sample, limiting its applicability in some cases.

### III. Indirect Methods for Determination of Bacterial Adhesion Forces

Techniques based on total internal reflection, while not direct force probes, are still very useful due to their ability to detect individual bacterial cells. The Quartz Crystal Microbalance represents another type of indirect technique, in which information on cellular adhesion forces can be obtained for cells or cellular biomolecules.

#### A. Total Internal Reflection Microscopy (TIRM)

1. Overview of TIRM

   Total internal reflection microscopy (TIRM) is a technique based on total internal reflection that can also be used to study bacterial interactions with surfaces (Figure 6A). When
light travels from a medium of higher refractive index to the other medium of lower refractive index, the ray of the light is both reflected and refracted until total internal reflection occurs, where the angle of incidence exceeds the critical angle. Under this condition, although the incident light beam is totally internally reflected at the interface, electromagnetic energy in the form of an evanescent wave appears in the medium with a lower refractive index (the liquid), and decays parallel to the surface (normal to the interface), as an exponential function of distance from the interface [97].

When a particle is in the pathway of the evanescent wave, it will absorb energy from the electromagnetic wave and scatter light (Figure 6B) [98]. Prieve and Walz. found that the scattered intensity by a micron-sized dielectric spheres dispersed in an aqueous medium decays as an exponential function of the separation distance between the sphere and the reflecting interface [99]. Under the influence of the forces between the particle and the substrate, including van der Waals forces, electrostatic forces, steric repulsion, and gravity, the particle will stay at a stable distance from the interface, in order to minimize its potential energy. Due to Brownian motion, the particle fluctuates around the equilibrium distance. Since the motion causes fluctuations in the scattered light intensity, a measurement of this latter property allows for the distance between the sphere and the plate to be calculated. Use of appropriate models allows the potential energy profile to be developed. The mean potential energy of interaction between the microscopic sphere and a flat plate in aqueous medium can be directly measured with a resolution of about 0.1 kT (or for forces as low as 0.01 pN) and with a distance resolution of 1 nm [98, 100]. An extensive review of the theory and principles of TIRM is found in ref. [101]. Forces acting on Brownian particles can be as small as $10^{-14}$-$10^{-11}$ N, which can be resolved with TIRM, but not AFM [101-103].
2. Biological Applications of TIRM

An early TIRM study probed the long range attractions between a protein (protein A) and immunoglobulin (IgG) from various animals (rabbit, horse, goat), showing species-specific attractions that were correlated with the known binding forces between the protein and antibody [104].

A next logical step was extension of TIRM to cellular systems. Robertson et al. used TIRM as a tool to investigate the nonspecific interactions between a glass plate and various biological entities, including cells (red blood cells and leukocytes) and liposomes [105]. The low refractive indices and flexible membranes hamper the practical use of TIRM on cells, making it difficult to measure absolute separation distances. As an alternate method to probe biological particles, Robertson and Bike created model cells, consisting of polystyrene beads coated with phospholipids [106], used to mimic the composition of the cell membrane composition while satisfying the optical requirements of TIRM. By measuring the distribution of the scattering intensity, a histogram of separation distances (Boltzmann distribution) was used to give the potential energy profile for the interactions of the model cells with glass. TIRM can be used to screen potential stabilizing molecules in the application of liposomes and biomimetic materials [106].

Another study used a form of TIRM, which was essentially optical tweezers combined with evanescent wave light scattering, to investigate the adhesion forces between two variants of *Staphylococcus aureus* and a glass plate [107]. The optical tweezers component was used to hold the bacterium in place and move it at the nanometer scale. Particle-surface interaction profiles were calculated with very high resolution, reportedly at the femtoNewton level [108]. *S. aureus* Smith diffuse strain, which has a polysaccharide capsular layer, experienced strong steric
repulsion with the glass plate. A non-encapsulated mutant strain, \textit{S. aureus} Smith compact, experienced only attractive forces for the glass [107].

3. Related Technique- Total Internal Reflection Microscopy Aqueous Fluorescence (TIRAF)

Alterations to the basic TIRM principles have been applied in order to achieve better resolution and to make the system more applicable to biological studies. If a fluorescent marker, such as rhodamine green, is added into the liquid medium, it can be excited by the evanescent wave and emit fluorescence, which takes place in the vicinity of the interface. The particles or cells close to the interface in the liquid medium can be easily elucidated in the fluorescence image since they do not take up the liquid or the fluorescent maker, forming the basis for total internal reflection aqueous fluorescence (TIRAF) microscopy [109].

Gingell et al. first described TIRAF microscopy and quantified the distance as a function of the ratio of the light intensity at the location of a spherical particle to the light intensity of the background at the same location. They also built a theory to calculate the thickness of the fluorescing layer and the surface contour (the membrane/substratum contact regions of adherent cell) of the particle [109].

\[ F = \frac{I(z_i)}{I_\infty} \] (1)

where \( I(z_i) \) and \( I_\infty \) are the light intensities in the presence and absence of the particles in the medium respectively. In the experiment, the ratio (i.e. F) is measured and the distance between the particle and interface is calculated based on Gingell’s equations, described elsewhere [109, 110]. Cells within \( \sim 100 \) nm of the surface can be detected [111].

Taking advantage of the high spatial and temporal resolution given by TIRAF, Geggier and Fuhr investigated the long-term (up to 585 min) dynamics of cell adhesion patterns for L929
mouse fibroblasts, and observed the formation of a single adhesion cluster that usually takes place within several seconds [112].

4. TIRAF in Bacterial Adhesion Studies

TIRAF has some unique applications in bacterial interaction studies, since unlike AFM and TIRM, TIRAF does not require immobilization of the cell or particle to be probed. In addition, since the fluorescent label is in the aqueous phase instead of on cell, the label-free approach eliminates the risk of damaging the cell surface or altering cellular properties. TIRAF is well suited for measuring separation distances between a bacterium and a plate, or for real-time imaging of the behavior of living bacteria under the influence of the interface.

Vigeant et al. used TIRAF to measure the nanometer-scale separation distances between *E. coli* and a glass plate [110]. The behavior of strain *E. coli* HCB437, a smooth-swimmer, and a non-motile variant (*E. coli* HCB137, which lacks flagella), was investigated. Both the motile and non-motile strain could be observed in the region < 80 nm from the surface. When the ionic strength was decreased, they observed that the motile cell was further from the quartz glass plate. This work demonstrated the utility of TIRAF for probing motile bacteria, since immobilization was not required. In a subsequent study, the separation distances between these two *E. coli* strains and glass surfaces with varying properties were probed [113]. Positively-charged glass and hydrophobically-coated quartz were also used as substrates in the TIRAF investigations. Cellular interactions with the surfaces could be described as occurring in three compartments: bulk, near-surface (bulk), and near-surface (constrained). The cells in the bulk did not “feel” the effect of the surface, and were apparently not affected by DLVO-type interactions. The near-surface (bulk) cells experienced hydrodynamic interactions, while the near-surface (constrained) cells experienced both hydrodynamic and surface (i.e. DLVO) interactions. During the
continuous process of trying to improve the applicability of TIRAF to bacterial adhesion studies, Smith et al. noted that the relative separation distance between *E. coli* and quartz can be determined with an uncertainty of ~12 nm \[114\]. They also noted that distances estimated by TIRAF may be overestimated by as much as 26 nm.

In brief, TIRM and TIRAF techniques are useful in measuring bacterial-surface separation distances and in calculating the potential energy for bacterial-surface interactions. Limited bacterial systems have been investigated to date, suggesting that there is ample opportunity to expand bacterial adhesion studies with these methods.

B. Quartz Crystal Microbalance (QCM)

1. Overview of QCM

   As a simple and high-resolution mass sensor, the Quartz Crystal Microbalance (QCM) finds many applications in biology and biotechnology, including molecular recognition \[115\], cell immobilization \[116\], and in drug delivery \[117\]. A detailed review on the application of QCM in biology is found in reference \[118\].

   QCM is an extremely sensitive mass sensor, capable of measuring mass changes in the nanogram range (Figure 7) \[119\]. The working mechanism is based on the fact that a piezoelectric crystal is caused to oscillate at a characteristic frequency, which is determined by the piezo material and the manner in which the crystals are sectioned. The quantitative relationship between the mass change and the induced frequency shift was put forth by Sauerbrey in 1959 \[120\].

   \[
   \Delta f = \frac{-2 \cdot \Delta m \cdot f_0^2}{A \cdot \sqrt{\mu_q \cdot \rho_q}}
   \]  
   \[\text{(2)}\]
where $\Delta f$ is the measured resonant frequency shift (Hz), $\Delta m$ is the mass change per unit area (g/cm$^2$), $f_0$ is the intrinsic resonant frequency of the crystal, $A$ is the electrode surface area, $\rho_q$ is the density of quartz (2.648 g/cm$^3$), and $\mu_q$ is the shear modulus of quartz ($2.947 \times 10^{11}$ g/cm$\cdot$s$^2$).

QCM can be operated in the gas or liquid phase. For biological systems, it is much more desirable to operate the QCM in liquids. QCM still has the extreme sensitivity to mass deposited on its surface and has the quantitative relationship between the mass change and the frequency shift when completely immersed in a solution [121]. However, when the QCM is applied in solution, both the bound mass and the solution properties (i.e. density and viscosity) contribute to the decrease of the frequency.

The common method of implementation is to immobilize a polymer or material of interest onto the QCM sensor, and bring it into contact with cells or biological samples in solution. By the recognition and interaction between materials of interest, the adsorption is monitored. Adsorbed material causes an increase in mass, which is detected as a decrease in frequency.

2. QCM in Biological Systems

Some difficulties arise in performing QCM for biological systems. The Sauerbrey equation is valid only when the added mass is rigidly deposited on the electrode surface, which is not the case for biological cells. Also, since QCM senses both the mass of absorbed film and the mass of liquid coupled to the system, any bound or trapped water can cause significant artifacts [122]. The absorbed mass values calculated from the frequency shift are hence generally higher than the real attachment of the biological materials of interest. Further, the QCM sensor is
sensitive to changes in the local viscoelastic environment and may be affected by changes in bulk fluid properties such as viscosity and/or pH [123].

Thus far, QCM has been applied to measure the adhesion of several types of eukaryotic cells, including epithelial, endothelial, and human platelets [123-129]. However, the inapplicability of the Sauerbray equation to cell attachment was demonstrated by comparing bound cell mass from the QCM with cell mass measured based on radiolabeling methods [130].

QCM can be particularly useful for studying the time-dependence of adhesion events, which is not as conveniently done with techniques like AFM. For example, the time dependence of the adhesive interaction between an endothelial cell and the QCM surface was probed for up to 24 hours [125, 127]. Interestingly, there was a 10 min lag between the appearance of endothelial cells at the QCM surface and the earliest changes in frequency of the quartz crystal [125], suggesting that this amount of time is needed for the cell to start forming attachments with the surface. Steady-state attachment of cells was reached after about 20 hours.

3. QCM for Bacterial Adhesion Studies

The QCM has seen less use in bacterial adhesion studies, although this represents another area where the potential exists to further exploit this measurement technique. An early study used QCM to monitor the development of biofilms on-line [131]. More recently, *E. coli* was investigated in a series of QCM experiments [132-135]. The adhesion of type-I fimbriated *E. coli* was compared with that of a mutant strain lacking in fimbrial expression, as a function of solution ionic strength [132]. The non-fimbriated cells were better able to contact the surface due to increased available surface area. Fimbriated cells maintained a more flexible contact with the surface. Also, the time-dependence of the adhesive interaction could be probed. Another study demonstrated that the number of *E. coli* cells attaching was decreased when the Cpx-
signaling pathway was disrupted. This regulatory process has been linked with responses to stress that lead to adhesion and invasion in the body [135]. Another set of experiments examined the role of the outer membrane protein OmpX in the adhesion of *E. coli* with type 1 fimbriae [133]. Deletion of OmpX was linked to increasing the contact of fimbriated bacteria with a surface. However, the non-fimbriated strains were also affected by the deletion of OmpX, in which their surface contact was decreased. The authors concluded that the gene coding for OmpX affects cell surface structures that mediate bacterial adhesion and contact with surfaces, including type 1 fimbriae, flagellae, and exopolysaccharides.

**IV. How to Relate Adhesion Measurements Based on Different Techniques**

In developing these various methodologies, it will be important to consider the relationship between bacterial adhesion measurements made with alternate techniques [136]. While direct measurements of adhesion forces can be achieved, such as with AFM or TIRAF tools, these measurements must still be related to the conditions under which bacterial attachment occurs in the various applications. Hydrodynamic conditions and the time-scales of the measurements are often different between the force measurement techniques and the attachment assays.

Although the AFM is essentially a static measurement under conditions of no shear, adhesion measurements have shown at least qualitative agreement with microbial attachment in systems where there is flow or other controlled hydrodynamics. For example, The adhesion forces measured by AFM between *Bacillus mycoides* spores and glass or modified glass were in qualitative agreement with the attachment results from a spinning disk [137]. Several studies have examined the relations between AFM adhesion measurements and bacterial retention in packed columns, as will be discussed in the next section.
V. Do Measurements of Adhesion Force Correlate with Microbial Retention in Environmental Systems?

A. Quantifying Bacterial Retention/Transport in Porous Media

It is desirable to correlate nanoscopic properties of bacteria gathered from AFM measurements with the macroscopic behavior of the bacteria in environmental systems. The transport of bacteria through packed soil columns is relatively easy to measure in the laboratory, and can be used to quantify the attachment of bacteria to soil. The porous media can be glass beads, silica sand, natural soil, etc. Bacterial solution is usually passed through the column with a pump, and the effluent material is collected, often as a function of time. The attached bacteria on the porous media or in the liquid phase can be measured using a number of techniques, such as using a radiolabel assay [138-140], growing cells from effluent samples on agar plates [141], or counting cells directly under a microscope [142].

A convenient way to quantify the transport of bacteria through packed columns is through use of the collision efficiency, the fraction of bacterial-collector collisions that result in successful attachment. The one-dimensional colloid filtration equation proposed by Yao et al. [143] describes the collision efficiency (\( \alpha \)) as

$$\alpha = \frac{-2d_c \ln(1 - F_R)}{3(1 - \theta) \eta L}$$

(3)

Where \( d_c \) is the collector diameter, \( \theta \) is the porosity of the medium, \( L \) is the length of the column, \( \alpha \) is the collector efficiency, and \( F_R \) is the fraction of bacteria retained in the packed column. If bacterial retention on the soil is to be calculated from a mass balance on the system and measuring the bacterial concentration in the liquid phase, then typically a steady-state breakthrough profile must be obtained. The steady-state breakthrough concentration of bacteria, \( C \), normalized to the influent concentration of bacteria entering the column, \( C_o \), is used to replace \((1-F_R)\) in equation 3.
The collector efficiency, which describes the fraction of approaching bacteria that make contact with the collectors, can be modeled by accounting for all of the transport mechanisms that the bacteria will experience, i.e. diffusion, interception, gravitational settling, van der Waals forces, etc. The most commonly used model has been the semi-empirical approach of Rajagopalan and Tien [144], although two alternate models have recently been proposed [145, 146].

B. Correlations of Bacterial Transport in Porous Media with AFM Force Measurements

Several studies have examined the comparative relations between bacterial retention to porous media and AFM adhesion forces. For example, the retention of *P. putida* KT2442 was quantified in batch and column assays, with glass and quartz media [147]. Some cells were treated with cellulase to break down cellulose and related macromolecules on the bacterial surfaces. AFM adhesion force measurements complemented the collision efficiencies obtained from the batch and column studies, and similar trends were observed with respect to cell preparation. Cells treated with cellulase had decreased collision efficiencies that were directly correlated with decreased adhesion force measurements from the AFM experiments.

Another example study considered the adhesion/retention behavior of *Burkholderia cepacia* strain G4, as well as a non-adhesive mutant form of this microbe, strain Env435 [148]. Strain Env435 has a different LPS structure than the parent strain, and is missing the O-antigen [149]. Bacterial retention in packed columns was quantified in column transport tests, with glass beads or hydrophobically modified glass beads as the column packing materials. The hydrophobically-derivatized surfaces presented higher collision efficiencies and higher overall adhesion forces from AFM studies than the bare surfaces. Strain G4 was not as affected by the hydrophobic surface modification as the mutant strain Env45, perhaps because the longer LPS molecules prevented silane from interacting with the bacterial surface.
Where data were available, we have compared the AFM-derived adhesion force measurements (from retraction curves) with measurements of the bacterial collision efficiencies from column assays (Table 3). For consistency, the collision efficiencies were calculated using the Rajagopalan and Tien model for the collector efficiency, although alternate mechanisms for summing contributions to bacterial collisions have been recently proposed [145, 146].

Due to the widespread appearance of steric interactions in AFM approach curves on microbial cells, we have found that adhesion force measurements from AFM retraction curves are more relevant in explaining bacterial attachment behavior [38]. However, AFM approach curves are still very useful in providing information on the properties of surface polymers on the microbial surfaces [40]. The available AFM data used either silicon nitride or glass microspheres for probing bacterial interactions, while the column data typically used quartz media. We previously showed that silicon nitride and quartz data are well correlated. For example, the attachment of *E. coli* JM109 to quartz directly correlated with AFM adhesion force measurements with silicon nitride [19], and energy profiles based on DLVO calculations between *E. coli*-quartz or *E. coli*-silicon nitride were identical [19]. Both quartz and silicon nitride have zeta potentials of -16 mV under similar conditions [150, 151], and so the profiles will be identical when electrostatic interactions dominate the DLVO profiles. We would expect differences in the Lifshitz-van der Waals (L-vdW) interactions between the bacterial cells and the two surfaces, but for the typical environmental conditions studied here, L-vdW forces were much smaller than the electrostatic components.

A direct correlation was observed between the adhesion force and the collision efficiency, when including all the microbial strains together (Figure 8). It appears that different strains or species of microbe may each have their own correlative relation between the adhesion
force and the collision efficiency, but the low number of data points available for some strains presents us from developing a more definitive relationship.

These results imply that AFM adhesion force measurements (i.e. from retraction curves) are very useful in predicting bacterial transport in porous media, although quantitative agreement cannot yet be obtained. One possible explanation for the lack of a better correlation is that the retraction curve data may be predicting the results of reversible bacterial attachment, while the colloid transport models most commonly used account only for irreversible attachment.

Further, a limitation of this analysis is that we cannot yet explain the interactions from a first-principles approach. In one study, the adhesion forces from AFM measurements on Aureobasidium pullulans were compared with attractive forces calculated from a modified van der Waals model that accounts for the roughness of the sample [38]. In this case, to model the interactions between A. pullulans cells and a silicon nitride AFM tip, the surface roughness of the microbial cell was used to provide characteristic length scales, rather than the radius of the microbe itself. In this case, the “rough” van der Waals model [152, 153] could predict trends in adhesion forces for cells from different growth phases or treated with varying protocols. The magnitude of the model-predicted forces, however, was approximately double the forces from the AFM measurements. Further work is directed at developing better quantitative explanations of AFM adhesion force data in the context of bacterial attachment and adhesion.

One very interesting approach towards the quantification of bacterial attachment based on AFM data has been brought forth by Cail and Hochella [46, 154]. The collision efficiency (termed “sticking efficiency” in this work) was derived on a theoretical basis from AFM approach curves. Their approach is to take an averaged force profile from numerous approach curves, and integrate this over the separation distance to determine the interaction energy. This
energy is used in the interaction force boundary layer (IFBL) model of Spielman and Friedlander [155], to determine the sticking efficiency of the colloidal particle. Sticking efficiencies for polystyrene particles interacting with silica glass (collectors) were calculated from the AFM data, and also compared with values based on DLVO calculations. Both the IFBL and DLVO models yielded very low sticking efficiencies at pH 6-7 and an ionic strength of 0.05 M, on the order of \(10^{-48} - 10^{-26}\) and \(10^{-142} - 10^{-32}\), for the IFBL/AFM and DLVO methods, respectively. Cail and Hochella reported that literature values of sticking efficiencies measured in column transport experiments at similar pH and ionic strength values are much higher, on the order of \(10^{-3} - 10^{-1}\). The reasons for the discrepancy comprise a number of factors, such as errors in estimating the absolute separation distance with the AFM, long-range repulsive forces observed with AFM, roughness, heterogeneity, surface polymer effects, the assumption of primary minimum deposition, and also inaccuracies in calculating the experimentally-derived sticking coefficients. At lower pH values, the agreement between the IFBL/AFM and empirical sticking coefficients was better.

This work on microspheres has also been extended to systems involving bacterial cells, namely *Enterococcus faecalis* [46]. Again, the authors noted discrepancies between DLVO-calculated sticking efficiencies and those calculated from AFM interaction force profiles, with those calculated by DLVO theory being many orders of magnitude lower, and being very far from agreement with empirical values. Although this technique has not yet provided agreement between empirically-obtained bacterial sticking coefficients and energy profiles obtained from the AFM, it represents an important step towards providing a theoretical framework for the direct prediction of bacterial transport from AFM data.
VI. Conclusions and Implications for Future Work

Few studies have addressed the way bacteria from different environments behave in terms of adhesion, but thus far, it does not appear that a unifying theory can be created to explain bacterial adhesion in relation to bacterial physicochemical properties. One such study compared the adhesion of bacteria from different origins (marine with biomedical), using both AFM and attachment studies (to polyurethane) [156]. Bacterial strains were chosen with a range of surface free energies. Interestingly, adhesion of the medical bacteria decreased with increasing surface free energy, whereas the marine strains showed the opposite behavior. The authors concluded that a general similarity or trend between differing bacteria does not normally exist, although bacteria from other environments should also be examined for comparing their behavior.

Although numerous experimental techniques are available for conducting measurements of bacterial adhesion at environmental interfaces, all have certain limitations or disadvantages, that must be taken into account depending on the researcher’s specific needs (i.e. Tables 1 and 2).

Work involving single-cell and single-molecule techniques, especially for techniques in which forces can be directly measured, such as the AFM, show great promise. Maurice et al. noted in 1996 that the time was right to apply the AFM to complex biogeochemical processes [49]. We can reiterate the point nearly ten years later, and note that the samples are still complicated and much work needs to be done in order to gain a better understanding of microbial adhesion at environmental interfaces.

Some parameters that would be extremely helpful in describing bacterial adhesion still cannot be measured well. The theoretical framework for calculating interfacial free energies provided by the models of van Oss et al. are somewhat underutilized (or not utilized in the best way) in this field because we do not have a good method for measuring the hydrophobicity of microbes. Several studies have demonstrated problems with measuring the contact angle of
dried microbial lawns, ranging from lack of reproducibility to lack of relevance since surface macromolecules are dried and reconfigured in the filtration process.

Many biological considerations are not yet fully appreciated. The next stage of bacterial adhesion research will likely involve studies that address the relationships between inner cellular metabolic and non-metabolic functions with the expression of specific proteins, synthesis of surface macromolecules, etc. that ultimately lead to biofilm formation. Environmental engineers and scientists are just beginning to incorporate molecular probes and genetics-based tools into this type of research. Clearly, the need for expertise in physicochemical surface analysis along with molecular biology will necessitate collaboration of scientists and engineers across disciplinary boundaries.

In addition, Dufrène recently pointed out that one of the biggest hindrances in the application of AFM based tools in microbiology is that limited studies have demonstrated the concrete (biological) benefits of its use [157]. This remains a challenge for researchers engaged in this field, in that they will be asked to demonstrate the applicability of their research at environmental interfaces towards the benefit of environmental problems and protection of the natural environment for the future.

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Notation:

\( I(z_i) \) : light intensities in the presence of the particles in the medium

\( I_\infty \) : light intensities in the absence of the particles in the medium

\( \Delta f \) : measured resonant frequency shift (Hz)

\( \Delta m \) : mass change per unit area (g/cm\(^2\))

\( f_0 \) : intrinsic resonant frequency of the crystal

\( A \) : electrode surface area

\( \rho_q \) : density of quartz (2.648 g/cm\(^3\))

\( \mu_q \) : shear modulus of quartz (2.947×10\(^{11}\) g/cm\(^2\)·s)

\( d_c \) : collector diameter (m)

\( \alpha \) : porosity of the medium (-)

\( L \) : length of the column (m)

\( F_R \) : fraction of bacteria retained in the packed column (-)

\( \alpha \) : collector efficiency (-)
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<td>TIRAF</td>
<td>Similar to TIRM</td>
<td>Cells or colloids within ~100 nm of the surface can be detected</td>
<td>[112]</td>
</tr>
<tr>
<td>QCM</td>
<td>Nanogram range, $10^{-14}$ to $10^{-12}$ g/cm$^2$·s in gas phase</td>
<td>Direct contact only</td>
<td>[120]</td>
</tr>
<tr>
<td>Technique</td>
<td>Advantages</td>
<td>Disadvantages</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
<td>---------------</td>
<td></td>
</tr>
<tr>
<td><strong>AFM</strong></td>
<td>Direct force measurement; Minimum requirement for the sample preparation; Technique works in air or various liquid environments; Provides high resolution imaging in addition to force information [22]</td>
<td>Requires chemical or physical immobilization of cells when measurements are made liquid, which may be destructive or cause artifacts in cell surface properties [42, 78]; Capillary forces in air lower the resolution and can cause artifacts in force measurements; No absolute measurement of distance, so calibration is required [40]</td>
<td></td>
</tr>
<tr>
<td><strong>Optical tweezers</strong></td>
<td>No immobilization requirement; Valid over narrow particle range; Works best for spherical particles Light must pass through the trapped sample [82, 84, 87]</td>
<td>Maximum force that can be measured is 150 picoNewtons [82, 84, 87]</td>
<td></td>
</tr>
<tr>
<td><strong>TIRM</strong></td>
<td>Forces as small as $10^{-14} – 10^{-10}$ N can be resolved [99, 101, 102]; Useful in measuring bacterial-surface separation distances</td>
<td>Cells should be labeled fluorescently or be intrinsically fluorescent; The low refractive indices and flexible membranes hamper the practical use of TIRM on cells</td>
<td></td>
</tr>
<tr>
<td><strong>TIRAF</strong></td>
<td>No immobilization requirement; Label-free; Real time imaging; Useful in measuring bacterial-surface separation distances [111]</td>
<td>Only bacteria-surface separation distance can be given, not force and mass information.</td>
<td></td>
</tr>
<tr>
<td><strong>QCM</strong></td>
<td>Particularly useful for studying the time-dependence of adhesion events [125]</td>
<td>The quantitative Sauerbrey equation only holds for measurements in air; In liquid, besides sensing absorbed film, QCM also senses bound or trapped solvent, which causes significant artifacts; Changes in bulk fluid properties such as viscosity and/or pH affect QCM measurements [125]</td>
<td></td>
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Figure Captions

Figure 1. Tapping mode AFM image of *Pseudomonas stutzeri* KC on a glass slide. Cells were prepared as described in reference [158]. Image obtained with the assistance of Bruce Logan, Pennsylvania State University.

Figure 2. Schematic of the operating principles of the atomic force microscope. A cell immobilized on a substrate is probed by a cantilever. The laser light reflected by the bended cantilever is collected by the four-quadrant photodiode detector. This signal is recorded and used to adjust the feedback loop.

Figure 3. Representative image of microbial biofilm on a glass slide: *Aureobasidium pullulans* imaged under water, using tapping mode AFM. Z scale corresponds to 400 nm. Cells were prepared as described in reference [38]. Image provided courtesy of Jill Pouliot, Ian Walton, and Matthew Nolen-Parkhouse, Worcester Polytechnic Institute.

Figure 4. Components of an AFM force analysis on a bacterial sample. A) The tip probes the bacterial sample, which contains surface macromolecules extending into solution. Force spectra are typically captured as a cycle of tip approach and tip retraction. Retraction portions of the cycles are described in detail here. B) Typically, the force ($F$) shows one or more adhesion peaks during the retraction portions of the AFM cycles. These peaks occur because macromolecules physisorb to the AFM tip, creating a weak bond. At some distance, the bond is broken, the macromolecule detaches, and the force returns to zero. Multiple peaks can indicate either the presence of multiple polymers, or may occur if several locations on a single polymer are probed in one cycle. These features have been described in the literature as “sawtooth” patterns, and are observed for various types of polymers [159, 160]. C) A distribution of polymer adhesion forces with the AFM probe will be obtained, and this is typically quantified.
via a histogram. D) The stretching portions of the retraction cycles can also be used to provide
information on polymer mechanical properties. The shaded (thicker) regions represent the
portions of the retraction curve which are considered in the context of polymer elasticity models.
E) The Wormlike Chain (WLC) model is commonly applied to polymer stretching data. In this
model, the persistence length ($L_p$) helps quantify the rigidity of the polymer. F) The Freely-
Jointed Chain (FJC) model also can be used to describe polymer elasticity. In this case, the
segment length ($a$) is used to represent the flexibility or rigidity of the polymer. In the FJC
model, each segment of the chain is envisioned to be independent, and can rotate freely in three
dimensions.

Figure 5. Schematic representation of optical tweezers. A cell in the solution is trapped by the
laser, which acts as optical tweezers. After passing through a high-quality microscope objective,
the laser beam is focused and optically holds the cell. Another objective (the top one) is used to
record the displacement of the trapped cell. This displacement can be used to calculate a force,
with the appropriate application of modeling.

Figure 6. A) Schematic of TIRM and B) amplified schematic, demonstrating the scattering of
the evanescent wave. For both images, the trapping laser from the objective lens brings the cell
close to the interface and then the laser (HeNe, for example), creates an evanescent wave. The
light scattered by the trapped cell is collected by the photomultiplier tube and recorded by the
computer.

Figure 7. Schematic of Quartz Crystal Microbalance. The quartz crystal, coated with a thin gold
electrode, is driven by an oscillator circuit. The frequency of the quartz crystal is recorded. A
solution with cells in it is passing through one electrode of QCM. After some cells adsorb, there
is a new frequency recorded, and this frequency change can be related to the change in mass. The adsorbed mass can be monitored with the time.

Figure 8. Correlation between average adhesion force measured from AFM retraction cycles with the collision efficiency from column transport experiments under flow conditions. Microbes are those listed in Table 3.
Figure 1
Figure 3
Figure 4

A) stretching of polymer

B) F vs. Distance

C) Adhesion Force vs. % of Polymers in Range

D) stretching of polymer

E) F vs. Distance

F)
Figure 5
Figure 6A
Figure 6B
Figure 8
Part II: Cranberries and Urinary Tract Infections
Chapter 3: Role of Cranberry Juice on Molecular-Scale Surface Characteristics and Adhesion Behavior of Escherichia coli

Abstract

Cranberry juice has long been believed to benefit the prevention and treatment of urinary tract infections (UTIs). As the first step in the development of infection, bacterial adhesion is of great research interest, yet few studies have addressed molecular level adhesion in this context. P-fimbriated *Escherichia coli* play a major role in the development of a serious type of UTI, acute pyelonephritis. Experiments were conducted to investigate the molecular-scale effects of cranberry juice on two *E. coli* strains: HB101, which has no fimbriae, and the mutant HB101pDC1 which expresses P-fimbriae. Atomic force microscopy (AFM) was used to investigate both bacterial surface characteristics and adhesion forces between a probe surface (silicon nitride) and the bacteria, providing a direct evaluation of bacterial adhesion and interaction forces. Cranberry juice affected bacterial surface polymer and adhesion behavior after a short exposure period (<3 hours). Cranberry juice affected the P-fimbriated bacteria by decreasing the adhesion forces between the bacterium and tip and by altering the conformation of the surface macromolecules on *E. coli* HB101pDC1. The equilibrium length of polymer (P-fimbriae) on this bacterium decreased from ~148 to ~48 nm upon being exposed to cranberry juice. Highly acidic conditions were not necessary for the prevention of bacterial adhesion, since neutralization of cranberry juice solutions to pH = 7.0 allowed us to observe differences in adhesion between the *E. coli* strains. Our results demonstrate molecular-level changes in the surfaces of P-fimbriated *E. coli* upon exposure to neutralized cranberry juice.
Introduction

Urinary tract infections (UTIs) refer to the presence of microorganisms in the bladder, prostate, collecting system, or kidney (Johnson 1991). UTIs are extremely prevalent, especially in females, the elderly and infants. Approximately eight million people per year experience UTIs in the U.S. (Cohn and Schaeffer 2004), resulting in annual estimated medical expenditures of $1.6 billion (Foxman 2002). By age 24, one-third of women will have at least one physician-diagnosed UTI that was treated with prescription medication, and the total cost over 20 years (from 1995) of treating UTIs by antibiotics are estimated to be as high as $25.5 billion (Foxman et al. 2000). Certain groups, especially women, are more prone to repeated infections (Dwyer and O'Reilly 2002). Recurrences frustrate the patient and may contribute to the development of bacterial antibiotic resistance.

UTIs are usually caused by Gram-negative bacteria, especially *Escherichia coli* (Johnson 2003). *E. coli* remains the predominant uropathogen (80%) isolated in acute community-acquired uncomplicated infections (Ronald 2003) and is the most prevalent pathogen associated with UTIs in young children (Sakran et al. 2003). As the first step of developing infections, bacteria must bind to the host cells and tissues, in most cases uroepithelial cells. For uropathogenic *E. coli*, Type 1 fimbriae (Bahrani-Mougeot et al. 2002) and P-fimbriae are proteinaceous macromolecules that facilitate the adhesion of *E. coli* to uroepithelial cells (Gunther et al. 2001; Mulvey 2002).

Due to continuing concern over antibiotic resistance in numerous types of infections (Wilson and Gaido 2004), growing research is directed at alternate solutions for infection treatment or prevention. Cranberry (*Vaccinium macrocarpon*) was being
used as a medicine by Native Americans before 1620 and has been utilized as a urinary antiseptic for more than 200 years (Gunn 1878).

Although only limited clinical studies have investigated the effects of cranberry product consumption on the presence of bacteria in the urine (bacteriuria) and/or the development of UTIs, promising results have been obtained in some cases, as reviewed in (Raz et al. 2004). For example, Avorn et al. supplied 300 mL doses (daily for 6 months) of cranberry juice cocktail vs. a placebo drink, in a study of 153 elderly women (Avorn et al. 1994). They found that bacteriuria and pyuria were significantly reduced in patients receiving the cranberry juice cocktail, compared to those receiving a placebo drink. Benefits have been found in clinical studies using cranberry juice cocktail (Avorn et al. 1994), cranberry mixed with water (Haverkorn and Mandigers 1994), cranberry-lingonberry concentrate (Kontiokari et al. 2001), pure cranberry juice (Papas et al. 1966), and cranberry capsules and tablets (Stothers 2002; Walker et al. 1997).

Although cranberry has been observed to promote a healthy urinary tract, a detailed understanding of how cranberry benefits the body is still lacking. Initially, it was believed that the acidity of cranberry (due to benzoic acid that becomes hippuric acid in the urine) imparted the antibacterial activity (Blatherwick 1923). However, more recent experiments have shown that the pH of urine (after cranberry consumption) is only slightly decreased and that the effect is transient (Sobota 1984; Walsh 1992). Since the early 1980s, researchers began presenting alternative antibacterial mechanisms for cranberry. A possible treatment strategy is to use agents to prevent or decrease bacterial attachment to epithelial cells, as has been suggested for cranberry (Sobota 1984). By impairing the adhesion step, the infection cannot develop.
The mechanisms by which cranberry alters the adhesion of *E. coli* are still poorly understood. Since the inhibition of adhesion of bacteria to eukaryotic cells in the presence of cranberry juice was first reported (Sobota 1984; Zafriri et al. 1989), limited studies have addressed the effect of cranberry juice or its components on adhesion of *E. coli* to eukaryotic cells or other surfaces. Interestingly, Sotoba showed that the urine from mice and humans (after consumption of cranberry or cranberry juice compounds) still contained the materials that could make *E. coli* less adhesive to epithelial cells (Sobota 1984). This suggests that the active compounds are not destroyed by the digestive system.

Very few in vitro studies have been performed to assess the adhesion of *E. coli* to a non-cell surface in the presence of cranberry. An extract from fresh cranberries decreased the strength of attachment of *E. coli* to glass coverslips when incubated together for 2 hours (Allison et al. 2000). Pre-conditioning of the surface prior to biofilm formation also weakened the strength of attached cells (Allison et al. 2000). The adhesion behavior was only qualitatively observed, as attachment was inferred from counting the number of colony forming units (CFUs) that transferred from the glass slide to a plate with fresh media. The type of fimbriae expressed by this strain of *E. coli* was not discussed.

Many questions remain unanswered with regard to the role of cranberry in mediating the adhesion of *E. coli*. No study has addressed the molecular-level interactions between cranberry and the *E. coli* surface using a nanoscale tool such as atomic force microscopy (AFM). In the present study, AFM experiments and modeling were used to probe the nanoscale interactions between a model surface (silicon nitride)
and carefully selected *E. coli* strains. The effect of cranberry on the conformation and adhesion properties of *E. coli* surfaces was quantified as a function of cranberry juice concentration.

**Materials and Methods**

Cultures. *E. coli* HB101 was obtained from the American Type Culture Collection (ATCC 33694). *E. coli* HB101 is a plasmid-less, non-fimbriated bacterium (Goodacre et al. 1991). *Escherichia coli* mutant HB101pDC1 expresses P-fimbriae only (Connell et al. 1996). The mutant strain was kindly provided by Professor C. Svanborg from the Department of Medical Microbiology, Lund University. Cultures were grown in Tryptic Soy Broth (TSB) at 37 °C and harvested in the mid-exponential growth phase. Bacterial cells were centrifuged for 15 minutes at 190g and resuspended in the desired media for the force measurements (described below). This low centrifugal force was chosen because previous results from our laboratory have shown that it is sufficient to pellet *E. coli*, but does so without causing any artifacts in cell surface characteristics and adhesion behavior (Bell and Camesano 2005), as can be found when high forces are applied to bacteria (Pembrey et al. 1999).

Cranberry juice. Consumer-grade cranberry juice cocktail (referred to hereafter as “cranberry juice”) was purchased (Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA). To exclude the effects of low pH on adhesion, the pH of cranberry juice was adjusted to 7.0 by adding 1 M NaOH solution. Solutions were diluted to 5, 10, and 20 wt. % cranberry juice in ultrapure water (Milli-Q water, Millipore Corp.). Since cranberry juice cocktail contains 27 wt.% cranberry juice, we considered that to be an
approximate upper limit to the cranberry concentration that a patient could be expected to
consume, and we chose other concentrations below that value.

Bacterial Cell Preparation for AFM. *E. coli* were immobilized on cleaned glass
slides using an EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide
Hydrochloride)/NHS (N-Hydroxysuccinimide) crosslinking reaction, as described
previously (Camesano et al. 2000). Slides were kept hydrated all the time before
performing AFM work. When doing AFM experiments, bacterial-coated slides were
immersed in ultrapure water, 5, 10 and 20 wt. % cranberry juice solutions. Typically, an
AFM experiment lasted for <3 hours. During this period, cells remain viable but are not
growing. Previous work has shown that the chemicals used to prepare bacteria for the
AFM experiment do not affect their viability (Camesano et al. 2000). Further, when the
glass slide is placed in fresh bacterial growth media (TSB) after the conclusion of an
AFM experiment, bacterial cells are seen to resume growth.

Force Measurements. Individual bacterial cells were probed by AFM (Digital
Instruments Dimension 3100 with Nanoscope III controller). The imaging and selection
of bacterial cells for force measurement has been described previously (Camesano and
Logan 2000). Measurements were carried out on five individual bacterial cells, 8 times
per bacterium per chemical condition studied. Silicon nitride AFM tips were used (DNPS,
Digital Instruments), with an average spring constant of 0.13±0.02 N/m, measured using
the method of Cleveland et al. and the correlation equations given in the manufacturer’s
software. Before using the cantilevers, they were exposed to UV light for 10 minutes to
remove any potential organic contamination.
Force Analysis. A force cycle yields 512 data points for each of the approach and retraction portions of the cycle. The data sets were converted to ASCII format and exported to a spreadsheet. Data were converted from deflection of the cantilever to forces using established procedures (Emerson and Cameano 2004).

Modeling of AFM Data. A steric model has been developed to quantify the interaction forces between a surface of relatively high coverage of grafted polymers and a bare surface. This steric model also can be applied to the interaction force between a polymer-bearing bacterium and the AFM tip. Adaptation of the model of Alexander (Alexander 1977) and de Gennes (De Gennes 1987) to AFM data was performed (Butt 1999), where the steric force, \( F_{st} \) is given by

\[
F_{st} = 50k_B T a L_0 \Gamma^{3/2} e^{-2dh/L_0} \tag{1}
\]

where \( k_B \) is the Boltzmann constant, \( T \) is temperature, \( a \) is tip radius, \( \Gamma \) is polymer density, \( h \) is sample-substrate separation distance, and \( L_0 \) is the equilibrium polymer length, describing how far the polymers extend into solution.

From the AFM force measurements, we know the interaction forces and the distances between the bacterial cell surfaces and the tip. By fitting the steric model, the grafted polymer density and the equilibrium polymer length can be calculated. For *E. coli* mutant HB101 pDC1, the P-fimbriae are expected to be the largest proteinaceous structure on the cell surface, and so the equilibrium length and density from the steric model should correspond with the length and density of P-fimbriae on the bacteria. For *E. coli* HB101, which does not express fimbriae on its surface, other (smaller) structures such as mannose receptors on the surface (Wang et al. 1998) or lipopolysaccharides from
the underlying cell membrane are expected to be demonstrated in the lengths and densities obtained from the steric model.

AFM Retraction curve analysis. Statistical analyses were used to analyze the AFM retraction profiles. After the AFM tip touches bacterial surface polymers, these molecules are compressed until they encounter the “compressing limitation”. At this point, the AFM tip still can “approach” the bacterial surface further. But this “approach” is due to the deformation of the AFM cantilever instead of the compression of the biomacromolecules, since a very weak cantilever is used. During the process, some biomacromolecules absorb on the AFM tip. When retracting the AFM tip, the absorbed biomacromolecules exert adhesive forces. The AFM tip must surmount the adhesive forces (called pull-off forces or retraction forces). Retraction peaks corresponding to these adhesion events are observed. The retraction peaks can be considered to be independent and random events.

For each bacterium and solution studied, the retraction peaks were combined (without any averaging) and the distributions of pull-off forces and pull-off distances were independently calculated. Due to the natural heterogeneity and variability in the data, one retraction peak is not meaningful. Rather, the statistical distribution of the pull-off forces and pull-off distances for a whole population is used to explain the behavior of the bacterial system under each condition.

As a precaution, our general protocol is to make a force measurement on clean glass, make a measurement on the bacterium, and then return to the clean glass. Comparing the final and initial measurements on the glass allows us to ensure that biomolecules that adsorb to the tip during the retraction part of the force cycle are
completely detached by the end of the cycle. Therefore, the tip is clean and a new cycle can begin.

**Results**

**Reproducibility of Force Cycle Data**

Repeated force measurements on a given bacterium were reproducible under a single set of conditions. Figure 1A shows illustrative data for the approach curves on one representative cell of *E. coli* HB101pDC1, which show little variability. When the steric model was applied to the approach curve data, and correlation coefficients ($R^2$ values) were used to evaluate the goodness of fit, all were >0.95 (discussed below in more detail).

The retraction curves (Figure 1B), show more variability, due to the dynamic nature of the bacterial surface polymers. In a single retraction curve, the tip may contact many biomolecules on the bacterial surface, and individual molecules may even contact the tip in multiple locations. Also, since these molecules are constantly moving and changing their conformations (due to Brownian motion and other intermolecular forces), it is not possible to contact the exact set of molecules in an identical conformation, even when a subsequent measurement is made a few seconds later. Despite the variability, statistical analyses were useful in combining and analyzing the retraction curve data from multiple force cycles. Multiple force cycles refers to repeated instances of the tip approaching and retracting from the bacterial surface.

**Surface Molecules on *E. coli* mutant HB101pDC1 (P-fimbriae)**

Data from the forty AFM force cycles per condition (8 measurements/cell, 5 cells/condition) were combined and the steric model was applied. The data for all forty
curves was used to determine average parameters for the equilibrium polymer length and polymer density. The equilibrium length of surface polymers, calculated from the steric model, (representative data shown in Figure 2) decreased from an average of 147±125 nm in the absence of cranberry juice treatment to an average of 53±21 nm, 48±26 nm and 48±45 nm in 5%, 10% and 20 wt. % cranberry juice solutions, respectively, when the P-fimbriated bacteria were in a solution cranberry juice.

The deviations reported are not indicative or “errors”, but of the spread in the data due to natural heterogeneity and the complexity of these microbial systems. However, due to this scatter, we could not rely on average values alone to explain variations in model parameters. In order to detect differences in polymer behavior among the four chemical solutions and distinguish differences due to the random variation, a statistical test, the one-way repeated ANOVA test, was performed. This test was first applied to the water data as a “control” and to the cranberry juice data as test conditions. Our analyses demonstrated that there is a statistically significant difference between the equilibrium length in ultrapure water and each of the values in cranberry juice. In comparing water versus the 5 wt. % cranberry juice solution, the equilibrium length of the polymers decreased, but the decrease in the equilibrium length appeared to plateau as a function of cranberry juice concentration when higher concentrations were tested (Figure 3A), since subsequent increases in the cranberry juice concentration did not further decrease the equilibrium polymer lengths. All of the cranberry juice concentrations had similar effects on the equilibrium length, and there were no significant differences among the equilibrium lengths in 5, 10 and 20 wt. % cranberry juice.
Fitting of the steric model to the approach curves also allowed us to calculate the polymer densities. In general, the density increased with a greater concentration of cranberry juice in solution (Figure 3B) The polymer densities increased from an average of $(9.24\pm8.55)\times10^{15}$ m$^{-2}$ in the absence of cranberry juice treatment to an average of $(8.01\pm2.32)\times10^{15}$ m$^{-2}$, $(1.63\pm1.01)\times10^{16}$ m$^{-2}$ and $(2.16\pm1.86)\times10^{16}$ m$^{-2}$ in 5%, 10% and 20 wt. % cranberry juice solutions, respectively, when the P-fimbriated bacteria were in a solution cranberry juice. Pure water and a cranberry juice concentration of 5 wt. % resulted in essentially identical values for the density. Further, statistical tests on the polymer densities revealed that there was no statistically significant difference between the grafted polymer density in ultrapure water and 5 wt. % cranberry juice, but that the densities in 5%, 10% and 20 wt. % cranberry juice solutions were different from one another. Further, we note that the measurements in cranberry are completely reversible when the solution is then replaced with water.

In this case, the density of the molecules represents an apparent density, and is dependent on the conformation of the molecules. When the fimbriae are in their most extended conformation, as appears in water and 5 wt.% cranberry juice, the apparent density is lower because there is less biopolymer near the cell wall. However, cranberry juice in increasing concentrations changes the conformation of the surface molecules. When higher cranberry juice concentrations were used, the fimbriae appeared to become more compressed near the cell wall. Therefore, the density in that region would be higher.

Surface Macromolecules on *E. coli* HB101 (no fimbriae)
The equilibrium length calculated from the steric model was not a function of the presence of cranberry juice or of the cranberry juice concentration for E. coli HB101. The equilibrium length of surface polymers, calculated from the steric model, (representative data shown in Figure 4A) varied from an average of 32±10 nm in the absence of cranberry juice treatment to an average of 43±27 nm, 22±5 nm and 30±28 nm in 5%, 10% and 20% cranberry juice solutions, respectively. In fact, the equilibrium length was nearly constant for all solutions studied (Figure 4A). ANOVA statistical tests confirmed that with water as the control group, there were no significant differences in the equilibrium polymer lengths for any of the cranberry juice solutions. Likewise, the grafted polymer density of E. coli HB101 calculated from the steric model was not dependent on the cranberry juice concentration (Figure 4B). The polymer densities varied from an average of (5.27±1.62)×10^{16} m^{-2} in the absence of cranberry juice treatment to an average of (3.78±1.74)×10^{16} m^{-2}, (4.03±1.05)×10^{16} m^{-2} and (4.26±1.89)×10^{16} m^{-2} in 5%, 10% and 20 wt. % cranberry juice solutions, respectively and no statistically significant differences were observed in the grafted polymer densities for E. coli HB101 in any of the cranberry juice solutions.

Analysis of Retraction Curve Data: Pull-off Forces and Pull-off Distances

E. coli mutant HB101pDC1

Unlike the data from AFM approach curves, the retraction curves show more variability, even on a single bacterium. This is mostly due to the dynamic and heterogeneous nature of the biomolecules on the bacterial surface, which means that the AFM tip is likely to make contact with a different portion of the biomolecule or a different biomolecule in subsequent measurements (Camesano and Abu-Lail 2002).
While a single retraction curve is not very meaningful, reliable results can be expected by integrating many adhesion events over multiple cells. In our analysis, we examined 40 retraction curves in one cranberry solution (eight force measurements on five cells). The data were combined through histograms and statistical analyses to help their interpretation.

For *E. coli* HB101pDC1, adhesion forces were inversely correlated with the cranberry juice concentration (Figure 5A). In ultrapure water or 5 wt. % cranberry, ~80% of the retraction forces were of an absolute magnitude >0.5 nN. In contrast, smaller adhesive forces were observed in the higher cranberry concentration solutions, with >40% of the retraction forces having an absolute magnitude <0.5 nN in the 20 wt. % cranberry juice.

The pull-off distances for *E. coli* HB101pDC1 decreased with increasing cranberry juice concentration (Figure 5B.). More than 60% retraction peaks in the cranberry juice (5%, 10% and 20 wt. %) showed up in the range <40 nm, while more than 60% retraction peaks in the ultrapure water appeared > 40nm.
Clear trends were not present for the effect of cranberry juice on the adhesion forces and pull-off distances for the non-fimbriated bacteria. The adhesive forces in any solution between the probe and *E. coli* HB101 were somewhat low, with more than 80% of the adhesion forces between *E. coli* HB101 and the probe having absolute magnitudes between zero and 0.5 nN, irrespective of the cranberry juice concentration (0-20 wt. %) (Figure 5C). Cranberry juice did not have much of an effect on the adhesive forces between the probe and this strain of *E. coli* HB101.

Similarly, the pull-off distances were not very sensitive to the cranberry juice concentration for HB101 (Figure 5D). More than 80% of the pull-off distances measured in any of the solutions were <60 nm. Most pull-off distances of *E. coli* HB101 in the cranberry juice (5%, 10% and 20 wt. %) and the ultra pure water occurred within the same range.

**Discussion**

**Mechanism of cranberry juice action on P fimbriated- *E. coli***

The biological role of fimbriae is to act as adhesins between bacteria and receptors on mammalian cells. While we did not study the binding between *E. coli* and mammalian cells, we showed that the *E. coli* itself can be affected by cranberry. Cranberry juice appears to affect fimbriae directly. Evidence for this is that cranberry juice affects the equilibrium lengths and density of the polymers on strain HB101pDC1, and the adhesion forces with a model surface, but these effects are not seen for the non-fimbriated strain, HB101.
We can consider several possible mechanisms to explain the interactions between cranberry juice and the surfaces of *E. coli*. These possibilities include i) cranberry juice alters the conformation of the P-fimbriae; ii) cranberry juice blocks the adhesive action of P-fimbriae; iii) cranberry juice removes P-fimbriae from the cells, and iv) cranberry juice causes genetic or phenotype-level changes in *E. coli* with P-fimbriae, causing non-expression of P-fimbriae. It is also possible that more than one mechanism is occurring simultaneously.

i) Role of cranberry juice on conformation of P-fimbriae

Exposure to cranberry juice resulted in a decrease in the equilibrium length of the polymers on the surface of *E. coli* HB101pDC1 from \(~148\) to \(~48\) nm. This appears to be a conformational change in the surface fimbriae, indicating the proteins are becoming more compressed on the bacterial surface when cranberry is present.

ii) Cranberry juice blocks adhesive action of P-fimbriae

Specific components in cranberry juice can bind to the P-fimbriae and inhibit the adhesion of P-fimbriae-acted bacteria to a surface. The components were identified as non-dialyzable material (NDM) with a high molecular mass constituent (12000-15000 Da) (Burger et al. 2000). Howell et al. considered the components to be proanthocyanidin compounds that have both hydrophilic and lipophilic moieties (Howell et al. 1998). Although research is ongoing to further identify and characterize the key components (Lila 2004; Lila and Raskin 2005; Smith et al. 2002), the active components that can alter bacterial adhesion apparently are hydrophilic. Proteins, including fimbriae, would be hydrophobic in their unaltered states. After the hydrophilic components bind to the P-fimbriae, the adhesion force between the “modified” P-fimbriae and the AFM silicon
nitride tip changes from the interaction between non-polar materials to that between the non-polar tip and the polar “modified” P-fimbriae. This results in a decrease in the adhesion forces between the model surface and the bacterium.

iii) Cranberry juice removes P-fimbriae from the *E. coli* surface

To reveal the relationship between the equilibrium lengths and the grafted polymer density, a further analysis was performed. According to mass conservation, the mass of surface polymers for a given strain should not change as a function of the cranberry juice concentration. The mass of the biomacromolecules on the outer surface of the cells is taken to be the product of the outer cell surface area, the equilibrium length, and the polymer density, respectively. Since the surface area does not change (verified by fluorescence microscopy experiments, data not shown), any variations in the equilibrium length and the polymer density should balance one another. Both in the presence and absence of cranberry juice, the product of the equilibrium length and the polymer density remains constant at \( \sim 1 \times 10^9 \text{ m}^{-1} \), and is independent of cranberry juice concentration. Further, the one way repeated ANOVA statistical tests showed that there were no statistically significant differences among the individual products from the three concentrations of cranberry juice. Therefore, the total mass of molecules on the bacterial surface does not change upon exposure to cranberry juice, and so it does not seem plausible that cranberry juice exposure is causing fimbriae to be removed.

iv) Cranberry juice causes loss of expression of P-fimbriae

Consistent with the above mass balance analysis, cranberry juice exposure cannot cause P-fimbriae to not be expressed, at least not over the time scales studied here. However, growth of the cells in cranberry-containing media could potentially cause a
different effect. Ahuja et al. reported P-fimbriated *E. coli* bacteria lost the P-fimbriae when growing in media mixed with liquid cranberry concentrate (unsweetened form of cranberry juice) (Ahuja et al. 1998). They hypothesized that some components in cranberry concentrate will interact with bacterial DNA and inhibit the expression of P-fimbriae. Our results neither can prove nor disprove this possible mode of action of cranberry. However, we have shown that growth in cranberry-containing media is not necessary for affecting the *E. coli* surface, since growth was not a factor in our studies. Exposure to cranberry juice for even a short time period (< 3 hrs. and during non-growth conditions) produces reversible yet important changes in surface properties.

Effects of cranberry juice pH on bacterial adhesion behavior

Acidification of urine was speculated to be responsible for the anti-bacterial properties of cranberry for more than 100 years, but recent research showed that this was not the reason for the anti-bacterial properties of cranberry juice (Sobota 1984; Zafriri et al. 1989). Therefore, we wanted to address whether the anti-adhesive response of *E. coli* to cranberry could be observed, even at non-acidic pH values. We adjusted the pH of the cranberry juice to 7.0 before the AFM experiments. The average equilibrium length of surface polymer, i.e. P-fimbriae on *E. coli* mutant HB101pDC1, decreased from ~148 nm in ultrapure water to ~48 nm in 20% cranberry juice. The cranberry juice after neutralization still has the capability to affect the adhesion and conformational behavior of *E. coli* mutant HB101pDC1. The finding that low pH is not necessary to prevent bacterial adhesion was verified by a direct approach.
Further, we can rule out electrostatic interactions as playing a dominant role in influencing the adhesion behavior between *E. coli* and the silicon nitride. Although water has the lowest ionic strength, all of the cranberry juice solutions have fairly low ionic concentrations. The cranberry juice cocktail (which is 27% cranberry) has an ionic strength of $\sim 9.55 \times 10^{-3}$ M. Therefore, the diluted juices have ionic strengths ranging from $\sim 0.001$ M (for the 5 wt.% solution) to $\sim 0.007$ M (for the 20% solution). If electrostatic forces were dominating the interaction between *E. coli* and the silicon nitride, we would expect to see decreased adhesion at the lowest ionic strength (i.e. pure water and 5 wt.% juice). Since the opposite trend was observed, it appears that non-electrostatic interactions are dominating in this system.

**Conclusions**

Cranberry juice components appear to affect P-fimbriae by altering their conformation and by binding of hydrophilic components. Both of these phenomena work to decrease the adhesion of P-fimbriated *E. coli* with a model surface. The AFM results show that cranberry juice has an immediate effect on the P-fimbriated *E. coli* bacteria. Some components in cranberry juice interacted with P-fimbriae directly, causing P-fimbriae to become compressed and less adhesive. A final note of interest is that most of the effects we saw occurred at a cranberry juice concentration between zero and 5%. Future work will be aimed at exploring the “critical concentration” of cranberry juice needed to alter the conformation and adhesion properties of P-fimbriae.
Acknowledgements

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References


Figure Captions

Figure 1. A) Representative approach curves on a single bacterium (*E. coli* HB101pDC1) in 5% cranberry juice. The eight measurements shown were performed on a single cell. For each condition, eight measurements were performed per cell, and five cells were examined. B) Representative retraction curves on a single bacterium (*E. coli* HB101pDC1) in 5% cranberry juice.

Figure 2. Representative example of a fit to the AFM approach curve data (symbols) with the steric model (solid line). *E. coli* HB101pDC1 in 5% cranberry juice. Based on the steric model fits, the equilibrium length is 48±26 nm and the polymer density is \((8.01±2.36) \times 10^{15} \text{ m}^{-2}\) \((R^2=0.99)\).

Figure 3. Steric model fits for *E. coli* HB101pDC1, as a function of cranberry juice concentration. \(R^2\geq0.9\), 5 cells per concentration, 8 force measurements per cell, i.e. \(n=40\). A) The average equilibrium length \(L_0\) of *E. coli* HB101pDC1 as a function of cranberry juice concentration. B) The average polymer density \(\Gamma\) of *E. coli* HB101pDC1 as a function of cranberry juice concentration. Error bars indicate one standard deviation.

Figure 4. Steric model parameters fit for *E. coli* HB101 as a function of cranberry juice concentration. \(R^2\geq0.9\), 5 cells per concentration, 8 force measurements per cell, i.e. \(n=40\). A) Equilibrium length \(L_0\), B) Polymer density. Error bars indicate one standard deviation.

Figure 5. Distribution of parameters from AFM retraction curves. A) Retraction forces for *E. coli* HB101pDC1 as a function of cranberry juice concentration, \(n\geq25\); B) Pull-off distances for *E. coli* HB101pDC1 as a function of cranberry juice concentration, \(n\geq25\); C) Retraction forces for *E. coli* HB101 as a function of cranberry juice concentration, \(n\geq25\);
D) Pull-off distance for *E. coli* HB101 as a function of cranberry juice concentration, \( n \geq 25 \).
Figure 1A

Figure 1B
Figure 2
Figure 3A

Figure 3B
Figure 4A

Figure 4B
Chapter 4: Cranberry Changes the Physicochemical Surface Properties of *E. coli* and Adhesion with Uroepithelial Cells

Abstract

Cranberries have been suggested to decrease the attachment of bacteria to uroepithelial cells (UC), thus preventing urinary tract infections, although the mechanisms are not well understood. A thermodynamic approach was used to calculate the Gibbs free energy of adhesion changes (ΔG<sub>adh</sub>) for bacteria-UC interactions, based on measuring contact angles with 3 probe liquids. Interfacial tensions and ΔG<sub>adh</sub> values were calculated for *E. coli* HB101pDC1 (P-fimbriated) and HB101 (non-fimbriated) exposed to cranberry juice (0-27 wt.%). HB101pDC1 can form strong bonds with the Gal-Gal disaccharide receptor on uroepithelial cells, while HB101-UC interactions are only non-specific. For HB101 interacting with UC, ΔG<sub>adh</sub> was always negative, suggesting favorable adhesion, and the values were insensitive to cranberry juice concentration. For the HB101pDC1-UC system, ΔG<sub>adh</sub> became positive at 27 wt.% cranberry juice, suggesting that adhesion was unfavorable. Acid-base interactions (AB) dominated the interfacial tensions, compared to Lifshitz-van der Waals interactions (LW). Exposure to cranberry juice increased the AB component of the interfacial tension of HB101pDC1. LW interactions were small and insensitive to cranberry juice concentration. The number of bacteria attached to UC was quantified in batch adhesion assays and quantitatively correlated with ΔG<sub>adh</sub>. Since the thermodynamic approach should not agree with experimental results when specific interactions are present, such as HB101pDC-UC ligand-receptor bonds, our results may suggest that cranberry juice
disrupts bacterial ligand-UC receptor binding. These results help form the mechanistic explanation of how cranberry products can be used to prevent bacterial attachment to host tissue, and may lead to the development of better therapies based on natural products.

Introduction

According to National Institutes of Health (NIH), urinary tract infections (UTIs) are the second most common type of infection in the body, particularly affecting women, the elderly, and infants. Indeed, one in three women will have at least one UTI in her lifetime [1], leading to 11.3 million women per year affected in the U.S. alone [1]. The Gram-negative bacterium *Escherichia coli* is the predominant uropathogen leading to UTIs [2]. Prescription antibiotics are the main methods to treat UTIs. However, due to the rapid development of bacterial antibiotic resistance and side effects, alternate solutions for infection prevention are of great interest [3]. The American Red Cranberry (*Vaccinium macrocarpon* Ait., family Ericaceae) has long been recognized for providing benefits in the maintenance of urinary tract health. Clinical studies have shown that consumption of cranberry juice or products can help prevent UTIs [4-8]. However, the mechanisms for cranberry to benefit urinary tract health are not completely understood. While earlier studies speculated that the benefits of cranberry were due to its acidity [9], some studies demonstrated that compounds in cranberry could prevent bacterial adhesion to host tissue [10-12].

Bacteria contain several virulence factors that aid in initiation of infection, such as P fimbriae, type 1 fimbriae, and hemolysin, which are all associated with acute pyelonephritis (bacterial infection of the kidney). Although P fimbriae are not the only factor related to this type of infection, over 95% of AP infections in children and 50-90%
of AP infections in adults are associated with P-fimbriated E. coli [13]. P fimbriae are flexible heteropolymeric structures possessing four subunit proteins, mainly of repeating PapE subunits with the receptor-binding adhesin PapG at its distal end [14]. The PapG adhesin binds to the α-D-galactopyranosyl-(1-4)-β-D-galactopyranose (Gal-Gal) receptor [14]. In a previous study, we combined atomic force microscopy (AFM) measurements with steric modeling and estimated that the average length of the P fimbriae on E. coli HB101pDC1 cells is 148 nm [15].

Research on the use of cranberry juice to prevent and treat UTIs by decreasing bacterial adhesion has focused on two areas. First, researchers are actively trying to characterize the anti-adhesive compounds in cranberry juice, refine the dose that would be needed, and identify metabolites [16-19]. A non-dialyzable material (NDM) [20-22] and A-type proanthocyanidin oligomers [18, 23] have been identified as active anti-adhesion compounds. A second research direction is to elucidate the anti-adhesion mechanism that cranberry juice imparts on bacteria and uroepithelial cells. Mechanisms that have been proposed include a shift in the distribution of zeta potentials [24] and cell shape changes, from rod to elongated, after culture in media containing cranberry juice [25]. Although these studies have provided insight into the action of cranberry juice on pathogenic bacteria, more research is needed to better understand cranberry’s actions on the bacteria/uroepithelial cell system. Previously, we measured the adhesion forces between P-fimbriated HB101pDC1 and human kidney epithelial cells via AFM and found the adhesion forces decreased from 9.64 nN to 0.5 nN after cranberry juice treatment [26]. We also observed changes in the morphology of P fimbriae. The average length of the fimbriae of E. coli HB101pDC1 decreased from 148 nm to 48 nm after
cranberry juice treatment, which may have contributed to the decrease in adhesion [15]. However, our knowledge of how cranberry blocks the adhesive ability of *E. coli* to uroepithelial cells is not complete.

The ability of bacteria to undergo the initial adhesion step to host tissue determines whether an infection can develop. Bacterial adhesion can be described as a combination of non-specific and specific interactions. Non-specific interactions include Lifshitz-van der Waals (LW) forces, electron-donor/electron-acceptor (also called Lewis acid/base or AB interactions), and electrostatic interactions. Specific interactions only exist between ligand-receptor pairs and are usually much stronger than the non-specific interactions. However, non-specific interactions can control whether bacteria will be able to come close enough to a surface for the specific interactions to become operative [27, 28]. Bacterial adhesion to surfaces can be modeled using a thermodynamic framework to predict the favorability of adhesion, and this is typically done following the van Oss-Chaudhury-Good approach, in which non-specific LW and AB interactions are summed [29, 30]. It is unnecessary to include electrostatic interactions to calculate the Gibbs free energy change upon adhesion (\( \Delta G_{\text{adh}} \)) at the separation distance assumed to represent physical contact (0.157 nm), since overlapping electrostatic double layers induce a charge balance [31]. While numerous prior studies have used the thermodynamic approach to evaluate bacterial adhesion to inert surfaces, including biomaterials [32-34], we are unaware of any previous studies to use this approach for a system of bacteria attaching to epithelial cells.

Although clinical studies have shown that consuming cranberry juice can help prevent bacterial adhesion, these studies can be difficult to relate to molecular
mechanisms due to the variation in individual samples and the lack of understanding of the cranberry metabolism process. The present study was conducted in well controlled in vitro conditions to eliminate uncertain factors and variations that usually occur in clinical studies to better approach the fundamental mechanisms. Therefore, the goal of the present study was to perform a more detailed thermodynamic modeling of the interfacial free energies governing the initial attachment of *E. coli* to uroepithelial cells in the presence of cranberry juice *in vitro* experiments. We related the interfacial energies and $\Delta G_{adh}$ values to experimental observations of the number of bacteria attached to uroepithelial cells (UC), as a function of cranberry juice treatment. Further, this work represents an *in vitro* model system for examining how exposure to cranberry juice affects bacterial adhesion at the molecular level.

**Materials and Methodology**

**Bacterial Culture**

Two isogenic strains of *Escherichia coli* were chosen to evaluate the role of P fimbriae on bacterial adhesion. *E. coli* HB101 (American Type Culture Collection; ATCC 33694) is plasmid-less and non-fimbriated [35]. A fimbriated variant of this strain was created by transformation of the non-fimbriated parent strain *E. coli* HB101 with a plasmid carrying the genes for P-fimbriae, and maintained under chloramphenicol selection (20 µg/ml). [36]. HB101pDC1 was generously provided by Prof. Majlis Svensson (Department of Medical Microbiology, Lund University, Sweden). Bacteria were precultured in Tryptic Soy Broth (TSB, 30 g/L, Sigma, St. Louis, MO) at 37°C
overnight and cultured in fresh TSB at 37 °C until reaching an absorbance at 600 nm of 0.7 – 0.8, corresponding to middle exponential growth phase.

Bacteria were centrifuged and washed three times with PBS (0.01 M) in a salt solution (pH 7.4, NaCl 0.138 M, KCl 0.0027 M, K₂HPO₄ 0.005 M, KH₂PO₄ 0.005 M), corresponding to a total solution ionic strength of 0.14 M. We hereafter refer to this solution as “PBS buffer”. PBS is widely used to mimic human biological conditions. In this case, we chose a buffer that was close in overall ionic strength to urine, since urine has an ionic strength of 0.15 M ionic strength {Gillenwater, 2002 #14}, consisting of mainly sodium ions (0.135-0.145 M). Due to the chemical similarity between urine and PBS, PBS can closely represent urine but also eliminates concern about sample variation that would be present in urine.

Uroepithelial Cell Culture

Human kidney uroepithelial cells (UC) were purchased (ATCC CRL 9520 VA) and kept in liquid nitrogen vapor phase. For the growth and subculturing media and protocols, we followed the specifications of ATCC. Briefly, cells were grown in Kaighn’s modification of Ham’s F12 medium and supplemented with 10% fetal bovine serum. Tissue culture flasks were kept in a 5% CO₂ in air atmosphere incubator at 37 ºC for 6-7 days where the media was replaced every other day. The cells were harvested by adding 0.25% (w/v) Trypsin- 0.03% (w/v) EDTA (Sigma-Aldrich Co., MO) to detach the cells from the culture flasks. After centrifugation (800 RPM) the cells were resuspended in 0, 5, 10 or 27 wt.% cranberry juice.

Cranberry Juice Treatment
Commercially available cranberry juice cocktail (Ocean Spray Cranberries, Inc. Lakeville-Middleboro, MA) (referred to hereafter as “cranberry juice”) was used. Cranberry juice cocktail contains 27 wt.% cranberry juice, and was the highest concentration of cranberry considered. We also used 5 and 10 wt.% cranberry juice solutions in 0.01 M PBS. All cranberry juice and solutions were neutralized to pH 7 with NaOH pellets, to exclude the effects of low pH. Bacteria and uroepithelial cells were incubated in PBS buffer, or 5, 10, and 27 wt.% cranberry juice solutions for 3 hr at 37°C. In preliminary experiments, we verified that bacteria and epithelial cells remained viable after exposure to cranberry juice for 3 hr by reculturing cells after cranberry juice treatment. We did not detect any loss in viability after this exposure time, which is consistent with other studies showing that cranberry is not biocidal to E. coli [37].

Contact Angle Measurements

The contact angles of droplets of ultrapure water (18.2 megohm·cm resistivity, Millipore Corp.; Billerica, MA), diiodomethane (99% pure, Alfa Aesar, Ward Hill, MA), and formamide (99.5% pure, J.T. Baker; Phillipsburg, NJ) were measured on cellular lawns using the sessile drop technique with a goniometer (Ramé-Hart, Netcong, NJ, USA) at room temperature and ambient humidity.

Bacteria (~6×10^9 cells) and uroepithelial cells (~7.5×10^6 cells) were deposited on 0.45-μm and 8-μm pore-size cellulose acetate filters (Millipore), respectively, via vacuum filtration. After a certain time, water contact angles reached a plateau and only the moisture retained by the cell surface structures remained, which is the correct state to use for liquid contact angle measurements [38]. At least 3 replicate contact angle
measurements were made per probe liquid per filter. At least 4 filters were analyzed per condition.

Interfacial Tensions and Gibbs Free Energies of Adhesion

The interfacial tensions of individual substrata were derived from the contact angles of the three probe liquids using the methodology described by van Oss and colleagues [29, 30, 39]. The total surface tension, \( \gamma \), can be considered the additive sum of the LW and AB components of the surface tension, given by

\[
\gamma = \gamma^{NW} + \gamma^{AB}
\]  

(1)

The AB component of the surface tension \( \gamma^{AB} \) is the geometric mean of the electron-donor \( \gamma^- \) and electron-acceptor \( \gamma^+ \) parameters for the applied liquid or the substrata, given by

\[
\gamma^{AB} = 2 \cdot \sqrt{\gamma^+ \cdot \gamma^-}
\]  

(2)

When a drop of a liquid (L) is deposited on a solid surface (S), the contact angle between the drop and the surface (\( \theta \)) is a function of the components and parameters of the surface tensions of the liquid and the solid. The Young-Dupré equation relates such magnitudes:

\[
\gamma_L (\cos \theta_L + 1) = 2 \cdot \sqrt{\gamma_L^{NW} \cdot \gamma_L^{NW}} + 2 \cdot \sqrt{\gamma_S^+ \cdot \gamma_L^-} + 2 \cdot \sqrt{\gamma_S^- \cdot \gamma_L^+}
\]

(3)

If \( \gamma_L^{NW} \), \( \gamma_L^- \), and \( \gamma_L^+ \) are known, then \( \gamma_S^{NW} \), \( \gamma_S^- \) and \( \gamma_S^+ \) can be calculated. Three equations are required to solve these three unknowns, thus three probe liquids with different polar properties must be used in the contact angle measurements. Typically, two polar liquids and one apolar liquid is chosen [40, 41]. By solving the three equations of three probe liquids simultaneously, we obtain:
\[
\begin{bmatrix}
\gamma_{LW}^S \\
\gamma_S^+ \\
\gamma_S^-
\end{bmatrix} = \left\{ 2 \cdot \begin{pmatrix}
\sqrt{\gamma_{LW}^W} & \sqrt{\gamma_{LW}^-} & \sqrt{\gamma_{LW}^+} \\
\sqrt{\gamma_{D}^-} & \sqrt{\gamma_{D}^+} \\
\sqrt{\gamma_{F}^-} & \sqrt{\gamma_{F}^+}
\end{pmatrix} \right\}^{-1} \cdot \left( \gamma_W \cdot [\cos(\theta_W) + 1] \right) \\
\gamma_D \cdot [\cos(\theta_D) + 1] \\
\gamma_F \cdot [\cos(\theta_F) + 1]\right\}^2
\]

where W, D, and F denote water, diiodomethane, and formamide.

If bacteria can adhere onto uroepithelial cells, a new interface (bacterium-uroepithelial cell: i.e. B-UC) will form at the expense of losing two old interfaces (bacterium-suspending liquid: B-L and uroepithelial cell-suspending liquid: UC-L). The Gibbs free energy of adhesion (\( \Delta G_{adh} \)) is the difference between the final state and initial energy states [30]:

\[
\Delta G_{adh} = \gamma_{B-UC} - \gamma_{B-L} - \gamma_{UC-L}.
\]

The interfacial free energy between subject 1 and subject 2 (i.e. \( \gamma_{B-UC}, \gamma_{B-L} \) and \( \gamma_{UC-L} \)) can be calculated as:

\[
\gamma_{12} = \left( \sqrt{\gamma_1^{LW}} - \sqrt{\gamma_2^{LW}} \right)^2 + 2 \cdot \left[ \sqrt{\gamma_1^+} - \sqrt{\gamma_2^+} \right] \cdot \left[ \sqrt{\gamma_1^-} - \sqrt{\gamma_2^-} \right]
\]

All of the LW terms and AB terms can each be collected so that an alternate way to represent the different contributions to the Gibbs free energy change upon adhesion in aqueous media can be written [30]:

\[
\Delta G_{adh} = \Delta G_{adh}^{LW} + \Delta G_{adh}^{AB}
\]

where

\[
\Delta G_{adh}^{LW} = \left( \sqrt{\gamma_{B}^{LW}} - \sqrt{\gamma_{UC}^{LW}} \right)^2 - \left( \sqrt{\gamma_{B}^{LW}} - \sqrt{\gamma_{W}^{LW}} \right)^2 - \left( \sqrt{\gamma_{UC}^{LW}} - \sqrt{\gamma_{W}^{LW}} \right)^2
\]

and
\[
\Delta G_{\text{adh}}^{AB} = 2 \cdot \left( \sqrt{\gamma_W^+} \cdot (\sqrt{\gamma_B^+} + \sqrt{\gamma_{UC}^-}) + \sqrt{\gamma_W^-} \cdot (\sqrt{\gamma_B^+} + \sqrt{\gamma_{UC}^+} - \sqrt{\gamma_W^+}) - \sqrt{\gamma_B^-} \cdot \gamma_{UC} - \sqrt{\gamma_B^-} \cdot \gamma_{UC}^- \right)
\]

Bacterial Attachment to Uroepithelial Cells

Bacteria were suspended in an aqueous solution containing 0, 5, 10, and 27 wt.% neutralized cranberry juice for 3 hr at 37 °C. UC were exposed to similar solutions. Next, bacteria (10^9 cells/mL) and UC (10^6 cells/mL) were incubated together in the same concentration cranberry juice solution in tissue culture flasks at 37 °C for 90 minutes. After incubation, loosely attached bacteria were removed by centrifugation at 100g [42]. Remaining bacteria and UC were resuspended in 200 μL PBS and wet mounts were prepared for cell counting. Slides were viewed with phase contrast microscopy (Nikon Eclipse E400 microscope; Tokyo, Japan; oil immersion 100x objective with 1.33 numerical aperture). Attached bacteria were counted for at least 20 UC per sample condition. This choice of sample size was determined through preliminary statistical analysis for a two-way ANOVA design. The sample size test included in the statistical software determined that 20 UC per sample condition would provide the desired sensitivity (\( \alpha = 0.05 \)).

Results

Contact Angles and Individual Surface Tensions

Contact angle measurements should be made on cells that have reached an equilibrium in their drying state, such that excess water is removed, but cells are still considered hydrated. The time at which this equilibrium is achieved was determined for each type of cell by measuring the contact angle over time. The equilibrium drying time for \textit{E. coli} HB101pDC1 was \( \sim 75 \) min (Figure 1A), and was similar for HB101 (not
shown). For uroepithelial cells, the drying time was ~45 min (Figure 1B). Similarly, equilibrium drying times were determined for bacteria and UC that had been treated with cranberry juice for 3 hr. These drying times increased by ~5-10 min (Figure 1C). All of the contact angle measurements that were used to calculate the surface tensions were measured on cells that had reached the plateau region of the contact angle vs. time plot. In order to truly dehydrate cells, much more time is needed to evaporate all cellular moisture. For cells dried for very long times, the contact angle measurements produce erroneous results. Video clips are provided in the Supplementary Information showing the contact angle measurements on bacteria before the equilibrium drying time, at the equilibrium drying time, and well past the proper drying time (~10 hr).

Using measured contact angles with the three probe liquids, the interfacial tension components were calculated for bacteria and UC, as a function of cranberry juice concentration (Table 1). For *E. coli* HB101pDC1, the water contact angles increased slightly as a function of exposure to cranberry juice (from 56 ± 2° in 0.01 M PBS to 63 - 65° in any of the cranberry juice solutions). The diiodomethane and formamide contact angles increased more than the water contact angles when comparing HB101pDC1 in buffer to these bacteria in any of the cranberry juice solutions. As a result, $\gamma^{LW}$ decreased from 28.8 to 18.4 mJ·m$^{-2}$ and $\gamma^{AB}$ increased by an order of magnitude, from 2.2 to 22.3 mJ·m$^{-2}$.

For *E. coli* HB101, the three liquid contact angles remained fairly constant as a function of cranberry juice concentration. Hence, the surface free energy components did not change significantly as a function of cranberry juice treatment (Table 1).
For uroepithelial cells, the water and formamide contact angles slightly increased at higher cranberry juice concentrations, while the diiodomethane contact angle decreased at higher cranberry juice concentration. (Table 1). As a result, $\gamma_{LW}$ increased from 19.5 mJ·m$^{-2}$ to 31.3 mJ·m$^{-2}$ and $\gamma^{AB}$ decreased from 12.5 mJ·m$^{-2}$ to 3.9 mJ·m$^{-2}$ upon exposure to increasing concentrations of cranberry juice.

Gibbs Free Energy Change of Adhesion, $\Delta G_{adh}$

Without exposure to cranberry juice, $\Delta G_{adh}$ was negative for either bacteria interacting with UC, indicating that adhesion would be favorable (Figure 2A and 2B; leftmost circle symbol in each plot). The magnitudes of the $\Delta G_{adh}$ values were similar for HB101 and HB101pDC1, suggesting that in the absence of cranberry juice, the thermodynamic model cannot discriminate between the two bacteria in terms of adhesion. As the cranberry juice concentration increased, $\Delta G_{adh}$ increased for HB101pDC1, eventually reaching a positive value for the highest juice concentration tested, 27 wt.%.

For HB101, $\Delta G_{adh}$ was always negative and was fairly insensitive to cranberry juice concentration.

In order to elucidate whether cranberry juice was acting on the bacteria, uroepithelial cells, or both, we tested how $\Delta G_{adh}$ would change when only one type of cell was exposed to juice. Although treating only *E. coli* HB101pDC1 caused an increase in $\Delta G_{adh}$ as a function of increasing cranberry juice concentration, the only way to achieve a positive $\Delta G_{adh}$ was by treating both the bacteria and the uroepithelial cells with 27 wt.% juice (squares in Figure 2A). $\Delta G_{adh}$ for the *E. coli* HB101 and UC system did not show an effect of cranberry juice treatment (Figure 2B).
Calculated Interfacial Free Energies

We examined the surface tensions or surface free energies, as a function of cranberry juice concentration for the three types of interfaces: bacteria-liquid (B-L), uroepithelial cell-liquid (UC-L), or bacteria-uroepithelial cell (B-UC) (Figure 3). For *E. coli* HB101pDC1, the new surface tension $\gamma_{B-UC}$ increased monotonically with increased cranberry juice concentration, from -18.2 mJ·m$^{-2}$ to 9.7 mJ·m$^{-2}$ (Figure 3A), which suggests that cranberry juice treatment disfavors the formation of the B-UC interface and is responsible for the increase in $\Delta G_{adh}$, according to equation (5). When the cranberry juice concentration was increased to 27 wt.%, $\gamma_{B-UC}$ reversed sign to positive. We do not have enough data to determine the exact concentration where the change occurred, but it appears to be at ~20 wt.%. The surface tension corresponding to the HB101pDC1-aqueous medium interface ($\gamma_{B-L}$) was not sensitive to cranberry juice treatment (Figure 3A), and all values were negative. The surface tension corresponding to the other original interface, between uroepithelial cells and the aqueous medium ($\gamma_{UC-L}$), increased monotonically with cranberry juice treatment, from 12.9 mJ·m$^{-2}$ to 23.9 mJ·m$^{-2}$. This was smaller than the increase of $\gamma_{B-UC}$ over the same range of cranberry juice concentrations. Since the change in $\gamma_{B-UC}$ was greater than that of the sum of the changes in $\gamma_{UC-L}$ and $\gamma_{B-L}$, the overall effect was that $\Delta G_{adh}$ was governed by the values of $\gamma_{B-UC}$.

For *E. coli* HB101, $\gamma_{B-L}$ values were all small and negative, and did not show any consistent trend with respect to cranberry concentration (Figure 3B). $\gamma_{B-UC}$ for HB101 increased as a function of increased concentration of cranberry juice, but this increase
was not as great as had been observed for $\gamma_{B-UC}$ corresponding to HB101pDC1, and the interfacial tensions never reached positive values.

Lifshitz- van der Waals and Acid/Base Components of $\Delta G_{adh}$

In order to compare the relative strengths of the LW and AB interactions, $\Delta G_{adh}$ was broken into components (Table 1). For the interaction of *E. coli* HB101pDC1 with uroepithelial cells, $\Delta G_{adh}^{AB}$ increased for treatments corresponding to higher cranberry juice concentrations. The $\Delta G_{adh}^{AB}$ values were greater and controlled the overall interaction, compared to the $\Delta G_{adh}^{LW}$ values, which were almost zero, suggesting that polar interactions facilitated *E. coli* HB101pDC1 adhesion to UC. At a high enough concentration (which we first observed at 27 wt.% cranberry juice), $\Delta G_{adh}^{AB}$ increased and became positive, resulting in an overall positive value for $\Delta G_{adh}$, suggesting that bacterial adhesion was unfavorable.

Bacterial Attachment to Uroepithelial Cells and Correlation with $\Delta G_{adh}$

The number of bacteria that attached to UC was quantified for all of the treatment conditions. In the absence of cranberry juice exposure, HB101 binds non-specifically but HB101pDC1 can undergo specific bonding with uroepithelial cells. For this case, the thermodynamic model did not distinguish between the $\Delta G_{adh}$ values for the two bacteria (the model predicted essentially the same $\Delta G_{adh}$ values); (Figure 2). However, after the bacteria were exposed to any cranberry juice, the model showed differences in the $\Delta G_{adh}$ values, which were more consistent with differences we observed in attachment. In the absence of cranberry treatment, *E. coli* HB101pDC1 presented a strong affinity for
uroepithelial cells, with an average 50.2 ± 22.9 bacteria per UC, compared to 8.2 ± 5.5 bacteria per UC for HB101 (Figure 4). With cranberry treatment, the number of adhered *E. coli* HB101pDC1 decreased to 13.6 ± 5.7, 9.3 ± 4.1 and 2.9 ± 1.5 per UC, corresponding to 5, 10 and 27 wt.% cranberry treatment (Figure 4A). The attachment of *E. coli* HB101 to UC was low and insensitive to cranberry treatment (Figure 4B).

We correlated $\Delta G_{adh}$ with the number of bacteria attached to the UC, as a function of the cranberry concentration used in the treatment. For HB101pDC1, there was a first order exponential decay relationship between $\Delta G_{adh}$ and the number of attached bacteria (Figure 4A). For HB101, both $\Delta G_{adh}$ and the number of attached bacteria remained steady with respect to cranberry treatment (Figure 4B).

**Discussion**

Comparing the Effects of Cranberry Juice on the LW and AB Components of $\Delta G_{adh}$

The advantage of applying the van Oss-Chaudhury-Good thermodynamic approach to model the interactions in these systems is that it allows for the energies to be decoupled into their component parts. Therefore, we could estimate the relative strengths of acid/base and Lifshitz-van der Waals interactions. For both *E. coli* HB101pDC1 and *E. coli* HB101, $\Delta G_{adh}^{LW}$ did not change significantly with cranberry treatment and all of the values were near zero. $\Delta G_{adh}^{LW}$ is the sum of non-polar interactions, including orienting dipole-dipole interactions, orienting dipole-induced dipole (or induction) interactions, and fluctuating dipole-induced dipole (or dispersion) interactions [30]. Only the dispersion interactions have a considerable impact between macroscopic bodies, especially in
aqueous media [30], and for this reason LW interactions are mainly determined by London-dispersion forces. In the present study, bacteria, uroepithelial cells and the suspending liquid each presented comparable $\gamma^{LW}$ values. Hence, $\Delta G_{adh}^{LW}$ was close to zero and was fairly constant with respect to cranberry juice treatment. Although higher cranberry juice concentrations slightly decreased $\gamma^{LW}$ for HB101pDC1 and slightly increased $\gamma^{LW}$ for the uroepithelial cells, the changes were too small to affect $\Delta G_{adh}^{LW}$.

In the case of AB interactions (i.e. the electron-donor and electron-acceptor or polar interactions), hydrogen bonding is the most important type of interaction occurring in aqueous media [30]. The short-range character of AB forces leads to their important role in biological molecular interactions, such as in amino acid or protein binding and in the specific recognition of other proteins or DNA molecules, with roles noted in many structural and molecular studies [43-45].

Correlation Between Gibbs Free Energy and Cell Adhesion

Prior work linked the action of cranberry juice to the behavior of P fimbriae, although these studies were not focused on molecular-level investigations [25, 46]. In our work, treatment with cranberry blocked the ability of P-fimbriaed bacteria to attach to uroepithelial cells. This result was illustrated through the thermodynamic model calculations. $\Delta G_{adh}$ values were well correlated with bacterial attachment for the HB101pDC1-uroepithelial cell system. At first, this result may be surprising since the thermodynamic modeling cannot account for specific types of interactions, such as those that should be present between the receptors on the uroepithelial cells and proteins on the P fimbriae of the bacteria. For example, we previously showed that trends in $\Delta G_{adh}$ could not predict bacterial attachment when ligand-receptor interactions were dominant,
such as for fibronectin binding with *Staphylococcus epidermidis* [28], since specific receptors on *S. epidermidis* can form ligand-receptor interactions with fibronectin. However, the thermodynamic model could correctly predict the non-specific adhesion between *S. epidermidis* and fetal bovine serum [28]. In the present study, *E. coli* HB101pDC1 and uroepithelial cells are expected to form ligand-receptor interactions between proteins on the P fimbriae and the globoseries of oligosaccharide receptors on the uroepithelial cells [47], while the HB101-UC interaction is only non-specific [48]. The fact that we found good agreement between the thermodynamic predictions and bacterial attachment suggests that there was a disruption in the ability of P-fimbriated *E. coli* to form ligand-receptor bonds with the uroepithelial cell receptors.

This supposition seems plausible, and is supported by previous work in this area. We demonstrated that the P fimbriae of *E. coli* HB101pDC1 were compressed by cranberry juice treatment, with the length decreasing from an average of 148 ± 125 nm in the absence of cranberry juice to an average of 53 ± 21, 48 ± 26, and 48 ± 25 nm in 5%, 10%, and 20 wt.% cranberry juice solutions [15]. Due in part to this morphology change, adhesion forces between P-fimbriae bacteria and the uroepithelial cells decreased from 9.64 nN to 0.50 nN after exposure to cranberry juice [26], which also suggested that the ligand-receptor system was affected. In the present study, when specific binding was no longer occurring due to cranberry exposure, then the $\Delta G_{adh}$ values were well correlated with the bacterial attachment results.

Further, we found that $\Delta G_{adh}$ values were also well correlated with the non-specific attachment of the non-fimbriated (control) bacteria, *E. coli* HB101, and uroepithelial cells (Figure 4). Cranberry juice treatment did not change $\Delta G_{adh}$
significantly, and the number of attached *E. coli* HB101 remained constant. In the absence of specific interactions, the two strains should behave the same. After treatment with a high concentration of cranberry juice (27 wt.%), the numbers of *E. coli* HB101pDC1 and *E. coli* HB101 that attached to uroepithelial cells were the same, even though HB101pDC1 attached much more in the absence of cranberry juice.

Dose-dependent Inhibition of Adhesion of P-fimbriated *E. coli*

Each of the changes we observed depended on the dose of cranberry juice, such as the increased $\Delta G_{adh}$ and the decrease in bacterial attachment to uroepithelial cells, as well as changes observed in previous work, such as the altered conformation of P fimbriae and the decrease in adhesion forces with uroepithelial cells. Even a cranberry juice concentration of 5 wt.% was sufficient to cause some changes. For example, the average length of P fimbriae decreased from 148 ± 125 to 53 ± 21 nm [49], and the adhesion force between *E. coli* and uroepithelial cells decreased from 9.64 to 0.50 nN upon exposure to 5 wt.% cranberry juice [26]. In the present study, $\Delta G_{adh}$ increased from -19.94 to -15.31 mJ·m$^{-2}$ and the number of attached bacteria per UC decreased from 13.6 ± 5.7 to 9.3 ± 4.1, when comparing zero and 5 wt.% solutions. Further, one-way repeated ANOVA statistical tests showed that there were significant differences in the Gibbs free energies of adhesion and number of bacteria attached for these two conditions (P<0.005). Since 27 wt.% cranberry juice corresponds to the amount of cranberry juice in commercial juice, and at least some of the compounds in cranberry will be changed or degraded in the body, then clearly 27 wt.% cranberry juice concentration is higher than *in vivo* conditions. However, we chose this to be the maximum cranberry concentration that the cells may encounter, and this served as a useful reference for our *in vivo* model.
We also note that even at a high concentration of 27 wt.% cranberry juice, the P fimbriae are not removed from the bacteria. In previous work, we showed that the total mass of fimbriae remained fairly consistent for *E. coli* exposed to cranberry juice in concentrations up to 20% [15], although the conformation of the P fimbriae was changed as a function of exposure to cranberry juice at different concentrations. Although some studies have showed that P fimbriae are altered by growth of *E. coli* in cranberry juice containing media [25], we did not directly address the issue of growth in cranberry media in this study. Further, we found that the effects of cranberry treatment on some physicochemical properties, such as adhesion forces measured with an atomic force microscope and the bacterial zeta potential, revert to their original values when bacteria are removed from cranberry juice and reintroduced to cranberry-free buffer solution (unpublished data from our lab, not shown).

Previous research has identified proanthocyanidins with A-type linkages (PACs) as active components of cranberry juice that are responsible for changes in bacterial adhesion [23]. Several studies have suggested that there exists a dose threshold necessary to inhibit bacterial adhesion [19]. Howell et al. reported that the concentration of PACs with at least one A-type linkage in commercially available cranberry juice cocktail is 0.346 mg/mL [18]. Accordingly, the 5 wt.% solution of cranberry juice we used should contain 64 µg/mL of A-type PACs. This dose value is very similar to the threshold value Howell et. al. reported as the lowest dose needed to exhibit *in vitro* bacterial anti-adhesion activity in the HRBC hemagglutination assay (60 µg/mL A-type PACs), which is a test commonly used to screen for adherence through P fimbriae [18]. Some research has identified another active anti-adhesion fraction or component of cranberries, which
has been termed non-dialyzable material (NDM). NDM was shown to selectively inhibit the adhesion of uropathogenic *E. coli* to animal cells, although the dose was not specified [50, 51]. In a related study, NDM at 100 µg/mL inhibited adhesion of *Helicobacter pylori* to human gastric mucus [52]. Since Bodet et. al. reported that NDM prepared from cranberry juice concentrate contains 65.1% PACs [22], we estimate that 100 µg/mL NDM is equivalent to 65.1 µg/mL PACs. Interestingly, this dose is similar to the threshold level needed to exhibit antiadhesion activity in the present work and in the studies of Howell and colleagues, although perhaps even lower doses could be tested.

The relationship between dose and activity has been found to be non-linear. For example, Gupta et. al. found a semi-logarithm relationship between P-fimbriated *E. coli* attachment to human mammalian cells over a PACs concentration of 5 to 75 µg/mL[10]. This is analogous to the exponential decay relationship we observed between the cranberry juice concentration and the number of attached bacteria (Figure 4), as well as qualitatively similar to the dose relationship based on previous studies of the adhesion forces (Supplementary Data Figure S.1A) and the length of P fimbriae (Supplementary Data Figure S.1B), spanning the dosage range of 0 to 345.8 µg/mL PACs (or 0 to 27 wt.% cranberry juice). In our results, we saw a significant transition between 0 and 64 g/mL PACs (or 0 and 5 wt.% cranberry juice). Although it is not yet known how these *in vitro* thresholds will translate to *in vivo* conditions, researchers are actively engaged in extending laboratory-scale mechanistic studies towards clinical trials [53, 54]. The scope of the present study was not a clinical approach towards the fundamental understanding of how cranberry juice prevents bacterial adhesion. Instead, we approached the mechanisms from a well controlled physical perspective. Even though we cannot
understand the chemical nature of all the important compounds, our work provides evidence of their effects on bacteria-uroepithelial cell interactions, and this should provide encouragement to chemists who are working on other aspects such as isolating, purifying and identifying the active compounds in cranberry juice of the overall problem.

In closing, we have presented a quantitative explanation for the role of cranberry juice in disrupting the binding between P-fimbriated *E. coli* and uroepithelial cell receptors through the use of a thermodynamic model. Future work is geared at identifying the critical dose necessary to impart an anti-adhesive effect, as well as further specifying the nature of active compounds in cranberry that are responsible for such effects.

**Acknowledgements**

This work was supported in part by the National Science Foundation (BES 0238627 and EEC 0452809). P.P.-A received Fellowship support from the American Association of University Women. A.M.G.M. was supported during her stay at WPI by projects 3PR05A021 and MOV05A022 from the “Consejerí de Infraestructuras y Desarrollo Tecnológico” of the “Junta de Extremadura”.
Table 1 Contact angles and surface free energy components of *E. coli* HB101, *E. coli* HB101pDC1 and Uroepithelial cells

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<thead>
<tr>
<th></th>
<th>Contact angle (^a) (Degree)</th>
<th>Parameter value (^b) (mJ·m(^{-2}))</th>
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<tr>
<td></td>
<td>(\theta_W)</td>
<td>(\theta_D)</td>
<td>(\theta_F)</td>
<td>(\gamma^{LW})</td>
<td>(\gamma^-)</td>
<td>(\gamma^+)</td>
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<td></td>
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<td>64±1</td>
<td>47±3</td>
<td>66±3</td>
<td>28.3±1.2</td>
<td>31.2±3.2</td>
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<td>49±1</td>
<td>63±1</td>
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<td>18.4±0.8</td>
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\(^a\) \(\theta_W, \theta_D, \theta_F\): contact angles of water, diiodomethane, and formamide

\(^b\) \(\gamma^{LW}\): Lifshitz-Van der Waals component of interfacial tension; \(\gamma^-, \gamma^+\): electron-donor and electron-acceptor components of interfacial tension; \(\gamma^{AB}\): Lewis acid-base component of interfacial tension, which is the geometric mean of \(\gamma^-\) and \(\gamma^+\); \(\gamma^{Total}\): total surface tension, which is the additive sum of \(\gamma^{LW}\) and \(\gamma^{AB}\).
Figure Captions

Figure 1. Water contact angles of cells as a function of drying time for A) *E. coli* HB101pDC1 with no cranberry juice treatment; B) *E. coli* HB101pDC1 with exposure to 27 wt.% cranberry juice; C) Uroepithelial cells with no cranberry juice treatment.

Figure 2. Effect of cranberry juice treatment on the Gibbs free energy changes upon adhesion for A) *E. coli* HB101pDC1; and B) *E. coli* HB101

Figure 3. Effect of cranberry juice treatment on the three interfaces, i.e. bacterium-uroepithelial cells (C-UC), bacterium-aqueous media (B-L), and uroepithelial cells-aqueous media (UC-L), quantified in terms of the interfacial tension for each pairing, $\gamma_{12}$. A) *E. coli* HB101pDC1; B) *E. coli* HB101

Figure 4. Correlation between Gibbs free energy change upon adhesion and the attachment of bacteria to uroepithelial cells, for A) *E. coli* HB101pDC1 and uroepithelial cells; and B) *E. coli* HB101 and uroepithelial cells.
Figure 1
Figure 2

(A) Cranberry Juice Concentration (pH=7, wt.%) vs. $\Delta G_{adh}$ (mJ/m$^2$)

- Both bacteria and uroepithelial cells treated
- E. coli HB101pDC1 treated only
- Uroepithelial cells treated only

(B) Cranberry Juice Concentration (pH=7, wt.%) vs. $\Delta G_{adh}$ (mJ/m$^2$)

- Both bacteria and uroepithelial cells treated
- E. coli HB101 treated only
- Uroepithelial Cells treated only
Figure 3
Figure 4

A

Exponential Decay fit:

\[ Y = 4.748 + 0.04e^{-x/2.766} \]

\[ R^2 = 0.99 \]

B

System interfacial free energy \( \Delta G_{\text{adh}} \) (mJ·m⁻²)
References

Chapter 5: Impact of Cranberry Juice and Proanthocyanidins (PACs) on the Zeta Potentials of Escherichia coli and Uroepithelial Cells

Abstract
Bacterial surface properties such as electrostatic potential play an important role in bacterial adhesion process, which is widely considered as the first step leading to infections. Cranberry juice and its compound A-type proanthocyanidins (PACs) were used to treat two isogenic uropathogenic E. coli strains and human kidney epithelial cells and the zeta potentials were measured at several cranberry juice or PACs concentrations. P fimbriae were shown to be slightly positively charged, which helps bacteria adhere onto mammalian cells. PACs significantly decreased the bacterial zeta potentials from -15.6 ± 0.9 mV to -41.5 ± 0.7 mV, which increased the electrostatic repulsion forces to mammalian cells. Cranberry juice treatment did not change bacterial zeta potentials significantly, ranging from -14.9 ± 1.8 mV to -16.3 ± 0.8 mV. The abundance of other compounds in cranberry juice other than PACs may have blocked the influence of PACs, considering the relatively small portion of PACs in cranberry juice.

Introduction
Bacterial adhesion is widely considered as the first step leading to infections, food surface contamination, and biofouling. For example, the adherence of uropathogenic E. coli bacteria to human urinary tract epithelial cells is a precursor to the development of urinary tract infections (UTIs). Initial bacterial adhesion is controlled by non-specific and specific forces (Busscher et al., 1992). Non-specific forces arise from intermolecular interactions and include Lifshitz-van der Waals (LW) forces (mainly dipole/induced dipole interactions), electron-donor/acceptor (or called Lewis acid/base or AB forces) and
electrostatic forces. Specific interactions only occur between ligand-receptor pairs. The non-specific forces play a role in determining the favorability of adhesion at a relatively long distance (from a few up to hundreds of nanometers) (Van Oss 1994). Specific interactions only can become operative at very close distances.

With the increasing concern of bacterial resistance and a growing body of patients with UTIs infected (11.3 million in the United States annually (Foxman et al., 2000)), there is much interest in developing alternative prevention and treatment remedies. The American Red Cranberry (*Vaccinium macrocarpon* Ait., family Ericaceae) has long been recognized for its health benefits in the prevention of UTIs, which has been confirmed through clinical studies (Avorn et al., 1994). Recently, A-type proanthocyanidins (PACs) have received a great deal of study, since this isolated compound found in cranberry juice can inhibit bacterial adhesion to bladder, vaginal and uroepithelial cells (Howell 2002; Vorsa et al., 2003; Howell et al., 2005; Howell 2007). However, the mechanisms for cranberry to benefit urinary tract health are not completely understood.

The attachment of bacteria to uroepithelial cells occurs when protein structures on the bacteria, called fimbriae, bind to receptors on uroepithelial cells. In previous work, we found that neutralized cranberry juice changed the P fimbriae on *E. coli* HB101pDC1 by decreasing its length (Liu et al., 2006). The cranberry juice also decreased adhesion between *E. coli* and uroepithelial cells (Camesano et al., 2007).

One method for characterizing the physicochemical properties of cells is through measurements of zeta potential. Zeta potential describes the surface charge of the cells in a given media, and helps explain the non-specific interactions. One study addressed the influences of adsorption of urinary components on the zeta potentials of uropathogen
surfaces (Habash et al., 2000). Urine was collected from volunteers following 3-day regimens of water, cranberry supplements, or ascorbic acid. For volunteers who had consumed cranberry supplements, the zeta potentials of bacteria in urine slightly increased in the positive direction, which was suggested to be due to fructose and tannin metabolites. However, it is not possible to relate the change in zeta potential to the amount of a particular type of compound in the urine, such as PACs, since it is not known how these compounds are metabolized by the body (Kim et al., 2004). Therefore, the aim of the present study was to determine how a known concentration of cranberry juice or PACs would alter the zeta potential of uropathogenic *E. coli*, and also to extend this work to include the zeta potential of uroepithelial cells.

Materials and Methods

Plasmidless, non-fimbriated *E. coli* HB101 (ATCC 33694) and P-fimbriated HB101pDC1 (Lund University, Sweden) were grown in tryptic soy broth at 37°C to middle exponential growth phase. *E. coli* HB101pDC1 were grown in the presence of 20 µg/ml chloramphenicol (Sigma) to ensure the expression of P-fimbriae. Bacteria were washed three times with phosphate buffered saline (PBS) solution (Gallardo-Moreno et al., 2006). According to recent analysis (Howell et al., 2005), PACs has a concentration of 345.8 µg/ml in 27 wt.% cranberry juice. Accordingly, A-type proanthocyanidins (PACs) concentrations are 64, 128 µg/ml in 5 and wt.% cranberry juice, respectively. Stock solutions of PACs were prepared at 345.8 µg/ml by gentle shaking in ultrapure water for ~12 hours. The 64 and 128 µg/ml PACs solutions were prepared from the PACs stock solution. Bacteria after washing were exposed to 0, 5, 10, and 27 wt.% neutralized,
filtered, cranberry juice cocktail (Ocean Spray Cranberries Inc, MA) or 0, 64, 128, and 345.8 µg/ml PACs solutions for 3 hours. PACs were kindly provided by Robin Roderick (Ocean Spray Cranberries Inc, MA).

Human uroepithelial cells (ATCC CRL 9520), were grown in Kailghn’s modification of Ham’s F12 medium and supplemented with 10% fetal bovine serum. Culture flasks were maintained in a 5% CO₂ in air atmosphere at 37 ºC for 7 days and the media was replaced every other day. Harvested uroepithelial cells (1x10⁶ cells/ml) received cranberry or PACs treatments at the same concentrations as were used for bacteria.

The surface charges of uroepithelial cells and bacteria were measured with a Zetasizer Nano ZS (Malvern Instruments, MA) and folded capillary cells (DTS1060), in PBS (pH=7.4). Zeta potential measurements were repeated three times for all samples.

Results and Discussion

Electrostatic properties of P fimbriae

The zeta potentials of *E. coli* HB101 and *E. coli* HB101pDC1 were -19.4 ± 1.6 mV and -15.6 ± 0.9 mV in 0.01 M PBS (Table 1). Due to the presence of protein and polymers, especially carboxylic groups on the outer surface of bacteria, bacteria are negatively charged at neutral pH. HB101pDC1 was less negatively charged than HB101. Since HB101pDC1 was created by inserting a plasmid encoding P fimbriae into HB101 (Connell et al., 1996), the only difference between the two strains is the expression of P fimbriae on the bacterial surface. Hence, the zeta potential difference can be attributed to the electrostatic property of P fimbriae, which suggests that P fimbriae have a positive
charge. P fimbriae are helical structures and contain mainly the proteins PapC, PapA, PapK (around 1000 copies, the major structural pilin), PapE, PapF, and PapG. PapG is the most distal protein of the P fimbriae and is considered as the adhesin (Hultgren et al., 1991; Mu and Bullitt 2006). The electrostatic properties of the adhesin PapG were characterized using isoelectrofocusing (Tewari et al., 1994). Two pI values of isolated PapG moiety were found, at pH values of 5.2 and 9.3. The authors inferred that PapG is an acidic protein with a net negative charge under neutral or even mildly alkaline conditions, but no measurements were made on whole fimbriae which comprise numerous proteins.

Some studies have addressed the electrostatic properties of fimbriae on other types of bacteria, such as the type IV fimbriae of *Pseudomonas aeruginosa*. For example, pilin monomers from *P. aeruginosa* K122-4 have a positive charge along the length of the fiber (Keizer et al., 2001), (Van Schaik et al., 2005). To our knowledge, ours is the first report of the electrostatic properties of intact P fimbriae.

The uroepithelial cells are negatively charged (-14.2 mV in our case; Table 1). The slightly positive charge of the P fimbriae may facilitate the binding of pathogenic bacteria to the negatively-charged host cells.

**Effects of cranberry juice and PACs on physicochemical properties of cells**

With increasing cranberry juice concentration from 5 to 27 wt.%, the zeta potentials of the bacteria or uroepithelial cells did not change significantly (Table 1). The cranberry juice was neutralized before these experiments because pH alone will change the zeta potentials of the cells. There may still be subtle changes in the cells due to
exposure to cranberry juice, as our previous work has suggested (Liu et al., 2007). However, these changes could not be detected with zeta potential since the zeta potential technique can only measure the charge of the outer region of the electrical double layer present on the surface of bacteria. This outer, diffuse, region is less stable since the ions in this layer are not firmly attached.

PACs have been shown to inhibit bacterial adhesion *in vitro* and are considered to be the active anti-adhesive compounds in cranberry. The zeta potentials of bacteria became more negative at higher PACs concentrations (Table 2). The PACs solutions were not neutralized since they were already very close to neutral, ranging from 6.81 to 7.09. Hence, the decrease in zeta potential was attributed to the adsorption of PACs molecules onto the bacterial surfaces. The decrease was not seen with cranberry juice, perhaps because other juice components coated the cell surfaces and blocked the PACs. Since both bacteria and uroepithelial cells became more negatively-charged after PACs treatment, the adsorption may help epithelial cells repel the approach of *E. coli* bacteria.

**Conclusions**

Our experiments showed that P fimbriae are slightly positively charged. The positive charge may help bacteria attach to negatively charged epithelial cells. Cranberry juice after neutralization did not change the electrostatic properties of bacteria or uroepithelial cells. Isolated PACs decreased the surface charge and hence increased the electrostatic repulsion forces between bacteria and uroepithelial cells.
Acknowledgements

Thanks to Robin Roderick (Ocean Spray Cranberries Inc.) for providing us with the A-type proanthocyanidins. This work was partially supported by the National Science Foundation (BES 0238627) and the Cranberry Institute and Wisconsin Cranberry Board.

Literature Cited


### Tables

#### Table 1. Effects of cranberry juice on bacteria and cell zeta potentials

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<th>Cranberry concentration (%)</th>
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#### Table 2. Effects of PACs on bacteria and cell zeta potentials

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<th>E. coli HB101pDC1</th>
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Chapter 6: Direct Adhesion Force Measurement Between Pathogenic 
\textit{E. coli} and Human Uroepithelial Cells Under the Influence of Cranberry

Abstract

Uropathogenic \textit{E. coli} bacteria are the main culprit responsible for urinary tract infections. Bacterial adhesion on uroepithelial cells is the first step of infection. With the increasing concern of antibiotic resistance, the benefits from cranberries towards prevention and treatment of urinary tract infections are of great importance for practical applications. However, the detailed especially the single cell scale mechanisms are still lacking. Adhesion forces between single bacterium and uroepithelial cells are the dominant factor deciding bacterial adhesion. The direct force measurements of such adhesion forces are still lacking. We developed a technique to functionalize AFM with a single intact \textit{E. coli} bacterium as force probe to direct measure the adhesion strength. Our results showed that cranberry juice can significantly decrease the adhesion forces between P-fimbriated uropathogenic \textit{E. coli} and uroepithelial cells. It provide the direct evidence of cranberry preventing UTIs and also offer an accurate method for exploring for further benefits of cranberry products.
Introduction

Urinary tract infections (UTIs) are the second most common infections in the U.S. according to National Institutes of Health. Female are more prone to UTIs than male. More than half of female population will have at least one UTI during there lifetime \(^1\). The annual rate of infection among women in the United States is 11.3 million symptomatic cases \(^2,3\) and over 10 million asymptomatic cases \(^2\). Worldwide, there are around 150 million people suffer UTIs per year, costing approximately $6 billion \(^4\). The Gram-negative bacterium *Escherichia coli* is the main culprit, responsible for 85-95% of cystitis cases and 90% of acute pyelonephritis cases \(^5\). Patients with diabetes mellitus have a higher prevalence of asymptomatic bacteriuria, UTIs, and a higher mortality outside the hospital compared with patients without diabetes mellitus.

Due to the evolution of bacteria, the over prescription and misuse of antibiotics, the bacterial resistance has posed an increasing threat to public health. In terms of UTIs, cotrimoxazole (trimethoprim/sulfamethoxazole), the current first-line treatment for uncomplicated UTIs has encounter a cotrimoxazole resistance exceeding 15% and can be as high as 25% in Canada and the U.S. \(^6\). In recent years, resistance has superseded this threshold in most regions of the US and Europe \(^1\).

Alternative strategies toward prevention or treatment of UTIs are of important applications. The development of UTIs can be divided into three steps: adhesion, invasion and multiplication. Antibiotics can be classified for either bactericidal or bacteriostatic purpose. Bactericidals are designed to invasion step by killing bacteria directly. Bacteriostatics are focused on multiplication step by inhibiting them from dividing. They are passive strategies and are prone to bacterial resistance. Bacterial
adhesion to host cells is the first step leading to following invasion and multiplication. Fundamental understanding of bacterial adhesion process is still lacking.

As one of three native North America berries, the American red cranberry (*Vaccinium macrocarpon* Ait., family Ericaceae) have been experientially recognized for their benefits of maintaining urinary tract health. Sotoba et al. found that preincubation of *E. coli* and uroepithelial cells in cranberry juice decreased bacterial adhesion two decades ago, leading to a paradigm shift in the understanding of the mechanism on bacterial adhesion from the acidity of the urine by consumption cranberries.

The studies of bacterial adhesion step of UTIs can be roughly divided into macro and micro scale. The macro scale studies include: (1) in vivo, such as clinical studies and animal model experiments; (2) in vitro, such as bacterial adhesion assay under static or flow conditions. The macro scale studies can provide a population based statistical evidence of bacterial adhesion and UTIs under various conditions. However, the results also depend on individual samples and naturally require large sample size to obtain statistically meaningful results. In order to understand the effects of consumption of cranberries on prevention and treatment of UTIs, systematic clinical studies started as early as 1979 and continue to present.

The micro scale studies include ultra surface visualization via atomic force microscopy (AFM), transmission electron microscopy (TEM) or modeling bacteria or host cell surface structures and physicochemical properties based on experimental data. Then correlate the changes at the micro scale to macro experiments. Compared to the macro scale experiments, the micro scale studies are more mechanism oriented. However, as a direct quantification of studying bacterial adhesion to...
uroepithelial cells, the direct adhesion force measurements between bacteria and epithelial cells has not been reported yet. Due to the magnitude of the tiny forces, there are mainly two techniques that can direct measure the force \(^{24}\), namely atomic force microscopy (AFM) and force measuring optical tweezers (OT). Recently adhesion force measurements by AFM and OT have been reported in other systems. OT has been used to measure the interaction between uropathogenic \(E. coli\) (fimbriae type not specified) and mannose-presenting self-assembled monolayers (SAMs) that was used to model the epithelial cell surface \(^{25}\). They estimated the force of detachment for a single, monovalent, \(\alpha\)-C-mannoside-pilus interaction is 1.7 pN. OP was also used to study the static and dynamic force response of P and type 1 fimbriae expressed by uropathogenic \(E. coli\) \(^{26}\). A bacterium was mounted on a 9.0 μm hydrophobic bead. Then a 3.0 μm hydrophobic bead was trapped by the OT and brought contact with the \(E. coli\) immobilized on the large bead. The data was acquired when the small bead was separated from \(E. coli\). They found a constant force level at 27 pN for one pilus detachment. The advantage of OT is the force detection sensitivity in the range of 0.1-200 pN range \(^{26}\), which is very suitable to study single molecule binding. However, the interaction between uropathogenic \(E. coli\) and host cells is polyvalent and the adhesins may recognize more than one type molecules. A single \(E. coli\) cell based direct force measurement is more appealing and needed to better study the adhesion between pathogen and host cells. As a good balance of sensitivity and magnitude, AFM can detect interactions range from picoNewtons to nanoNewtons. Besides, molecules \(^{27}\) \(^{28}\) and bacteria \(^{29}\) functionalized AFM tip can be reproducibly obtained, which make it possible to direct measure adhesion forces at the molecule and cell level. AFM tips have been coated with \(Pseudomonas aeruginosa\) \(^{30}\),
Staphylococcus epidermidis, Streptococcus mitis to measure the interactions with poly (ethylene oxide) (PEO)-brush, serum proteins deposited surfaces, and tooth enamel, respectively. However, the force measurement between bacteria and mammalian cells is still lacking.

The aim of present study was firstly to measure the interactive forces between uropathogenic E. coli and human uroepithelial cells. Then evaluate the effects of cranberry juice on the adhesion forces.

Materials and Methods

Bacterial strains and growth conditions

In order to evaluate the role of P fimbriae on bacterial adhesion, two isogenic Escherichia coli strains were chosen. E. coli HB101 (American Type Culture Collection; ATCC 33694) is plasmid-less and non-fimbriated. Escherichia coli mutant HB101pDC1 was created by inserting a plasmid expressing P fimbriae (maintained under chloramphenicol selection, 20 µg/ml). HB101pDC1 was generously provided by Prof. Majlis Svensson (Department of Medical Microbiology, Lund University, Sweden). Bacteria were precultured in Tryptic Soy Broth (TSB, 30 g/L, Sigma, St. Louis, MO) at 37°C overnight and cultured in fresh TSB at 37 °C until reaching an absorbance at 600 nm of 0.7 – 0.8, corresponding to middle exponential growth phase.

Bacteria were centrifuged and washed three times in 0.01 M PBS with a total solution ionic strength of 0.14 M. according to previously reported protocol. Due to the chemical similarity between urine and PBS, PBS was chosen to represent urine and to eliminate sample variation that would be present in urine.
Uroepithelial cells and growth conditions

Human uroepithelial cells were purchased from ATCC (CRL 9520 VA) and kept in liquid nitrogen. Cells were cultured as previously reported. For the force measurement, cells were cultured on petri dish under the same culture conditions.

Cranberry juice and treatment

Cranberry juice cocktail (Ocean Spray Cranberries, Inc. Lakeville-Middleboro, MA) (referred to hereafter as “cranberry juice”) was purchased from a local grocery store. It contains 27 wt.% cranberry juice, and was used to prepare 2.5%, 5.0% and 10 wt.% cranberry juice solutions in 0.01 M PBS. Prior to use, cranberry juice was neutralized to pH 7 to exclude the influence of low pH. Bacteria were incubated in PBS buffer, or 2.5%, 5.0%, 10.0%, and 27 wt.% cranberry juice solutions for 3 hr at 37°C. This exposure time did not cause any loss in viability as detected in our lab and reported by other groups.

AFM force measurements

The interaction forces between E. coli and uroepithelial cells were directly measured by an AFM (Dimension 3100 with Nanoscope IIIa controller, Veeco Metrology, Inc., Santa Barbara, CA) using silicon nitride AFM tips on a triangular cantilever. Spring constants were measured using a thermal technique. They were in the range of 0.09-0.25 N/m, with an error of less than 10% per tip.

The AFM tip was carefully coated with a bacterium with the help of AFM itself using a technique developed in our laboratory. Briefly, the AFM tip was brought to contact with poly-L-lysine (0.1% w/v in water, Sigma, USA) for 5 minutes. Bacteria
pellet after the corresponding cranberry juice treatment was placed on a section of parafilm on a glass slide to form a very thin film of bacteria. The poly-L-lysine-treated AFM tip was positioned over the bacterial film with the help of the equipped optical microscope. Preferably, the AFM tip was above the bacteria film and the cantilever was above bare parafilm, which requires the positioning over the boundary of bacteria film and bare parafilm. Prior to contact with bacteria film, AFM setting parameters such as scan size, scan rate and deflection set point, etc., were adjusted to minimize the lateral movement of AFM tip and the indentation onto the bacteria film. The AFM tip was engaged on the bacteria layer and allowed to contact bacteria for 1-3 minutes.

Confluent uroepithelial cells in the Petri dish were washed with fresh culture medium to remove the suspended dead cells and then replaced with PBS, 2.5%, 5% and 10% cranberry juice solutions right prior to force measurement. The optical microscope was used to position bacterium functionalized AFM tip over uroepithelial cells. A force cycle of 512 data points for each approach and retraction curves were collected. The adhesion forces were calculated from the retraction curves using the established procedures.

**Results and discussion**

Functionalization of AFM tip with intact *E. coli* bacteria

In order to direct measure the tiny biological interaction forces between bacteria and host cells, AFM provides a sensitive diction range suitable for such purposes. Then how to keep the cell surface structure intact remains the big challenge. The coating technique developed in our lab can provide an unmodified bacteria surface for force
measurements as specified in our previous study. There are three methods can be used to verify the successful coating. Namely, resonance frequency shift, characteristic force curves and SEM imaging. The first two techniques have been discussed in detail. Figure 1 A show the intact uroepithelial cells grown in confluence region. Figure 1 A show the SEM imaging of a single E. coli HB101pDC1 coated AFM tip.

Interaction time dependence of adhesion forces between P-fimbriated E. coli and uroepithelial cells

The biological interactions depend on many factors such as surface chemistry, orientation and the residence time. In order to measure the adhesion forces between P-fimbriated E. coli and uroepithelial cells, an appropriate residence time has to be resolved first. AFM not only provides accurate and sensitive force diction when functionalized with bacteria, but also offers a wide range of residence time. The residence time between P-fimbriated E. coli and uroepithelial cells were set as: 1 μs, 10 μs, 100 μs, 1 ms, 10 ms, 100 ms, 1 s, 10 s and 100 s. The corresponding adhesion forces were measured and shown in Figure 2. Among the micro second residence time, i.e. 1 μs, 10 μs, 100 μs, there is no statistically significant difference (P=0.300). The 1 ms and 10 ms residence time did not show statistically significant difference (P=0.985). The 10 ms and 100 ms residence time did not show statistically significant difference either (P=0.068). However, the 1 ms and 100 residence time differed significantly (P=0.006). The 100 ms and 1 s residence time did not show statistically significant difference either (P=0.100). The 1 s and 10 s residence time did not show statistically significant difference (P=0.009). However, both of them showed statistically significant difference with 100 s residence time (P<0.001).
At the presence of ligand-receptor interactions, *E. coli* HB101pDC1 should possess higher adhesion with uroepithelial cells; however, the difference did not show at low residence time (from 1 μs to 10 ms). For *E. coli* HB101pDC1, there is no statistically significant difference between 100 ms and 1 s residence time. The variation characterized by the percentage of standard deviation over the mean value was much smaller for 1 s residence time (25.41%) than the one for 100 ms residence time (70.06%). Hence, 1 s residence time was chosen as the measuring time.

Adhesion forces between *E. coli* bacteria and uroepithelial cells.

P-fimbriated uropathogenic *E. coli* HB101pDC1 is more virulent than non-fimbriated *E. coli* HB101. In vitro bacterial adhesion assay and other macro scale methods such as clinical studies have shown that P-fimbriated *E. coli* have stronger ability in adhering onto host cells. The force measurement results in current study also support these phenomena from the cell scale. The adhesion force between *E. coli* HB101pDC1 and uroepithelial cells is 9.32 ± 2.37 nN; while only 0.74 ± 0.18 nN between *E. coli* HB101 and uroepithelial cells. Many in vitro bacterial adhesion assay and clinical studies have shown that cranberry juice can decrease the P-fimbriated bacterial adhesion.

After cranberry treatment, the adhesion forces between *E. coli* HB101pDC1 and uroepithelial cells decreased to 5.91 ± 1.42 nN, 3.21 ± 0.80 nN, 0.76 ± 0.24 nN, and 0.75 ± 0.19 nN in 2.5%, 5.0%, 10.0% and 27.0 wt.% cranberry juice, respectively. Except 10.0% and 27.0% (P=0.893), the adhesion forces between *E. coli* HB101pDC1 and UC cells differed from other conditions significantly (P<0.001). The adhesion forces between *E. coli* HB101 and uroepithelial cells were 0.74 ± 0.26 nN, 0.78 ± 0.24 nN, 0.72 ± 0.26 nN, and 0.78 ± 0.18 nN in 2.5%, 5.0%, 10.0% and 27.0 wt.% cranberry juice,
respectively. The adhesion forces did not change significantly for *E. coli* HB101 (P=0.794).

**Conclusions**

The current study presented the first direct force measurement of adhesion force between uropathogenic *E. coli* and uroepithelial cells and showed that cranberry treatment can decrease the adhesion forces between P-fimbriated *E. coli* and uroepithelial cells.
References


Figure Captions

**Fig 1** Confluent layer of uroepithelial cells and a single *E. coli* functionalized AFM tip
A) Uroepithelial cells
B) *E. coli* functionalized AFM tip

**Fig 2** Adhesion forces as a function of interaction time

**Fig 3** Adhesion forces between *E. coli* HB101pDC1 and uroepithelial cells under the influence of cranberry juice of various concentrations

**Fig 4** Adhesion forces between *E. coli* HB101 and uroepithelial cells under the influence of cranberry juice of various concentrations
Figure 1

A) Uroepithelial cells

B) *E. coli* functionalized AFM tip
Figure 2

Exponential Fit:
\[ Y = 0.08452 e^{x/1.28915} + 0.17558 \]
\[ R^2 = 0.97 \]
Figure 3

Adhesion force (mN)

Neutralized cranberry juice concentration (wt.%)
Figure 4

Adhesion force (mN)

Neutralized cranberry juice concentration (wt.%)

PBS  2.5%  5.0%  10.0%  27.0%
Chapter 7: Cranberry Prevents the Adhesion of Bacteria: Overview of Relevant Health Benefits

Abstract

With growing antibiotic resistance, alternative therapies and preventions are needed to help control microbial infections in hospital and community environments. By altering the ability of bacteria to adhere to cells in the body, anti-adhesion therapies are a promising complementary method to control infections. Research suggests that ingestion of cranberry compounds can serve as an anti-adhesion treatment for bacteria, bringing benefits in several ways: urinary tract and kidney health, maintaining a healthy oral environment, and prevention of ulcers due to Helicobacter pylori infections. We review the mechanisms by which cranberries alter the ability of bacteria to adhere and lead to health benefits for these body systems. A focus is placed on new molecular level understandings of the role of cranberry on the interactions between Escherichia coli surfaces and uroepithelial cells.

Introduction

Overview of Bacterial Adhesion

The adherence of bacteria to cells or tissues in the body is the propagating step in infections. Bacterial surfaces contain several types of molecules that help them attach to cells, such as proteinaceous fimbriae or pili, flagella, lipopolysaccharides, and capsular polysaccharide molecules. When the bacterial structures find their complementary receptors on mammalian cells, the two bind tightly. In the case of urinary tract infections, fimbriae expressed by *Escherichia coli* (*E. coli*) must bind to receptors on uroepithelial cells. Compounds that prevent this adhesion represent an alternative therapy to the use of antibiotics, since the anti-adhesion molecules do not kill or impair the growth of the
bacteria, yet they are able to prevent the infection from developing. A similar mechanism exists in gastric ulcers. In the case of a *Helicobacter pylori* infection, which can lead to the development of a gastroduodenal ulcer, bacteria must attach to human gastric mucosal cells for the infection to develop. A third bacterial infection that develops following adhesion of bacteria is related to periodontitis, an inflammatory disorder of tooth-supporting tissues. Gram-negative bacteria, such as *Porphyromonas gingivalis* can colonize teeth, gingival epithelial cells, and red blood cells, or interact with other oral bacteria and proteins in the mouth through receptors on their surfaces (1).

In each of these three systems, cranberry compounds have been implicated in preventing the bacterial adhesion process, thus presenting a complementary or alternative methodology to prevent urinary tract infections (2, 3), *H. pylori* infections (4, 5), and periodontitis (1). We present a brief overview of the current knowledge of how cranberry is beneficial for these three systems.

Cranberries and UTIs

The American red cranberry (*Vaccinium macrocarpon* Ait., family Ericaceae) has long been recognized for benefits to maintenance of a healthy urinary tract. This is especially a concern for women, 1/3 of whom will have at least one UTI in their lifetime (6), leading to the infection of 11.3 million women per year in the U.S. alone (7). Elderly women are also extremely prone to UTIs, with some women over 65 experiencing at least one UTI per year (8, 9).

UTIs are caused when bacteria attach to and colonize mucosa surfaces in the urinary system (10). The resulting infection can range from cystitis (bladder infection) to
a more serious illness, acute pyelonephritis (kidney infection). The Gram-negative bacterium *E. coli* is implicated in 85-95% of cystitis and 90% of pyelonephritis infections in women (11). If untreated, UTIs can cause kidney failure, and in some cases death (10, 12, 13).

Cranberry effects on *E. coli*: *In vivo* studies of urinary tract health

The pioneering clinical trial of Avorn et al. (3) was the first to conclusively demonstrate that consumption of cranberry juice helped prevent recurrent urinary tract infections in women. This study was conducted on female residents of a long-term care facility. The women drank 300 mL/day of artificially sweetened cranberry juice or a placebo with similar color and taste for a period of 6 months. After one month, the prevalence of bacteria in the urine of the cranberry juice drinkers was significantly decreased.

Kontiokari et al. studied 150 university women (mean age of 30) who presented to the Finnish University of Oulu’s student health center or occupational clinic, and had clinically documented *E. coli* UTIs (14). The three groups received either 1) cranberry-lingonberry juice concentrate (50 mL/day for 6 months), 2) 100 mL of a probiotic *Lactobacillus* GG drink, five times per week, for one year, or 3) a control group who did not receive any intervention. The rate of recurrence of UTIs in the 12 months following the study was statistically different among these treatment groups. The overall absolute risk of recurrence of UTI was reduced by 20% for the cranberry group compared to the control group, but a benefit was not seen due to lingonberry (14).

Stothers et al. (15) studied 150 women (ages 21 to 72) who had prior histories of UTIs (≥ 2 in previous year), and provided them with either cranberry juice (250 mL at
three times per day + placebo tablet), cranberry extract in pill form + placebo juice, or both juice and pills that were non-cranberry containing placebos, and followed the women for one year. The tablet group had the least recurrence of UTI in the following year (18%), with the cranberry juice group having a similar but significantly different recurrence rate of 20%. Both the tablet and juice groups had much lower recurrence than the non-cranberry placebo group, where 32% infection recurrence was observed.

In a pilot study of five women with culture-confirmed UTIs, participants who ate sweetened dried cranberries (SDC) in a single dose exhibited anti-adherence properties in their urine that were comparable to consuming a cranberry juice cocktail drink (16). More data from this and other clinical investigations will help demonstrate if SDCs can be used for prevention of UTIs in the same way as cranberry juice cocktail (CJC).

Cranberry effects on *E. coli*: *In vitro* studies related to urinary tract health

While the earliest studies suggested that acidification of urine was responsible for cranberry’s benefits towards UT health (17), research since the 1980s has focused on the anti-adhesive properties of cranberry juice, and recent studies demonstrated that the pH of urine (after cranberry consumption) is only slightly decreased and that the effect is transient (18, 19), or showed no decrease in urine pH (2).

All uropathogenic *E. coli* (UPEC) isolates express protein molecules on their surfaces, known as fimbriae. These molecules include the nearly universally expressed type 1 fimbriae, which bind to a lectin on uroepithelial cells (20), and P fimbriae, which are associated with 23% of cystitis infections and nearly all pyelonephritis infections (21). Type 1 fimbriae are mannose sensitive, meaning that any mannose type sugar (i.e. fructose, common to all fruit juices) can block this protein from being able to attach to
eukaryotic cells (22). P fimbriae are mannose resistant, but their binding to uroepithelial cells can be blocked by other compounds found in cranberries (23).

The ground-breaking studies demonstrating an in vivo effect of cranberry juice on bacterial adhesion to epithelial cells were performed in the 1980s, although the bacterial surface fimbriae were not investigated in these initial studies (19, 22, 24). Next, researchers began to characterize how cranberry affected bacteria with specific types of fimbriae. Zafriri et al. were the first to postulate that different compounds in cranberry could affect P and type 1 fimbriae, with their studies showing that fructose inhibited the adhesion of bacteria with type 1 fimbriae only (22). In a follow up study, these researchers tried to characterize the material that was effective against type P-fimbriated bacteria, and they determined that a high molecular weight, non-dialyzable material (NDM) inhibited the adhesion of UPEC to epithelial cells (25). A breakthrough came in 1998, when Howell et al. identified through directed fractionation, specific proanthocyanidin compounds in cranberry that caused P-fimbriated *E. coli* to exhibit anti-adhesion properties (23). The chemical structure of these compounds was further elucidated (26, 27). The studies of these two independent groups suggest that perhaps multiple mechanisms of anti-adhesive properties can be demonstrated against bacteria, and different compounds could be responsible for the different effects.

Current laboratory research in this area approaches the problems from multiple perspectives, including: characterization of the types of proanthocyanidins in terms of their chemical structures (28); determination of whether the beneficial compounds in cranberries are degraded by the body and elucidating their ultimate form in urine (2), microbiological studies focusing on the genes responsible for the production of fimbriae,
and the role of particular fimbrial proteins in determining adhesion of the *E. coli* to uroepithelial cells (29), physical characterizations of the conformation and morphology of bacterial fimbriae (30), and physical interaction force measurements between *E. coli* bacteria and uroepithelial cells (31).
Better Specification of Beneficial Compounds

In order to identify precisely which compounds have anti-adhesion activity, Howell et al. conducted in vitro studies of the anti-adhesive capability of A-type and B-type proanthocyanidins isolated from cranberry, and other foods, including grape and apple juices, green tea, and dark chocolate (28). The type-A proanthocyanidins isolated from cranberry juice had anti-adherence activity at the lowest dose of 60 µg/mL. The B-type proanthocyanidins from grape had some activity at a much higher dose (1200 µg/mL), while none of the other B-type proanthocyanidins had any in vitro anti-adherence activity.

Are Beneficial Compounds Degraded in Urine?

DiMartino et al. studied 10 healthy men and 10 healthy women (21-25 years old) in a placebo-controlled double-blind investigation of cranberry consumption on in vitro bacterial adherence (2). Volunteers consumed a single dose of between 0 and 750 mL of cranberry juice, or an appropriate dilution with mineral water or placebo beverage. All of the participants eventually consumed all four of the tested doses in random order, with a “washout” period of >6 days in between. UPEC strains were grown in their urine and the ability of these *E. coli* to attach to bladder epithelial cells was then characterized. A statistically significant decrease in bacterial adherence was noted for the cranberry groups compared to the control groups, and this was dose dependent. These results suggest that growth in cranberry can condition bacteria and change their properties, making them less able to adhere to epithelial cells.
Physical and Morphological Effects of Cranberry on *E. coli* Bacteria

Some laboratory studies have concentrated on understanding how cranberry compounds affect fimbriae on *E. coli*, especially P fimbriae. For example, Ahuja et al. suggested that growth in media containing cranberry caused *E. coli* to be unable to express their P fimbriae (32). In another study where bacteria were not grown in cranberry juice but exposed to it after growth in normal media, Liu et al. used atomic force microscopy (AFM) to probe the physical conformation of P fimbriae on *E. coli* HB101pDC1 that were exposed to cranberry juice cocktail (CJC) in concentrations ranging from 0 to 20% CJC (30). They found that CJC caused the P fimbriae to collapse on the surface of the *E. coli* cells, decreasing the protein’s height and ability to extend from the surface of the bacteria. Molecular adhesion forces between the *E. coli* cells with collapsed fimbriae were significantly decreased compared to the molecular adhesion forces between the control (i.e. non-infective) strain of *E. coli*. This was the first study to quantify the molecular adhesion forces for *E. coli* treated with cranberry juice.

Recent Progress in Development of Molecular Mechanisms of Cranberry Action Against *E. coli*

Recently, we have investigated the molecular scale effects of cranberry compounds on *E. coli* bacteria (31). We examined the morphology and cellular membrane properties of *E. coli* HB101 cells grown in culture media (tryptic soy broth; TSB) supplemented with cranberry juice, compared to *E. coli* grown in only TSB. The cranberry juice was neutralized to pH 7.0 before the bacterial growth experiments. The growth rate of the bacteria changed in an unpredictable manner when their growth media was supplemented with 10% CJC. Initially the bacterial growth rate decreased, but then
after some time of acclimation, they resumed normal growth rates. In addition, Gram staining of the bacterial membrane revealed that culture in media supplemented with CJC changed the cellular membrane of the *E. coli*. For example, Figure 1A shows *E. coli* HB101 bacteria grown in only TSB, and stained with a Gram stain. The *E. coli* appear pink, which is characteristic for Gram-negative bacteria. For the *E. coli* bacteria that had been grown in media supplemented with CJC, some of the cells stained pink while some stained purple (Figure 1B). The purple appearance is an indication of Gram-positive bacteria and is an unusual finding for *E. coli*. While the mechanism of action is not yet clear, we speculate that some compounds from the cranberry juice are altering either the peptidoglycan layer or lipopolysaccharide layer of the *E. coli*, causing these apparent changes in the cell wall organization.

![Figure 1](image_url)

**Figure 1.** A) *E. coli* HB101 grown in TSB. B) *E. coli* HB101 grown in TSB supplemented with 10% neutralized CJC.

In addition, we are using a nanotechnology-based tool, atomic force microscopy (AFM), to measure the nanoscale adhesion forces between *E. coli* bacteria and uroepithelial cells. By combining nanoscopic force measurements with calculations of the interaction energies surrounding bacteria and uroepithelial cells, we have found that
cranberry juice affects the nature of the *E. coli*-uroepithelial cell in several ways: 1) cranberry juice causes P fimbriae on the *E. coli* to collapse, thus being unable to form attachments to uroepithelial cells (30), 2) cranberry juice causes an “energy barrier” to build up around the *E. coli* and the uroepithelial cells, thus making it unfavourable for the two to make contact with one another (31), and 3) cranberry juice decreases the forces of adhesion between P fimbriated *E. coli* and urinary tract cells from 9.64 nN (in buffer alone) to 0.50 nN (in buffer plus 10% cranberry juice; Figure 2) (31). Our nanoscale measurements can help researchers elucidate the mechanisms by which cranberry compounds can block the adhesion of *E. coli* bacteria to uroepithelial cells.

![Graph showing adhesion force between *E. coli* HB101pDC1 and uroepithelial cells.]  
*Figure 2. Adhesion force between individual bacterial cells of *E. coli* HB101pDC1 (expresses P fimbriae) and uroepithelial cells, probed in phosphate buffered saline (PBS), or in buffer supplemented with CJC at concentrations of 2.5, 5.0 or 10.0 %.*

Cranberry and *H. pylori*
A high molecular weight, non-dialyzable material (NDM) isolated from cranberry juice inhibited the adhesion of three different strains of *H. pylori* to human erythrocytes and human gastric mucous (33). These bacterial strains were found to have a sialic-acid specific adhesin on their surface. It is hypothesized that the compounds from cranberry blocked the ability of this adhesin to attach to receptors on the immobilized human mucus. A follow-up study examined the adhesion behaviour of 83 strains of *H. pylori*, and confirmed that 0.2 mg/mL of NDM was sufficient to inhibit adhesion of 53/83 of the strains (63.86%) to gastric cells (5). This research suggested that consumption of cranberry would make it more difficult for *H. pylori* to colonize the mucus and the epithelium of the gut, thus representing a possible preventive measure against peptic ulcers caused by *H. pylori*. It may be possible to use cranberry in combination with antibiotics to prevent infections from recurring.

A randomized, double-blind, placebo-controlled clinical study investigated 189 adults infected with *H. pylori* (4). The cranberry juice group drank two boxes containing 150 mL cranberry juice per day for 90 days, while the control group received a placebo beverage at the same frequency and duration. At both 35 and 90 days after intervention, 14 of 97 participants (14.43%) from the cranberry group and 5 of 92 participants (5.43%) from the control group were free of *H. pylori*, as determined by a $^{13}$C-urea breath test.

**Cranberry’s Action Against Oral Bacteria**

Cranberry can also act against oral bacteria. For example, a high-molecular weight NDM of cranberry juice inhibited coaggregation of oral bacteria (25, 34) and reduced salivary counts of oral bacteria (34). Further, this NDM inhibited the ability of *P. gingivalis* to form biofilms, and prevented the microbes from attaching to surfaces.
coated with proteins, such as type I collagen, fibrinogen, and human serum, which represent periodontal sites (1). A pilot-type clinical study showed that six weeks of daily use of a mouthwash containing cranberry NDM reduced counts of mutans streptococci and total bacteria in saliva, compared to a control group receiving placebo mouthwash (35). Due to these encouraging results, it is likely that more clinical studies will follow.

Future Research Needs

The use of cranberry as an anti-adhesive therapy for preventing a wide range of infections has great potential. Clinical studies have focused mainly on urinary tract health (3, 14), and a few recent trials related to H. pylori (4) and oral bacteria (35), but future studies will likely be performed. Scientifically and clinically, more questions need to be answered so that the appropriate dose, frequency, and duration of cranberry needed to bring about these benefits can be identified. Further, scientists can continue to search for other infections where cranberry can be of some benefit. A combination of molecular level characterization of bacterial interactions with genetic techniques to identify the genes responsible for the adhesion process can help in determining the mechanisms of bacterial adhesion for each system. These laboratory studies will be combined with clinical studies that seek to establish the optimal conditions for providing benefits.
Reference and Notes

17. Blatherwick, N.R., Archives of Internal Medicine, 14, 409-450, (1914).
Part III: Implanted Medical Device Related Infections and Biomaterial Development
Chapter 8: Thermodynamic Investigation of Staphylococcus epidermidis Interactions with Protein-Coated Substrata

Abstract

We evaluated self-assembled monolayers (SAMs) as potential coatings to prevent bacterial adhesion to biomaterials. Bacterial retention experiments were conducted on SAMs, some of which were coated with the model proteins fetal bovine serum (FBS) and fibronectin (FN). A thermodynamic approach was applied to calculate the Gibbs free energy changes of adhesion ($\Delta G_{adh}$) of Staphylococcus epidermidis interacting with the substrates. When only non-specific interactions controlled bacterial attachment, such as for the non-protein coated substrates or the FBS-substrates, the correlation between the thermodynamic predictions and measured values of bacterial retention was strong. However, when FN was adsorbed to the surfaces, the thermodynamic modeling underestimated bacterial adhesion, presumably since specific interactions between proteins of S. epidermidis and FN led to stronger attachment. Bacterial viability on the substrates was correlated with thermodynamic properties. For example, although bacteria attached more to surfaces having negative $\Delta G_{adh}$ values, these cells experienced the greatest loss of viability, presumably since strongly attached bacteria were unable to divide and grow. When the $\Delta G_{adh}$ values were decoupled into their components, we saw that acid-base interactions due to hydrogen bonding dominated the interactions of bacteria and proteins with each other and with the substrates in aqueous media. Finally, we discuss concerns regarding the use of the thermodynamic model to predict bacterial adhesion behavior in biomaterials systems.
Introduction

The formation of biofilms on implanted medical devices represents a serious concern since biofilm-associated infections may not be treatable with antibiotics and can require implant removal. A practical approach to infection prevention is to inhibit the establishment of biofilms by blocking the initial bacterial adhesion steps. The objective of a growing number of biomedical studies is to find promising implant materials that can minimize bacterial colonization. For example, self-assembled monolayers (SAMs) that include poly(ethylene oxide) and other polymers have demonstrated the ability to prevent the retention of bacteria to gold-coated substrates by altering substrate characteristics such as wettability, roughness, and surface free energy. SAMs provide a uniform layer of molecules with the same terminal group and points of orientation, properties that facilitate their use in materials research. While many studies have focused on the materials and chemistry aspects of antimicrobial SAM developments, few studies have fully considered how the material will be changed by the deposition of serum proteins when the substrate is placed in the body, although the need to evaluate biomaterials in the presence of serum proteins has been noted. Since serum proteins will readily adsorb to biomaterials upon device implantation, biomaterials must be evaluated for their interactions with proteins as well as with bacteria in order to better represent the physiological situation.

Long-range interactions that govern whether bacteria can contact a substrate are determined by physicochemical properties like charge and hydrophobicity. If charge is neglected due to the induced balance charge of double layer superimposition, then a thermodynamic approach can be used to describe the non-specific adhesion of bacteria to
a surface by evaluating the surface free energies of microbes and substrates, as reviewed in \(^9\). The most widely used thermodynamic approach is to consider the Gibbs free energy change upon bacterial adhesion to a substrate as the interfacial tension of the new interface (bacteria/substrate) minus the interfacial tensions of the old interfaces (bacteria/liquid and substrate/liquid). The surface component approach was first suggested by Fowkes in the 1960s \(^{10, 11}\), although the version proposed by van Oss, Chaudhury, and Good (VCG) has been the most widely applied in the bacterial adhesion literature \(^9, 12, 13\). In the VCG model, LW and AB interactions are added together, and AB interactions are further subdivided into electron donor and electron acceptor contributions.

The intermolecular forces described in colloidal interaction models or thermodynamic predictions such as these do not account for stronger, specific types of interactions that can occur between bacterial ligands and certain receptor molecules. These specific interactions represent the second step of bacterial adhesion, and can only occur after the non-specific interactions bring bacteria close enough to the substrate of interest. For example, molecules on the surface of \textit{S. epidermidis} can form tight bonds with certain proteins, including fibronectin (FN) \(^{14, 15}\).

The goal of this study was to predict the adhesion behavior of \textit{S. epidermidis} interacting with protein coated and non-protein coated SAMs, using the VCG thermodynamic approach. The Gibbs free energy analysis was correlated with bacterial retention and viability. Based on the interfacial free energy analysis, predictions are made of substrate properties that would lead to the optimal design of anti-adhesive and antimicrobial biomaterials.
Materials and Methods

Bacterial Cultures

The Gram-positive bacterium, *Staphylococcus epidermidis*, a clinical isolate, was a gift from Dr. Stephen Heard (Department of Anesthesiology, University of Massachusetts Medical School, Worcester, MA, USA). Bacteria were precultured in 20 mL of Tryptic Soy Broth (TSB) solution (30 g/L) (Sigma, St. Louis, MO) in the incubator at 37°C overnight. One mL of precultured bacteria solution was added to 30-50 mL of TSB at 37°C in a water shaker bath. Bacteria were grown to the middle exponential phase, monitored by a spectrophotometer at 600 nm. Cells were harvested by centrifuging at 1400 g for 15 min (Fisher Scientific, Pittsburgh, PA) and washed three times with 0.1 M 2-(N-Morpholino)ethanesulfonic acid (MES) buffer solution at pH=7.1. MES buffer can provide a mimic physiological condition.

Substrata

SAMs terminating in isophthalic acid (IPA) and isophthalic acid with silver (IAG) were created on gold-coated glass slides (Figure 1) and characterized as previously described\(^7,16\). The substrata considered were bare gold and SAMs on gold, and for some experiments the SAMs were covered with either of two model proteins, fetal bovine serum (FBS) or fibronectin (FN). In a previous study, we determined the optimal serum protein concentration and deposition conditions to be used\(^16\). Briefly, substrata were immersed in 10 vol% FBS in the incubator at 37°C for 80 min, or for FN, substrata were immersed in 5 μg/ml FN solution in the incubator at 37°C for 24 hrs. The morphology of the protein layers was characterized by atomic force microscopy (AFM), as we reported on previously\(^16\).
Bacterial Retention Experiments

A batch assay was used to quantify bacterial retention on the substrates, and a dual-staining technique was used to characterize the viability of the retained cells. Each substrate was immersed in bacterial solution in the incubator at 37°C for 30 min. Substrates were removed from solution and rinsed with MES using a micropipettor to discard loosely attached bacteria. Retained cells on the substrata were stained with a BacLight™ live/dead kit with final concentrations of 0.835 μm propidium iodide (PI) and 0.5 μm Syto 9™ in bacterial solution (Molecular Probes, Faraday, CA) and examined with fluorescence microscopy (Nikon Eclipse 400 fluorescence microscope, Tokyo, Japan with a mounted 18.2 Color Mosaic Camera; Diagnostic Instruments, Inc., Sterling Heights, MI), using a 100X oil immersion lens. Texas Red and FITC filters were used to observe bacteria stained with PI (red; stains membrane compromised cells only) and Syto 9 (green; stains all cells). At least 15 random locations were examined per slide and at least 4 slides were analyzed for each substrate. We previously verified that the dual stain technique as applied here gives reliable results that are consistent with the information obtained by counting colonies of bacteria formed on agar plates.

Contact Angle Experiments

Surface hydrophobicities for bacteria and substrates were characterized by measuring the contact angles of three probe liquids with different polarities, using ultrapure water, diiodomethane (99% pure, Alfa Aesar, Ward Hill, MA), and formamide (99.5% pure, J.T. Baker; Phillipsburg, NJ). Measurements were made at room temperature and ambient humidity using the sessile drop technique with a goniometer.
(Ramé-Hart, Netcong, NJ, USA). The deposition of 2 μl droplets on each substrate was recorded in a video and analyzed to obtain the contact angles.

Bare substrata were cleaned and dried under nitrogen gas as described previously, immediately prior to contact angle measurements. For serum protein-adsorbed substrata, the contact angle was measured on a thin layer of air-dried proteins.

Three to four ml of bacterial solution at ~1.5×10^9 cells/ml (after washing), were deposited onto 0.45-μm cellulose acetate membranes (Millipore, Billerica, MA) using negative pressure, which corresponds to 60-90 layers bacteria coverage onto the filter membrane. An appropriate multiple layers of bacteria coverage is important to yield accurate contact angle measurements. Water contact angles were measured on the bacteria deposited filters as a function of time to pre-determine the drying time. Previous research in our laboratory suggested that ~ 50 min are needed for evaporation of the loosely held moisture associated with S. epidermidis (unpublished data). When the water contact angles reached a plateau, the moisture among the cellular exterior evaporated while the cells were not dehydrated. At the time, cell surface structures such as lipopolysaccharides and proteins retained their moisture, and therefore accurate contact angles of each probe liquid on the microbial lawns could be measured.

At least 6 random locations were examined per slide and at least 5 slides were analyzed for each substrate or bacteria.

Calculation of Interfacial Free Energies

The surface free energies of individual substrata are derived from the contact angles of the three probe liquids using the methodology described by van Oss and
colleagues. The total surface tension, \( \gamma \), can be considered the additive sum of the LW and AB components of the surface tension, given by

\[
\gamma = \gamma_{LW} + \gamma_{AB}
\]  

(1)

The AB component of the surface tension (\( \gamma_{AB} \)) is the geometric mean of the electron- donor (\( \gamma^- \)) and electron-acceptor (\( \gamma^+ \)) parameters for the applied liquid or the substrata, given by

\[
\gamma_{AB} = 2 \cdot \sqrt{\gamma^+ \cdot \gamma^-}
\]  

(2)

The relationships between the three components of the surface tensions of a solid surface, i.e. \( \gamma_{LW} \), \( \gamma^- \) and \( \gamma^+ \), and the known surface tensions of the probe liquids, are calculated by the Young-Dupré equation:

\[
\gamma_L (\cos \theta_L + 1) = 2 \cdot \sqrt{\gamma_{LW} \cdot \gamma_{LW}^L} + 2 \cdot \sqrt{\gamma_i^+ \cdot \gamma_L^-} + 2 \cdot \sqrt{\gamma_i^- \cdot \gamma_L^+}
\]  

(3)

where the subscript \( i \) can refer to any of the solid substrates probed or the bacterial lawn, considered to be a solid surface, and \( L \) can refer to probe liquids. Since there are three unknowns in equation (3), three equations are required to solve for the three unknowns. Hence three probe liquids with different polarity need to be chosen. Water is always been chosen. Apolar molecule, diiodomethane is also chose. Polar molecule, such as formamide or glycerol either can be chosen. In our study, formamide was chosen. Thus L denotes water (W), diiodomethane (D), or formamide (F). Reference values for the surface tensions of the probe liquids were taken from ref. After measuring contact angles on all of the solid surfaces with the three probe liquids, eq. 3 was solved for each liquid, so that the three unknown interfacial tension components were obtained.
Using eq. 4, we calculated $\gamma_{i}^{LW}$, $\gamma_{i}^{+}$, and $\gamma_{i}^{-}$ corresponding to $i = S$ (for all of the solid surfaces, i.e. gold, the SAMs, surfaces with adsorbed proteins, etc.) and for $i = B$ (for the bacterial lawn as a solid surface in contact with the liquid).

We next calculated the Gibbs free energy change upon bacteria-substrate adhesion in aqueous media, $\Delta G_{adh}$, which was based on the interfacial tensions for bacteria/substrate, bacterial/water, and substrate/water\(^{18}\), as

$$\Delta G_{adh} = \gamma_{BS} - \gamma_{BW} - \gamma_{SW} \tag{5}$$

where B, S, and W correspond to bacteria, solid substrates, and water, respectively.

When calculating protein-liquid medium-protein system, the equation (5) is applied again.

$$\Delta G_{adh} = \gamma_{PP_{j}} - \gamma_{PW} - \gamma_{P_{j}W} \tag{5a}$$

where $P_{i}$, $P_{j}$, and W denote protein in the liquid (FBS or FN), protein on the substrata (FBS or FN), and liquid medium, respectively.

The interfacial tensions in eq. 5 were calculated using the general form

$$\gamma_{12} = \left(\sqrt{\gamma_{1}^{LW} - \gamma_{2}^{LW}}\right)^{2} + 2 \cdot \left[\sqrt{\gamma_{1}^{+}} - \sqrt{\gamma_{2}^{+}}\right] \cdot \left[\sqrt{\gamma_{1}^{-}} - \sqrt{\gamma_{2}^{-}}\right] \tag{6}$$

where 1 and 2 can refer to B, S, and W.

All of the LW terms and AB terms can each be collected so that an alternate way to represent the different contributions to the Gibbs free energy change upon adhesion in
aqueous media can be written. By inserting eq. 6 into eq. 5 and collecting terms, we obtain \(^\text{18}\):

\[
\Delta G_{\text{adh}} = \Delta G_{\text{adh}}^L \Delta G_{\text{adh}}^B
\]  

(7)

where these contributions are calculated according to

\[
\Delta G_{\text{adh}}^L = \left( \sqrt{\gamma_{B}^L} - \sqrt{\gamma_{S}^L} \right)^2 - \left( \sqrt{\gamma_{B}^L} - \sqrt{\gamma_{W}^L} \right)^2 - \left( \sqrt{\gamma_{S}^L} - \sqrt{\gamma_{W}^L} \right)^2
\]  

(8)

and

\[
\Delta G_{\text{adh}}^B = 2 \cdot \left[ \sqrt{\gamma_{W}^L} \cdot \left( \sqrt{\gamma_{B}^L} \sqrt{\gamma_{W}^L} - \sqrt{\gamma_{S}^L} \sqrt{\gamma_{W}^L} \right) + \sqrt{\gamma_{W}^L} \cdot \left( \sqrt{\gamma_{B}^L} + \sqrt{\gamma_{S}^L} - \sqrt{\gamma_{W}^L} \right) - \sqrt{\gamma_{B}^L} \gamma_{S}^L - \sqrt{\gamma_{B}^L} \gamma_{S}^L \right]
\]  

(9)

Surface free energy and surface tension, interfacial free energy and interracial tension are used interchangeably in this paper.

Results

Bacterial retention and viability on substrata

Batch assays were used to characterize the retention and viability of \(S. \text{epidermidis}\) interacting with each surface (Table 1). Gold slides retained the most bacteria and had the greatest effect on cellular viability (82.7 ± 14.3% of retained cells were non-viable), with lower retention and less loss of viability on IPA and IAG. A one-way ANOVA test showed significant differences in bacterial retention on either SAM (IPA or IAG) compared to bare gold (p<0.05).

Bacteria were retained less on any FBS-coated substrate compared to the corresponding uncoated surface (Table 1). The FBS also appeared to provide some protection for the cells, with all of the FBS-adsorbed substrates showing lower percentages of non-viable cells than for the corresponding substrata without proteins. However, once the protein was present, the underlying effects of substrate properties on
bacterial retention could not be discriminated. A one-way ANOVA test showed no significant difference in bacterial retention for gold, IPA, and IAG after FBS deposition (p>0.05).

Bacterial retention was favorable on the FN-coated substrata, compared with either the corresponding uncoated or FBS-coated surfaces (Table 1). (The exception is for FN on gold, which resulted in lower bacterial retention than bare gold). More than an order-of-magnitude difference in bacterial retention was observed when comparing the surfaces coated with FN compared to FBS, even though the added concentration of bacteria was identical. A one-way ANOVA test showed significant differences in bacterial retention for FN adsorbed to IPA and IAG compared with FBS adsorbed to IPA and IAG, and also when comparing with bare IPA and IAG substrates (p<0.05). Although IAG resulted in a large decrease in cellular viability compared to IPA, even with FN in the system. This suggests that some silver ions may have migrated through the FN protein layer. In contrast, most cells were still viable when retained on IAG + FBS (~97%), suggesting that the silver ions did not migrate through the FBS layer.

Surface thermodynamic properties of bacteria and substrata

**Bacterial properties.** The components of the interfacial tensions for each substratum and *S. epidermidis* cells were derived (Table 2). The LW component of the surface tension of *S. epidermidis* was 31.979 mJ·m⁻². This value does not vary greatly among bacterial strains and is within 10% of 40 mJ/m² for >140 bacterial strains that have been studied, with a few exceptions. The electron-donor component of the surface tension for *S. epidermidis*, γ⁻ (5.484 mJ·m⁻²) is much greater than the electron-acceptor component of the surface tension, γ⁺ (3.984x10⁻⁶ mJ·m⁻²), which may suggest
that the bacteria has a strongly monopolar surface, or favors the electron-donating or Lewis base properties. Some researchers have cautioned that comparison of the magnitude of the Lewis acid and Lewis base surface tension components for a given surface are not valid, since the choice of reference values assigned to water in the original theory affect the magnitude of the numbers calculated \(^\text{21, 22}\). It is considered meaningful, however, to compare the acidic components of several different substrates among one another, and likewise to compare the basic components of several substrates \(^\text{21, 22}\).

**Substrata properties.** The substrates showed varying degrees of hydrophobicity (Table 2). When the contact angle of water on gold was measured immediately after cleaning (within 1 min), the surface appeared hydrophilic (water contact angle of 36 ± 0°). In practice it was very difficult to maintain this level of hydrophilicity for the gold since particles suspended in the air immediately adsorbed to the surface. Therefore, it is unlikely that the gold surfaces that the bacteria saw in the retention assays were hydrophilic. When the contact angles were measured on gold after exposure to air for a few minutes, the values were consistently higher (89 ± 2°). Indeed, research from the 1960s has shown that metal surfaces, including gold, are non-wetting when cleaned in ultrahigh vacuum but appear hydrophobic under typical laboratory conditions \(^\text{23}\).

The contact angles on the SAMs (IPA and IAG) were similar to one another for all probe liquids. IPA showed a higher \(\gamma\) than IAG, which can be correlated with the surface structure. IPA has two carboxylic terminals per molecule while for IAG, the two negatively charged carboxylic terminals are partially neutralized by silver ions (Figure 1) \(^7\).
Adsorbed FBS and FN. After FBS deposition, the substrates were more hydrophilic and more monopolar (i.e. greater separation in the $\tilde{\gamma}^-/\tilde{\gamma}^+$ values relative to one another; Table 2). The FBS-coated SAMs were more basic than the FBS-coated gold, and the dispersion forces ($\tilde{\gamma}^{\text{LW}}$) had a decreased relative contribution to the total interfacial tension on the SAMs compared to the FBS-gold substrate. For the FN-adsorbed substrates, the surfaces became more hydrophilic and more basic than the non-protein coated substrata (Table 2). Comparing the two proteins, the water contact angles and the derived interfacial tensions were similar except for on the gold slides, where the FN-gold substrate was more hydrophilic than the FBS-gold substrate.

Gibbs free energy change upon bacterial adhesion to substrate ($\Delta G_{\text{adh}}$)

Correlation between $\Delta G_{\text{adh}}$ and bacterial retention. Based on the surface tensions of individual substrata, S. epidermidis, and the aqueous media, $\Delta G_{\text{adh}}$ values in water were calculated and correlated with bacterial retention (Figure 2). The Gibbs free energy of adhesion was strongly correlated with the mean number of bacteria retained on bare substrates and FBS-adsorbed substrates (Figure 2A). Negative $\Delta G_{\text{adh}}$ values corresponded to significant retained S. epidermidis cells from the batch assays, such as for gold and the SAMs. While fewer cells were retained for systems having positive $\Delta G_{\text{adh}}$ values, the trend did not hold for the FN-adsorbed surfaces, where bacterial retention was high despite very positive $\Delta G_{\text{adh}}$ values (Figure 2B).

The Gibbs free energy change upon adhesion was also related to cellular viability. A strong negative correlation was observed between $\Delta G_{\text{adh}}$ and the percentage of non-
viable bacteria, regardless of the substrate or whether proteins were present (Figure 3). The only outlier was for the FN + IAG substrate.

Interfacial tensions for the three different interfaces. In order to compare the energetic favorability of existing in the two old interfaces (bacteria/liquid, substrata/liquid) compared with the new interface (bacteria/substrata), each interfacial tension component was calculated according to eq. (5), following the VCG approach. The interfacial tensions $\gamma_{BS}$ and $\gamma_{SL}$, along with the Gibbs free energy change of adhesion are shown in Figure 4 for each substrate and each condition studied (i.e. protein-coated and non-protein coated substrates). For the bacterial-substrate interactions, gold and the SAMs behaved similarly with very low interfacial tensions, while adding either protein resulted in an increase in the interfacial tension (Figure 4A). The substrate-liquid interfacial tension was very high for gold, and positive but not as high for the SAMs (IAG and IPA) (Figure 4B). However, the addition of proteins to each of these substrates caused the substrate-liquid interfacial tension to become negative, suggesting that the proteins are stable in the presence of water. When these interfacial tensions are taken together, with consideration also of $\gamma_{BL}$ (a constant for our system since only a single bacterium-liquid combination was examined, 28.2 mJ/m$^2$), the $\Delta G_{adh}$ values of the non-protein coated surfaces are different in magnitude and sign compared to the corresponding values for the protein-coated surfaces (Figure 4C). Adhesion is predicted to be favorable only for the non-protein coated surfaces.

AB and LW components of the Gibbs free energy changes upon adhesion. The $\Delta G_{adh}$ values were decoupled into apolar (LW) and polar (AB) components. Since the values of the LW components were small and did not differ appreciably for the various
substrates (Figure 5), $\Delta G_{\text{adh}}^{AB}$ controlled the overall adhesion. The $\Delta G_{\text{adh}}^{AB}$ values for bare gold and the SAMs were highly negative, leading to overall $\Delta G_{\text{adh}}$ values that were negative, thus favoring bacterial adhesion. For the protein-deposited surfaces, the increased polar interactions (i.e. positive values of $\Delta G_{\text{adh}}^{AB}$) caused the overall system to be unfavorable for bacterial adhesion.

*Competition between bacteria and proteins for substrate.* With the presence of serum protein and bacteria in the liquid media, the adhesion on the substrata comprises some combination of the interactions of protein-protein, bacteria-protein and bacteria-bacteria. In order to compare the affinity of these different combinations, the $\Delta G_{\text{adh}}$ value for each type of pairing was calculated (Figure 6). This analysis allows us to suggest behavior patterns beyond what was measured experimentally. For example, the $\Delta G_{\text{adh}}$ for the interaction of *S. epidermidis* with *S. epidermidis* is highly favorable, suggesting that co-aggregation of bacteria is possible. Therefore, some bacteria attaching to the substrates may already be in aggregated form. Proteins, on the other hand, are favored to adsorb to the bare gold and SAMs compared to the surfaces already coated with proteins.
**Discussion**

Bacterial Deposition on Protein Coated Substrates

Researchers seek to design novel implant materials that can prevent or minimize bacterial adhesion and thus prevent biofilm development. In our previous work, IPA and IAG-terminating SAMs showed promise for preventing the adhesion/retention of *S. epidermidis*, compared with other aliphatic and aromatic molecules considered, but our past studies and other biomaterial studies have often not accounted for the behavior of the biomaterial in the presence of serum proteins \(^7, 8, 16\). For both uncoated substrates and FBS-adsorbed substrata, there was a strong correlation between the Gibbs free energy change of adhesion and bacterial retention (Figure 2A), although a non-linear relationship was observed. For convenience, a first-order exponential decay function was used to correlate \(\Delta G_{adh}\) and the retained cell count, but we do not have a physical explanation as to why the exponential relationship was so strong.

All non-protein coated substrata had negative \(\Delta G_{adh}\) values, suggesting that bacterial retention would be favored. FBS deposition caused \(\Delta G_{adh}\) to become positive, which was correlated with the significantly lower numbers of bacteria retained on the slides in the experimental batch assays. Thus, the serum proteins inhibited bacterial retention. Previous researchers have also found that bovine serum or plasma can inhibit *S. epidermidis* adhesion on serum-covered surfaces \(^25, 26\). One group fractionated bovine serum in order to determine that a specific protein component, apo-transferrin, was responsible for decreasing the adhesion of *S. epidermidis* to polyurethane materials by a factor of five \(^27\).
The thermodynamic predictions, however, failed to describe the experimentally observed bacterial retention for experiments with fibronectin (Figure 2B), since positive values of $\Delta G_{adh}$ still yielded high concentrations of retained bacteria. The likely explanation is that bacteria being retained to the FN-adsorbed substrates are experiencing specific ligand-receptor interactions, aiding their retention to the substrates in a way that cannot be described by the thermodynamic approach used here. Indeed, some studies have shown that FN or other proteins such as fibrinogen and thrombospondin can increase bacterial binding $^{28-30}$. *S. epidermidis* cells have specific receptors that bind to FN and some other proteins, while any interactions with FBS are mainly non-specific $^{31}$. The thermodynamic approach can still be used to predict the initial steps of bacterial adhesion, but specific interactions also must be considered when evaluating the promise of a biomaterial.

Upon implantation, both bacteria and serum proteins have the potential to adsorb on the surface. Protein adsorption on biomaterials and interaction with other cells are crucial issues in fields such as bacterial and eukaryotic cell adhesion $^{32,33}$, tumor cell migration $^{34}$, tissue and blood compatibility with implants $^{35}$, inflammation $^{36}$ and infections $^{37}$. Twenty years ago, Gristina et al. first described this “race for the surface” that occurs between bacterial pathogens and other cells, microbes, and proteins in the body during biomaterial implantation $^{38,39}$. Despite recognition of the importance of this topic for some time, a detailed understanding of the interactions between microbes, proteins, cells and biomaterials is still lacking, thus limiting our ability to prevent implant infections.
Protein adsorption to surfaces. FN is an adhesive glycoprotein that plays an important role in both bacterial and mammalian cell adhesion, tumor cell migration, and cell differentiation. FBS comprises different types of proteins including a small fraction of fibronectin, and proteins known for their role in cell adhesion, such as vitronectin and apo-transferrin. When considering how each of the proteins interacted with the substrates, FBS had a slightly negative $\Delta G_{adh}$ for gold slides and slightly positive $\Delta G_{adh}$ for the rest of the non-protein coated substrata, but the $\Delta G_{adh}$ values became large and positive for FBS interacting with all of the protein-coated surfaces (Figure 6), suggesting that FBS interactions were most unfavorable for surfaces already coated with some proteins.

FN also had an unfavorable interaction with all of the surfaces, with the least affinity for surfaces already coated with either protein (Figure 6). The somewhat low affinity of the proteins for the substrate (compared to bacterial affinity) may help explain why it is so difficult for surgeons to achieve tissue integration of an implant before bacterial infection develops.

Aggregation of proteins or bacteria. FBS and FN would not be predicted to self-aggregate, given $\Delta G_{adh}$ values that range from 40-100 mJ/m$^2$. However, S. epidermidis cells displayed a strong affinity to themselves (i.e. $\Delta G_{adh} = -56.4$ mJ/m$^2$). Indeed, aggregation is very common for S. epidermidis, as we often observe qualitatively in our experiments. After a few S. epidermidis adhere on the substrata, the retained bacteria mask the surface and make it easier for other bacteria to attach, similar to the process known as filter ripening observed in environmental systems. With time, more bacteria
can attach to the biomaterial, which can easily lead to multilayer biofilm formation under *in vivo* conditions, an underlying cause of catheter-related bloodstream infections \(^{43}\).

Correlation between interfacial free energy change \(\Delta G_{\text{adhesion}}\) and cellular viability

We observed a strong correlation between cellular viability and \(\Delta G_{\text{adh}}\) (Figure 3). For the bacterial systems in which the highest adhesion energy was predicted with a substrate, there was the greatest impact on cellular viability, except for one case (FN-adsorbed onto IAG slides). Although these results are preliminary, one explanation is that the high affinity between the bacterium and substrate could make it more difficult for cells to divide. A few studies have shown that when bacteria were strongly adhered to a biomaterial surface, they were less able to grow, and a correlation between bacterial surface growth rate and \(\Delta G_{\text{adh}}\) has been noted \(^{44-46}\). Although the design of our studies was slightly different, in that bacteria were not given time to grow into biofilms on the surfaces, we speculate that the bacteria would still be trying to divide during the time course of this experiment, and therefore a similar type of mechanism was probably responsible for their loss of viability on the high energy surfaces.

The anomalous data to this trend was for FN-adsorbed IAG, where the cellular viability was very similar to that observed for non-protein coated IAG slides. A possible explanation is that disassociated silver ions from IAG killed the bacteria. Silver ions are recognized for their ability to kill bacteria and silver-based antimicrobial compounds and solutions have been widely used as antimicrobial agents \(^{47, 48}\). However, the FBS-adsorbed IAG slides did not present a high percentage of non-viable cells. This difference may be due to the way that FBS and FN each coat the surface. Our previous studies on the adsorption of proteins on the SAMs (under identical conditions) showed that the
deposition of FBS on gold forms thicker structures than FN. From AFM imaging experiments, the FBS layer on gold had a mean thickness of 258.67 ± 124.30 nm, while the thickness of FN was 110.53 ± 16.17 nm. Possibly, the thicker layer of FBS prevents the silver ions from reaching the bacteria. Implantable materials containing antimicrobial agents are currently being studied for several applications, but not a lot is known about how to control the diffusion of the antimicrobial compound from the biomaterial. Our results suggest that in some cases, silver ions may be able to reach the target microbes and have an antimicrobial effect, even if proteins from the body adsorb to the biomaterial and cover it to some extent. This work also serves to emphasize that biomaterial development studies need to consider the interactions of the material with in vivo proteins, as well as with bacterial pathogens.

Contributions of LW and AB interactions to \( \Delta G_{adh} \)

LW interactions comprise orienting dipole-dipole interactions, orienting dipole-induced dipole (or induction) interactions, and fluctuating dipole-induced dipole (or dispersion) interactions, which have been described by Keesom, Debye, and London, respectively. Since only the dispersion interactions have considerable impacts between macroscopic bodies in aqueous media, (London) dispersion forces are expected to dominate the LW interactions for our system. Positive correlations were previously observed between the attachment of *S. epidermidis* and *E. coli* to five different polymers and the dispersive component of the free energy, while a negative correlation was observed between the bacterial retention and the basic component.

In our study, the bacteria, media, substrates, and protein-coated substrates presented relatively similar values for LW forces, while the AB interactions presented a
lot of surface-dependent variability. So although dispersion forces are the dominant type of LW interaction, these forces were still very small in comparison to acid/base interactions, which controlled the overall values of the Gibbs free energy changes upon adhesion.

AB interactions were also found to vary as a function of the substrate and protein presence (Figure 5). Hydrogen-bonding is the dominant component of AB interactions in aqueous media and hydrogen bonding is important for many biological systems, such as amino acid binding, specific recognition of proteins and DNA, and in other structural and molecular biological applications.

There are a few different methods others have used to examine the importance of hydrogen bonding on bacterial adhesion and retention on surfaces. Infrared spectroscopy was used to determine that the O-antigens of Gram-negative bacterial lipopolysaccharides formed hydrogen bonds with surface-bound water molecules associated with mineral oxide surfaces, mediating the attachment of bacteria to such surfaces. More recently, AFM was used to probe the molecular adhesion forces between E. coli and silicon nitride. A Poisson statistical analysis on AFM force profiles in aqueous media was used to decompose AB interactions from LW interactions. The hydrogen bond energy for E. coli with silicon nitride in water was determined to be ~ 46.6 kJ/mol. Since hydrogen bonding is such a strong mechanism by which bacteria can attach to surfaces, it is a natural step to explore the development of biomaterials that resist bacterial colonization through prevention of hydrogen bonds. For example, thin films of polyamines could be functionalized with acyl chlorides so that hydrogen donor groups were blocked, thus...
inhibiting the attachment of proteins and bacteria to the surfaces via hydrogen bonding. This will likely be a fruitful avenue to pursue in the further development of biomaterials.

Comments on the Thermodynamic Approach to Evaluate $\Delta G_{adh}$

Although the VGC methodology to predict surface free energies and describe adhesion of bacteria and numerous other colloidal systems has been widely applied with much utility, some concerns have been raised regarding the optimal application of the theory for bacterial and other systems. We will not attempt to summarize all such criticisms but will address the ones deemed most relevant in our work. Specifically, the biggest challenges for applying this towards bacterial adhesion to biomaterials are discrepancies in evaluating contact angles and the overwhelmingly “basic” nature of almost all polymers and materials evaluated as substrates for bacterial adhesion.

By making use of the sessile drop technique and evaluating the drying time of the bacteria, it should be possible to make accurate and reproducible measurements of contact angles on bacterial lawns. Certainly, the use of a video camera to evaluate the droplet and computer-assisted image analysis can also help. Due to the non-linear relationships in the thermodynamic calculations, we wanted to verify that small variations or errors in contact angle measurements would not affect our conclusions. We modified each measured contact angle value by $\pm 2^\circ$ and calculated the effect of such variation on the resulting $\Delta G_{adh}$ for all the surfaces in this study. We also examined the effect on having this error for just one probe liquid (i.e. water, formamide, diiodomethane), two probe liquids, or in all three probe liquids. Based on examining all possible combinations of errors, we found that the Gibbs free energy change never was altered by more than $\sim 5-8 \text{ mJ/m}^2$, and this was found only when the maximum error was
assumed for the contact angles with all three probe liquids. When the surfaces examined yielded such a large range of $\Delta G_{adh}$ values (from -70 to +50 mJ/m$^2$), we consider it unlikely that errors or variability in contact angle measurements can alter our conclusions.

The concern regarding the overall basic nature of nearly all polar solid substrates has been much discussed in the literature. While some researchers have questioned whether this is physically realistic $^{59,61}$, van Oss et al.$^{62}$ suggested that there may be a physical explanation. Since the Earth’s lower atmosphere and surface are each very enriched in oxygen, then this enrichment could explain why all biopolymers, minerals, oxides, etc. found in the natural environment are predominantly electron donating $^{62}$. Lee sought to evaluate whether the overwhelmingly basic nature of surfaces was due to the reference values chosen for the probe liquids in VCG theory, especially in the assumption that $\gamma^+ = \gamma^- = -25.5$ mJ/m$^2$ for water $^{63}$. An analysis based on linear solvation energy relationships (LSER) determined that a ratio of $\gamma^+ / \gamma^-$ =1.8 for water was more consistent with other information on how water is able to form hydrogen bonds. Using corrected values proposed for $\gamma^+ / \gamma^-$ for water and formamide, we again carried out the same thermodynamic calculations. While the individual components of interfacial tension changed, the effect on $\Delta G_{adh}$ or on any of the trends we observed in this study was negligible (Figure 7). Therefore, we concur with other researchers that contact angle measurements and surface free energy calculations are a powerful method for evaluating bacterial adhesion processes.
Conclusions

System Gibbs free energy changes upon adhesion were well correlated with bacterial retention results on various substrata coated with SAMs and FBS, suggesting that this approach can be used to screen for biomaterials with properties that will inhibit bacterial colonization. However, when specific interactions between bacteria and serum proteins were considered, such as for fibronectin interacting with \textit{S. epidermidis}, the thermodynamic analysis underestimated bacterial retention. The $\Delta G_{adh}$ values were further decoupled into LW and AB components, and the AB term was further described in terms of electron-donor and electron-acceptor contributions. AB interactions due to hydrogen bonding were determined to be the dominant physical force affecting bacteria/protein/substrate interactions in liquid media. The competition between serum proteins and bacteria for biomaterial surfaces, as well as bacteria/bacteria and protein/protein interactions, must be considered in future studies of antimicrobial surface design.

Acknowledgements

We would like to thank Dr. W. Grant McGimpsey, Mr. Eftim Milkani and Mr. Jean-Roland Pascault for their help with the contact angle measurements. We also thank Dr. Stephen Heard, Dr. Cuong Vuong, and Dr. Ray Emerson for helpful discussions regarding this work.

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References

Table 1. *Staphylococcus epidermidis* retention and viability on different substrata

<table>
<thead>
<tr>
<th>Substrate (n ≥ 6)</th>
<th>Retained cells/mm²</th>
<th>Viable cells (%)</th>
<th>Non-viable cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental Gold</td>
<td>2685 ± 556</td>
<td>17.3 ± 14.3</td>
<td>82.7 ± 14.3</td>
</tr>
<tr>
<td>IPA</td>
<td>1204 ± 741</td>
<td>63.5 ± 16.4</td>
<td>36.5 ± 16.4</td>
</tr>
<tr>
<td>IAG</td>
<td>833 ± 750</td>
<td>36.3 ± 28.4</td>
<td>63.7 ± 28.4</td>
</tr>
<tr>
<td>FBS + gold</td>
<td>83 ± 172</td>
<td>78.0 ± 37.0</td>
<td>22.0 ± 37.0</td>
</tr>
<tr>
<td>FBS + IPA</td>
<td>63 ± 82</td>
<td>86.1 ± 12.7</td>
<td>13.9 ± 12.7</td>
</tr>
<tr>
<td>FBS + IAG</td>
<td>48 ± 55</td>
<td>93.7 ± 8.9</td>
<td>6.3 ± 8.9</td>
</tr>
<tr>
<td>FN + gold</td>
<td>1805± 897</td>
<td>90.3 ± 9.0</td>
<td>9.7 ± 9.0</td>
</tr>
<tr>
<td>FN + IPA</td>
<td>1580 ± 1414</td>
<td>93.4 ± 5.5</td>
<td>6.6 ± 5.5</td>
</tr>
<tr>
<td>FN + IAG</td>
<td>1230 ± 761</td>
<td>27.7 ± 27.2</td>
<td>72.3 ± 27.2</td>
</tr>
</tbody>
</table>

*a* mean number of cells retained ± standard deviation
Table 2. Contact angles and surface free energy components for *S. epidermidis* and different substrata

<table>
<thead>
<tr>
<th></th>
<th>Contact angle (n ≥ 30) (Degrees)</th>
<th>Parameter value (mJ/m²)</th>
<th>θ_W</th>
<th>θ_D</th>
<th>θ_F</th>
<th>γ_LW</th>
<th>γ_F</th>
<th>γ_AB</th>
<th>γ_Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. epidermidis</em></td>
<td>87±3</td>
<td>48±1</td>
<td>70±3</td>
<td>31.979</td>
<td>5.484</td>
<td>3.984x10⁻⁶</td>
<td>0.009</td>
<td>31.988</td>
<td></td>
</tr>
<tr>
<td>Cleaned Gold^a</td>
<td>36±0</td>
<td>29±1</td>
<td>60±0</td>
<td>31.945</td>
<td>73.213</td>
<td>0.645</td>
<td>13.744</td>
<td>45.689</td>
<td></td>
</tr>
<tr>
<td>Exp. Gold^1</td>
<td>89±2</td>
<td>29±1</td>
<td>63±4</td>
<td>42.327</td>
<td>2.023</td>
<td>0.019</td>
<td>0.388</td>
<td>42.715</td>
<td></td>
</tr>
<tr>
<td>IPA</td>
<td>72±5</td>
<td>27±0</td>
<td>51±2</td>
<td>40.215</td>
<td>9.930</td>
<td>0.187</td>
<td>2.729</td>
<td>42.944</td>
<td></td>
</tr>
<tr>
<td>IAG</td>
<td>75±4</td>
<td>28±4</td>
<td>52±3</td>
<td>40.579</td>
<td>6.881</td>
<td>0.246</td>
<td>2.601</td>
<td>43.180</td>
<td></td>
</tr>
<tr>
<td>FBS + Gold</td>
<td>51±5</td>
<td>43±2</td>
<td>73±5</td>
<td>26.976</td>
<td>64.210</td>
<td>1.282</td>
<td>18.148</td>
<td>45.122</td>
<td></td>
</tr>
<tr>
<td>FBS + IPA</td>
<td>23±3</td>
<td>82±0</td>
<td>79±0</td>
<td>7.410</td>
<td>126.030</td>
<td>0.002</td>
<td>1.010</td>
<td>8.420</td>
<td></td>
</tr>
<tr>
<td>FBS + IAG</td>
<td>29±6</td>
<td>82±0</td>
<td>82±0</td>
<td>7.530</td>
<td>123.090</td>
<td>0.025</td>
<td>3.500</td>
<td>11.023</td>
<td></td>
</tr>
<tr>
<td>FN + Gold</td>
<td>25±3</td>
<td>42±4</td>
<td>70±7</td>
<td>24.767</td>
<td>107.800</td>
<td>1.667</td>
<td>26.811</td>
<td>51.578</td>
<td></td>
</tr>
<tr>
<td>FN + IPA</td>
<td>31±2</td>
<td>41±4</td>
<td>68±4</td>
<td>25.929</td>
<td>95.624</td>
<td>1.227</td>
<td>21.667</td>
<td>47.596</td>
<td></td>
</tr>
<tr>
<td>FN + IAG</td>
<td>33±7</td>
<td>41±0</td>
<td>75±7</td>
<td>25.296</td>
<td>104.797</td>
<td>2.783</td>
<td>34.158</td>
<td>59.454</td>
<td></td>
</tr>
</tbody>
</table>

^aGold slides whose contact angle is measured immediately after cleaning (referred to as “Cleaned Gold” and measured within 1 min of cleaning) are hydrophilic. In practice it is very difficult to maintain this level of hydrophilicity since small organic and inorganic particles suspended in the air adsorb to the surface almost immediately. Therefore, “Exp. Gold^1” refers to the gold slides used in the experiments, in which they were exposed to normal air for > 1 min.
Figure Captions

Figure 1. Schematic of the SAMs on gold slides.

Figure 2. Correlation between retention experiments and system interfacial free energy calculations of *Staphylococcus epidermidis* and different substrata; A) Correlation between retention experiments and system interfacial free energy calculations of *Staphylococcus epidermidis* and bare or FBS absorbed substrata; B) Correlation between retention experiments and system interfacial free energy calculations of *Staphylococcus epidermidis* and FN absorbed substrata; C) System interfacial surface change.

Figure 3. Correlation between interfacial free energy and retention cellular viability.

Figure 4. Bacteria-Substrate interfacial tension vs. Substrate-Aqueous medium interfacial tension; A) Bacteria-Substrate interfacial tension of different substrata; B) Substrate-Aqueous medium interfacial tension.

Figure 5. Interfacial free energy: Lifshitz-van der Waals (LW) contributions vs. Acid-Base (AB) interactions contributions.

Figure 6. Competition between serum proteins deposition and bacterial adhesion.

Figure 7. Sensitivity of thermodynamic calculations to choice of reference values used for the probe liquids. Open symbols are the original calculations (i.e. $\gamma^+/\gamma^-$ for water =1.0, same as Figure 5), while closed symbols were calculated using $\gamma^+/\gamma^- =1.8$ for water and $\gamma^+/\gamma^- =1.1$ for formamide, according to $^63$. 


Figure 1
Figure 2

A) Exponential Decay fit:
\[ Y = 165.66e^{-(x+24.36)} \]
\[ R^2 = 0.98 \]
Figure 3

Non-viable cells (%)

Linear Regression except (FN+IAG) data

Linear Regression fit
\[ Y = -0.5962X + 28.522 \]
\[ R^2 = 0.87 \]

Gibbs free energy change \( \Delta G_{\text{adhesion}} \) (mJ/m²)

1. Gold
2. IAG
3. IPA
4. FBS + Gold
5. FBS + IPA
6. FBS + IAG
7. FN + Gold
8. FN + IPA
9. FN + IAG
Figure 4
Figure 5
Figure 6
Figure 7
Chapter 9: Adhesion Forces Between *Staphylococcus epidermidis* and Surfaces Bearing Self-Assembled Monolayers in the Presence of Model Proteins

Abstract

Self-assembled monolayers (SAMs) are being developed into coatings to reduce microbial biofilm formation on biomaterials. To test anti-adhesion properties, SAMs can be easily constructed on gold, and used to represent a coated biomaterial. However, coatings that prevent bacterial adhesion must also resist protein adsorption. We explored the competitive effects of bacteria and protein for adsorption to SAMs, choosing fetal bovine serum (FBS) to represent protein non-specific binding, and fibronectin (FN) to evaluate ligand/receptor binding. *Staphylococcus epidermidis* cells were immobilized on an atomic force microscope (AFM) tip and used as a force probe to detect the interaction forces between bacteria and gold-coated SAMs. The SAMs tested were alkanethiol molecules terminating in isophthalic acid (IPA) or isophthalic acid with silver (IAG). While *S. epidermidis* showed weak interactions with FBS, the bacteria showed strong adhesion with FN, due to ligand/receptor binding. Bacterial retention and viability experiments were correlated with the force measurements. *S. epidermidis* interacting with IAG SAMs showed a loss of viability, due to the mobility of silver ions. For most substrata, there was a link between high adhesion forces with bacteria and a high percentage of dead cells being retained on that substratum (even in the absence of a specific biocidal effect, such as silver). This may suggest that high adhesion forces can cause stress to the bacterial cells, which contributed to their death. The relationship between highly adhesive SAMs and bacterial inactivation may be useful in future
biomaterial design. When evaluating coatings for biomaterials, it is important to consider the interplay between bacteria, proteins, and the coating material.

**Introduction**

*Staphylococcus epidermidis* is one of the most frequently isolated nosocomial sepsis pathogens associated with infections of implanted medical devices [1]. Due to the formation of biofilms, microbial infections on biomaterials are difficult to treat, and may require surgical replacement of the implant. Biofilms form when bacteria attach to a solid surface, proliferate, and develop into multilayers. Some biofilm-forming strains can produce a polymeric matrix that encloses the bacterial community and protects it from antimicrobial agents and phagocytosis, although some biofilms are not associated with such matrices [2-7].

One strategy to prevent biofilms from forming on biomaterials is to coat antimicrobial agents onto the implanted materials. For example, stainless steel or titanium rods coated with the antiseptic dye gendine were able to prevent biofilms from forming when implanted into bovine bone soaked in donor calf serum *in vitro* for two weeks [8]. In another study, some success was realized using a plasma membrane of poly(butyl methacrylate) that released ciprofloxacin™ to prevent accumulation and growth of *S. epidermidis* and *Pseudomonas aeruginosa* for 48 hrs [9]. However, a challenge of these studies is that it is difficult to maintain a steady release of the active agent from the biomaterial over long periods. Popat et al. showed that gentamicin-loaded nanotubes were effective in minimizing bacterial adhesion, but this required the precise design of nanotube length and diameter, in order to control antibiotic loading and release rates [10]. More strategies and examples were reviewed recently by Wu et al. [11].
The increasing existence of bacteria that are resistant to antibiotics adds to this challenge. Silver ions have shown broad-spectrum antibacterial activity at very low concentrations (parts per billion level) [12,13]. Bacteria are unable to develop a resistance to silver’s antimicrobial abilities, reportedly due to the numerous silver target sites within a bacterium [14,15]. Stobie et al. used a low-temperature processed silver-doped phenyltriethoxysilane sol-gel coating to prevent *S. epidermidis* biofilm formation. The release of silver ions from the sol-gel coating inhibited the development of the *S. epidermidis* biofilm over a period of 10 days [16]. In addition, silver is nontoxic to mammalian cells at similar concentrations, or up to 4 μg/ml [17].

An alternate strategy to the use of biocides is to design a material that prevents infection by inhibiting the initial adhesion of bacteria, thus preventing biofilms from forming. Polymers such as poly(ethylene glycol) derivatives and phosphorylcholine (PC)-based materials have been able to prevent bacterial adhesion for short times. However, such materials can degrade, especially in the presence of oxygen and transition metal ions [18,19]. More examples can be found in a recent review by Qiu et al. [20].

Self-assembled monolayers (SAMs) can provide a uniform layer of molecules with the same terminal group and orientation, which can facilitate mechanistic studies to identify target groups for incorporation into potential coatings. Although most studies of SAMs use gold substrata, recent studies also show that SAMs can form on polymers [21,22]. However, it is still more convenient to construct SAMs on gold than on polymers, and this method can be useful for the initial screening of functional groups that will resist microbial adhesion. Recently, Hou et al. tested the *E. coli* biofilm inhibition properties of SAMs constructed on gold. SAMs terminated with tri(ethylene glycol) were resistant to
*E. coli* biofilm formation for 48 hours [23]. SAMs can prevent the retention of bacteria to gold-coated substrata by altering substratum physicochemical properties such as wettability, roughness, and surface free energy [24-27]. The thickness of the SAM is also very important in controlling whether a coating will remain nonfouling over an extended period of time, since some surfaces may perform well for short periods, but become altered under *in vivo* conditions. Alterations in performance can be due to host cell extracellular matrix protein adsorption, such as fibronectin, in early stages [28], and due to exopolysaccharide production by bacteria in later stages of biofilm formation [18]. Researchers studying bacterial adhesion to biomaterials are beginning to consider how the material will be changed by the deposition of serum proteins when the material is placed *in vivo* [29,30].

Protein adsorption and bacterial adhesion on implanted materials are mediated by numerous processes, such as dispersion forces among different molecules, Lewis acid/base forces between electron-donor and electron acceptor pairs (including hydrogen bonding), and electrostatic forces generated from surface charges. Although the molecular origin of specific and non-specific forces is the same [31,32], it is often convenient to distinguish between the two when describing bacterial adhesion to a given substratum. Different parameters have been used to characterize bacterial surfaces to help predict their interactions, such as measuring water contact angle on bacterial layers, and zeta potential of bacterial solutions. However, correlations between physicochemical characteristics, such as wettability and zeta potential, with protein and bacterial adhesion have been inconsistent [25,26,33,34]. Another approach is use a model to calculate the non-specific interactions accounting for the bacteria, substratum, and media, such as the
thermodynamic framework proposed by van Oss and colleagues [35]. We previously used this methodology to explain the interactions between S. epidermidis and protein-coated SAMs [36]. One advantage of applying this model is that interactions can be broken into fundamental components, leading to a mechanistic-based understanding of bacterial and protein adhesion.

When materials are implanted into the body, there is a competition between bacteria, plasma proteins, and tissue to adhere to the biomaterial [37]. Both non-specific forces and specific forces (ligand/receptor bonding) will contribute to the adhesion process. Some proteins such as bovine serum albumin (BSA) do not form ligand/receptor bonds with bacteria, and interact only non-specifically. However, proteins such as fibronectin (FN) and vitronectin can form specific ligand/receptor bonds with Staphylococci. While the binding sites between FN and S. aureus has been identified and well-characterized [28,38-40], the binding sites between FN and S. epidermidis have not been conclusively identified [41]. Fewer studies have addressed S. epidermidis attachment to protein-coated surfaces, especially at the molecular level [41-43].

The goal of this study was to demonstrate how molecular-level adhesion forces between bacteria and substrata are modified when proteins are present. Atomic force microscopy (AFM) is a powerful tool for directly characterizing the interaction forces between bacteria and substrata, including potential biomaterials [26,44-46]. To this end, we used AFM to characterize forces between S. epidermidis and protein-coated SAMs.
**Materials and Methods**

**Bacterial cultures**

A clinical isolate of *S. epidermidis* from a patient experiencing catheter-related bloodstream infection was provided by Dr. Stephen Heard (Department of Anesthesiology, University of Massachusetts Medical School, Worcester, MA). Bacteria were precultured in 20 mL Tryptic Soy Broth (TSB) solution (30 g/L) (Sigma, St. Louis, MO) at 37 °C overnight. Precultured bacteria (1 mL) were added to 30-50 mL TSB and cultured at 37 °C in a water shaker bath, until mid-exponential growth phase. Bacteria were centrifuged at 1400g for 15 minutes (Fisher Scientific, Pittsburgh, PA) and washed three times with 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer at pH 7.1.

**Serum proteins**

Two common serum proteins were chosen as models, fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA), which is a mixture of serum proteins; and fibronectin (FN) (BD, Bedford, MA), which can form ligand-receptor interactions with *S. epidermidis* [41,47].

**Substrata**

SAMs terminating in isophthalic acid (IPA) and isophthalic acid with silver (IAG) were created on gold-coated glass slides, shown schematically in Supplementary Information Figure S.1. SAMs were characterized as previously described [26]. We examined bare gold, gold substrata with SAMs, and gold substrata with SAMs plus one of the two model proteins (FBS or FN). In a previous study, we determined the optimal serum protein concentration and deposition conditions to be used [48,49]. Briefly, substrata were immersed in 10 % v/v FBS diluted in 0.1 M MES in the incubator at 37°C.
for 80 minutes. For FN, substrata were immersed in 5 μg/ml FN solution in the incubator at 37°C for 24 hrs. AFM was used to verify protein deposition under these conditions (Supplementary Information Figure S.2), which we also described in another manuscript [48].

AFM force measurements

The interaction forces were measured with a Dimension 3100 atomic force microscope with Nanoscope IIIa controller (Veeco Metrology, Inc., Santa Barbara, CA) with silicon AFM tips (Mikromasch USA; CSC38 Cantilever Type A), on a rectangular cantilever. Spring constants were measured using a thermal technique [50], and found to be in the range of 0.05 – 0.19 N/m, with an error of <10% per tip. Bacteria were coated onto the AFM tips using a technique developed in our laboratory [51]. The AFM tip was carefully treated with poly-L-lysine (0.1% w/v in water, Sigma, USA) for 5 minutes. The bacteria pellet was placed on a section of parafilm on a glass slide, to form a thin film of bacteria. The poly-L-lysine-treated AFM tip was positioned over the bacterial film using the optical microscope. The scan size was decreased to zero and the scan rate was set to 0.1 Hz, in order to minimize the lateral movement of AFM tip during engagement. By adjusting the distance and deflection set point, the AFM tip was engaged on the bacteria layer and allowed to contact bacteria for 1-3 minutes.

The presence of bacteria on the AFM tip was checked by scanning electron microscopy (SEM; JEOL JSM-840), measurement of the resonance frequency shift of the cantilever, and by inspection of characteristic force curves for coated and uncoated tips. For the SEM measurements, the bacteria coated AFM tip was kept in a clean closed AFM tip box and allowed to dry under ambient conditions. After drying, the coated tip was
immobilized on an SEM specimen stub using double-sided carbon tape (Electron Microscopy Science, Washington, PA). Due to the conductivity difference between bacteria and AFM tip material, no metal coating was needed for SEM imaging, which was operated at 15 kV.

Force measurements were conducted in 0.1 M MES buffer, which is close to the ionic strength of physiological phosphate buffered saline (PBS) that is often used in studies of bacterial adhesion. In the past, we have used MES rather than PBS because phosphate ions have sometimes interfered with our ability to immobilize bacteria for AFM measurements [26]. Each substratum was probed with a non-bacteria coated probe first, to ensure that the surface was uniform and to set a baseline for comparing the force measurements with the biologically functionalized probes. At each position, at least 10 force curves were recorded. At least three replicate areas of the slide were examined for each condition. One force measurement cycle consists of an approach and retraction portion, each containing 512 data points. We tabulated the adhesion peaks corresponding to all adhesion events and constructed histograms to compare their distributions. A normal distribution was also calculated corresponding to the adhesion force data, and two statistical tests for normality were applied, the Anderson- Darling and Shapiro-Wilk tests.

Correlation of bacterial adhesion forces with Gibbs free energies of adhesion

We correlated bacterial adhesion force data with Gibbs free energy changes of adhesion values that we reported previously [36,52]. Specifically, Gibbs free energy of adhesion values were calculated for bacteria interacting with gold, SAMs on gold, and those substrata with adsorbed FBS or FN.

Bacterial retention experiments
A batch assay was used to quantify the number of bacteria retained on each substratum, and a dual-staining technique was used to determine the percentage of bacteria that were viable, as described previously [36,48]. Each substratum was immersed in bacterial solution (10⁹ cells/ml) in the incubator at 37°C for 30 minutes. Substrata were briefly removed from solution and rinsed with 0.1 M MES to remove loosely attached bacteria. Retained cells on the substrata were stained with a BacLight™ live/dead kit with final concentrations of 0.835 μm propidium iodide and 0.5 μm Syto 9™ (Invitrogen, Faraday, CA) and examined with fluorescence microscopy (Nikon Eclipse 400 fluorescence microscope, Tokyo, Japan with a mounted 18.2 Color Mosaic Camera; Diagnostic Instruments, Inc., Sterling Heights, MI), using a 100X oil immersion lens. Texas Red and FITC filters were used to observe bacteria stained with propidium iodide (red; stains membrane-compromised cells only) and Syto 9 (green; stains all cells). At least 15 random locations were examined per slide and at least 4 slides were analyzed for each substratum. The retention results were averaged based on at least 60 images per condition.

**Results**

**Verification of S. epidermidis force probe creation**

Our ability to use the AFM-based technique to screen potential biomaterials relies on the accuracy and reproducibility of making bacterial probes. Three methods were used to verify that the tips were successfully coated. At the conclusion of an experiment or for probes prepared similar to those used for AFM experiments, we imaged the probes with scanning electron microscopy (SEM), and attached bacteria could clearly be seen (Figure 1).
A more quantitative way to verify that the tip was coated with bacteria was to characterize the changes in the cantilever’s resonance frequency. The shift in frequency was monitored as the mass on the probe increased. We compared the resonance frequency of a bare tip, a tip coated only with poly-L-lysine, and of a tip coated with bacteria.

When the AFM cantilever is tuned using the piezoactuator, the resonance frequency has a quantitative relationship with the spring constant and effective mass of the cantilever, as given by [49]

\[
f_1 = \frac{1}{2\pi} \sqrt{\frac{k}{M}} \tag{1}
\]

\[
f_2 = \frac{1}{2\pi} \sqrt{\frac{k}{M + \Delta m_c}} \tag{2}
\]

\[
f_3 = \frac{1}{2\pi} \sqrt{\frac{k}{M + \Delta m_c + \Delta m_B}} \tag{3}
\]

where \(M\) denotes the effective mass of the cantilever, \(\Delta m_c\) is the mass of added poly-L-lysine, and \(\Delta m_B\) is the mass of added bacteria. The terms \(f_1, f_2,\) and \(f_3\) denote resonance frequencies of the cantilever of the bare tip, chemically modified tip, and bacteria coated tip. The three unknowns \((M, \Delta m_C,\) and \(\Delta m_B)\) can be found by combining equations (1) - (3), as

\[
\Delta m_B = \frac{k}{4\pi^2} \left( \frac{1}{f_3^2} - \frac{1}{f_2^2} \right) \tag{4}
\]

Typical values we observed were \(f_1=12.41 - 13.27\) kHz, \(f_2=11.62 - 12.85\) kHz, and \(f_3=9.42 - 10.55\) kHz. Therefore, the coated bacteria mass is \(~2 - 9\) pg. The variation of the coated bacteria mass occurs because we do not always capture exactly the same number of bacteria on the tip. However, we observed that clusters of \(~8 - 12\) cells were
typically attached. While we previously showed that a single cell of *E. coli* could be attached to an AFM probe [51], we could not achieve this sensitivity with *S. epidermidis* because cell-cell interactions are favorable and the bacteria always aggregate into a small cluster. However, we reproducibly created clusters with a small grouping of bacterial cells for *S. epidermidis*.

**AFM force measurements**

Adhesion forces of bacteria with gold were high and showed long range adhesion peaks (Figure 2A). This is consistent with previous adhesion force measurements with gold and *S. epidermidis*, in which very high adhesion forces were observed [26]. When either SAM was added to the gold, adhesion forces decreased due to the surface coating. The average adhesion force between *S. epidermidis* and uncoated gold was $1.21 \pm 0.41$ nN. A one-way repeated ANOVA test showed no significant differences between the average adhesion forces of *S. epidermidis*/IPA ($0.66 \pm 0.19$ nN) and *S. epidermidis*/IAG ($0.73 \pm 0.32$ nN) ($p=0.388$), although both SAMs showed significant differences with the adhesion forces of *S. epidermidis*/gold ($1.21 \pm 0.41$) ($p<0.001$).

After FBS coating of gold or the SAMs, the adhesion forces decreased for all three substrata, and were very similar to one another, suggesting that FBS masks the properties of all underlying substrata (Figure 2B). The average adhesion forces were $0.23 \pm 0.09$ nN, $0.18 \pm 0.03$ nN, and $0.17 \pm 0.06$ nN for FBS coated gold, IPA and IAG substrata, respectively, which were all significantly different compared to the corresponding bare substrata ($p<0.001$).

When FN was adsorbed to the surfaces, we saw a similar behavior to FBS in that protein could mask the surface properties (Figure 2C). However, we did not see as
noticeable a change in the adhesion forces as we saw for the FBS-coated surfaces. Compared to FBS, all of the adhesion forces for FN-coated surfaces were higher, and all FBS surfaces were significantly different from FN surfaces (p<0.001). Pull-off distances were also lower for FBS than FN (Figures 3A and 3B). The force magnitude between *S. epidermidis* and FN is very similar to the reported adhesion forces between *S. aureus* and FN measured via AFM [53].

One way that we quantified the nature of the protein layer was to characterize whether the histogram of adhesion forces between bacteria and protein showed a normal distribution. The normality or lack of normality for these distributions was quantified via the Anderson-Darling and Shapiro-Wilk tests. For the Anderson-Darling (AD) test, a score <1.092 passes the normality test. For the Shapiro-Wilk (SW) test, a score of >0.89 is considered normal. Adhesion forces for FBS on any surface were very similar and were normally distributed (Figures 3C and 3D), suggesting that no orientation on the surface was preferred for any of the three substrata (gold or either SAM on gold). Both the AD and SW tests showed the results were normal, with the AD score being 0.75 and the SW score being 0.90 on gold. For the SAMs, the FBS data passed the normality test using either criteria, with the AD score being 0.409 (< 1.092 is normal), and the SW score being 0.976 (> 0.89 is normal.) For FN, normal adhesion forces were observed on each of the SAMs, but not on gold (Figures 3E and 3F). For FN on gold, the AD test failed, with a score of 1.65 (< 1.092 is normal), although the SW test suggested the distribution was normal, with a score of 0.94 (>0.89 passes). For FN adsorbed to either SAM, both tests showed that the distribution of adhesion forces was normal, with the AD score being 0.387 (< 1.092 passes), and the SW score being 0.973 (>0.89 passes). The
latter finding suggests that the FN can adsorb to gold with at least two different orientations.

Correlation between force measurements and bacterial retention

The mean adhesion forces for the nine different conditions were correlated with the results of the bacterial retention experiments (Figure 4), and they showed a linear correlation ($R^2=0.96$). Although mean values do not fully characterize the distribution of adhesion forces for all conditions (such as were shown in Figure 3), the use of the mean values allows for convenient comparisons to be made across all conditions. Large adhesion forces corresponded to more retained bacteria, such as for gold and the FN-coated SAMs. FBS-coated SAMs showed small adhesion forces and low retention. Statistical tests were used to help support these comparisons. Pairwise comparisons between all treatments were performed using the Kruskal-Wallis One Way Analysis of Variance on Ranks. Based on pairwise comparisons, each of the 9 treatments was significantly different from the other eight treatments ($p<0.001$).

Correlation between force measurements and Gibbs free energy calculations

In a previous study, we calculated the Gibbs free energy changes of adhesion corresponding to the same SAMs, proteins, and bacteria [36]. Gibbs free energy changes can be used to correlate and predict biological adhesion behavior in the absence of ligand-receptor interactions. Probe liquids (typically three liquids that span a range of polarities) are used to calculate the surface tensions of individual substrate and then used to determine system Gibbs free energy changes. Gibbs free energy calculations significantly underestimate the adhesion behavior of bacteria in the presence of ligand-receptor interactions because specific interactions can give rise to adhesion forces that are
much greater than those attributable to non-specific interactions. In the present study, we determined if there was a correlation between these previously calculated Gibbs free energies of adhesion and the forces of adhesion we measured with the AFM (Figure 5). When FN-coated SAMs were excluded, adhesion forces showed a linear correlation with the Gibbs free energy changes of adhesion ($R^2=0.86$). However, this trend did not hold for FN-coated SAMs. Although FN-coated SAMs presented large adhesion forces, the Gibbs free energy changes were positive.

Correlation between force measurements and viability of retained cells on substrata

With the help of the dual staining technique, we could quantify viable and non-viable *S. epidermidis*. The adhesion forces between *S. epidermidis* and the substrate showed a linear correlation with the number of non-viable retained bacteria (Figure 5). For most of the conditions, this was a direct relationship, such that FBS-coated SAMs presented the lowest adhesion forces and corresponded to lowest dead cell percentages. The exception was for some of the FN-coated surfaces, where highly adhesive cells were still viable. When FN-coated gold and IPA were excluded, there was a positive correlation between adhesion forces and loss of cellular viability ($R^2=0.93$).

**Discussion**

Binding between *S. epidermidis* and proteins

Understanding the nature of the binding sites between bacteria and proteins is useful for numerous applications, including biosensor design, biomaterial development, environmental protection, and controlling and preventing bacterial adhesion for industrial applications. We chose these two model proteins because we expected to find differences in how they interact with bacteria.
Bovine serum albumin is a major component in FBS and has been shown to provide anti-adhesion abilities to proteins and bacteria [54,55]. In addition to the absence of ligand-receptor binding, the low adhesion forces between \textit{S. epidermidis} and the FBS-coated substrata could be due to an inhibitory effect of albumin.

FBS adsorbs without a preferred orientation on all of the surfaces. Based on our experiments, we also considered the interaction of FN with gold to lack a preferred orientation. Considering the chemistry involved, none of the molecules of FN within the Arg-Gly-Asp domain or at either terminus can form a preferential bond with gold. FN on the SAMs can adsorb randomly but is more likely to interact with the functional groups on the IPA or IAG to adsorb with either the C- or N-terminus linked to the SAM. Au-S bonds are difficult to form from proteins because steric hindrance due to the bulky protein body prevents the bond formation, and only cysteine residues can be readily used for binding [56]. Au-S bonds can form from either S\(^{-2}\), SH\(^{-1}\) or SH\(_2\) in aqueous electrolytes solutions [57-59], but Au-S bonds from disulphide (as contained in FN) can form only in the gas phase [60]. In the absence of a preferred bond arrangement, there can be multiple arrangements for the FN to bind with the gold, and this may be why we did not observe a repeatable or normal distribution for the adhesion forces of bacteria with the FN-coated gold.

Studying the behavior of the FN adsorbed to the SAMs allows us to gain a better understand the binding sites between FN and \textit{S. epidermidis}. The binding sites of FN to a related bacterium, \textit{S. aureus}, have been extensively studied [61-63], showing that the N-terminal domain of FN forms part of the binding site for FN-binding proteins from \textit{S. aureus} [64,65]. However, the binding mechanism between FN and \textit{S. epidermidis} is still
unclear. Recombinant phage display studies identified a FN-binding domain for *S. epidermidis*, but this was not the same site as the 30 kDa N-terminal region that has previously been shown to bind with *S. aureus*, suggesting that the two bacteria have different binding domains for FN [28,66,67]. A novel study employed anti-FN monoclonal antibodies that were attached to either the C-terminus or N-terminus of FN, so that one end of the molecule could be confirmed to be blocked [41]. An increase in *S. epidermidis* attachment (retention) to the FN-coated surface was shown with C-terminus bound. The authors suggested that this indicates a preference for bacteria binding at the N-terminus, which was free, although it is also possible that the binding domain is in the middle of the molecule somewhere. Although another recent study suggested that the binding domain between FN and *S. epidermidis* is located near the C-terminus, this group did not verify the orientation of FN on the substrata [68].

In our studies, the orientation of FN was controlled on the SAMs, but not on gold. Carboxyl groups on FN molecules (C-terminal domain) can form hydrogen bonds with carboxyl groups on IPA and IAG SAMs. If we assume that FN adsorbs to the IPA and IAG surfaces via hydrogen bonds to the carboxyl groups, then the N-terminus would have been exposed. Thus, the *S. epidermidis* on the AFM tip was more likely to have interacted with the N-terminus than the C-terminus. The high adhesion forces we observed between *S. epidermidis* and FN may suggest that the preferred binding is at the N-terminus of FN.

Using SAMs to evaluate functional groups with anti-adhesion and anti-bacterial properties
SAMs are useful models for biomaterials due to their uniform construction and the ease with which the terminal functional group can be changed. For example, SAMs terminating in oligo (ethylene glycol), carboxyl, amine, and methyl groups have been used to modify substrata properties, such as hydrophobicity, roughness, and surface charge, in order to find surfaces that resist bacterial adhesion [18,69,70]. We previously measured low adhesion forces between *S. epidermidis* and IPA or IAG SAMs [26], but the current study extends that work to demonstrate how bacterial adhesion changes in the presence of serum proteins. After the deposition of proteins (FBS-covered surfaces and FN-covered surfaces), both AFM force measurements and bacterial retention assays suggest that bacteria cannot distinguish the properties of the underlying substratum in terms of adhesion. Bacterium-bacterium interactions may also be favorable and attached bacteria can attract more bacteria to the surface, as we showed through free energy calculations in a prior study [36]. With time, the substrata would potentially be covered by proteins and *S. epidermidis*. These results suggest that even though some substrata can prevent bacteria and protein adhesion at the early phases due to their surface properties, most differences in substrata properties are masked once proteins coat the surfaces. Perhaps a multi-tiered approach will allow for improved development of biomaterials. For short-term implantation of biomaterials, development of anti-adhesive coatings might be worthwhile. However, for longer-term implantation, it might be beneficial to work towards developing materials that can inactivate bacteria or break down bacterial biofilms. For example, MBEC™ pegs were incubated with engineered bacteriophages that produce an enzyme to simultaneously attack bacteria and break down the matrix of the biofilm [71]. Without incorporating a biocide, it seems unlikely that a truly non-
fouling coating can be developed for *in vivo* conditions, due to the favorability of bacteria-serum protein interactions.

Relationship between cellular viability and adhesion

We saw a link between loss of cell viability and high adhesion forces for the systems in which non-specific interactions were dominant. Apoptosis is one the main types of programmed cell death and has been primarily studied in eukaryotes, although limited studies have recently described the existence of apoptosis-like cell death in prokaryotes, such as bacteria. Various stresses including antibiotics, high temperature, and DNA-damaging agents can trigger bacteria death pathways. Sahoo et al. were the first group to propose that shear stress (mechanical force) can introduce programmed death in *Bacillus subtilis*, although this phenomena was noted earlier for large-scale cell culture bioreactors [72,73]. The agitation and aeration in the reactor can create strong hydrodynamic stress, which produces damages to animal cells, plant cells, and bacteria. However, the shear forces are not lysing the bacteria. For example, *B. subtilis* exposed to mechanical forces did not release intracellular components, yet the bacteria died [72]. Instead, they found that the increased shear stress increased the activity of a plasma membrane bound enzyme, (NADH-oxidase), which caused increased production of intracellular reactive oxygen species [72]. Although this was shown only for bulk mechanical forces, our work suggests that perhaps forces acting on individual bacterial cells may also cause stress that can lead to reductions in viability. While more experiments would be necessary to confirm this suggestion, the findings may be of interest in terms of potential biomaterial development.
As an exception in the relationship between adhesion force and cell viability, a high percentage of bacteria retained on FN-deposited surfaces were viable. The different behavior may be due to the nature of the interactions between each type of protein and the bacteria. FN can form strong specific binding with receptors on the cell walls of *S. epidermidis*, but this is a localized force that occurs at discrete locations on the cell surface. In contrast, the non-specific interactions that are operative between FBS and *S. epidermidis* occur everywhere over the cell body. This suggests that stress can impact viability, but it depends on exactly how this force is localized.

**Biocidal effect of silver**

The antimicrobial properties of silver were demonstrated previously [74,75]. The SAMs terminating in isophthalic acid with silver (IAG) showed antimicrobial properties, resulting in inactivation of 60-70% of attached bacteria. Specifically, for bacteria retained to SAMs terminating in isophthalic acid plus silver (IAG), 63.7% $\pm$ 28.4% of *S. epidermidis* cells lost viability, compared with 36.5% $\pm$ 16.4% on IPA slides. Even after the deposition of FN on IAG slides, 72.3% $\pm$ 27.2% of retained *S. epidermidis* cells lost viability. Under identical conditions, we previously showed that the thickness of FN on the IAG surface is ~110 nm. Thus, these results suggest that the silver ions are able to penetrate over 100 nm in order to reach and inactivate the bacteria. We did not see as strong of an antimicrobial effect for IAG when FBS was present, and this may be because the FBS layer was much thicker, reaching ~260 nm. Silver is water insoluble and only a minimal diffusion rate of antimicrobially active silver ions can be observed [76]. Silver-bearing SAMs may show promise as potential biomaterials for short-term use, but a better release mechanism is needed to resist protein adsorption for longer times.
Conclusions

Characterizing bacterial interactions is important in biomaterial development. This work aimed to directly characterize the adhesion forces measurements between an S. *epidermidis*-functionalized AFM tip and substrata. In particular, we determined how bacterial adhesion was affected by the presence of proteins that could participate in specific or non-specific binding with the bacteria. Molecular adhesion forces between *S. epidermidis* and FN were much greater than the forces between the bacterium and FBS, due to ligand/receptor binding that can occur with FN. SAMs with deposited proteins were more favorable for bacterial adhesion than non-protein coated SAMs. Further, we found a strong correlation between high adhesion forces and the retention of non-viable bacteria, suggesting that certain types of mechanical forces can lead to stress that deactivates bacteria. Although silver ions incorporated into SAMs help decrease bacterial viability, this effect was limited to relatively thin protein layers. This study re-emphasizes that future studies of biomaterial design should consider how bacteria will interact with physiological proteins. In later stages, the biocompatibility of the SAMs and the interaction of SAMs with other proteins *in vivo* could be investigated.
Nomenclature

$M$ effective mass of the cantilever

$\Delta m_c$ mass of added poly-L-lysine

$\Delta m_B$ mass of added bacteria

$k$ spring constant of AFM tip

$f_1$ resonance frequency of the cantilever of the bare AFM tip

$f_2$ resonance frequency of chemically-modified AFM tip

$f_3$ resonance frequency of bacteria-coated AFM tip

AFM atomic force microscopy

SEM scanning electron microscopy

FBS fetal bovine serum

FN fibronectin

BSA bovine serum albumin

IAG isophthalic acid with silver

IPA isophthalic acid

SAM self-assembled monolayer

MES 2-(N-morpholino)ethanesulfonic acid

TSB tryptic soy broth
Acknowledgments

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Figure Captions

Figure 1. Representative SEM images of biological probes, for *Staphylococcus epidermidis* coated onto AFM tip. A) One Coated AFM tip and cantilever; B) Closer view of coated AFM tip showing bacterial cells.

Figure 2. Representative retraction force profiles for the interaction between *Staphylococcus epidermidis* and A) bare substrata (gold or SAMs on gold); B) FBS-deposited substrata; and C) FN-deposited substrata.

Figure 3. A) Pull-off distance distribution histograms of FBS-deposited substrata and FN-deposited substrata; B) Adhesion force distribution histograms of FBS-deposited substrata; (C) Normality test for adhesion force distribution of FBS on gold. For the Anderson-Darling (AD) test, a score <1.092 passes the normality test. For the Shapiro-Wilk (SW) test, a score of >0.89 is considered normal. Both tests showed the results were normal, with the AD score being 0.75 and the SW score being 0.90; (D) Normality tests for FBS on the SAMs (both IAG and IPA data were grouped together, since they were very similar). Data passes the normality test using either criteria, with the Anderson-Darling score being 0.409 (< 1.092 is normal), and the Shapiro-Wilk score being 0.976 (> 0.89 is normal); (E): Normality tests for FN adsorbed on gold. While the Anderson-Darling test failed, with a score of 1.65 (< 1.092 is normal), the Shapiro-Wilk test suggested the distribution is normal, with a score of 0.94 (>0.89 passes); (F) For FN adsorbed to either SAM, both tests showed the distribution of adhesion forces is normal, with the AD score being 0.387 (< 1.092 passes), and the SW score being 0.973 (>0.89 passes).
Figure 4. Correlation between number of bacteria retained on each substrata and the mean adhesion force measured in AFM experiments, for *S. epidermidis* interacting with each of the 9 substrata. Although mean values do not fully characterize the distribution of adhesion forces for all conditions (such as were shown in Figure 3), the use of the mean values allows for convenient comparisons to be made across all conditions. Statistical tests were used to help support these comparisons. Pairwise comparisons between all treatments performed using the Kruskal-Wallis One Way Analysis of Variance on Ranks showed that each treatment is significantly different from every other treatment (p<0.001 needed).

Figure 5. Correlation between mean adhesion forces between *S. epidermidis* and each of the 9 substrata with the Gibbs free energy change of adhesion. Values for the Gibbs free energy changes of adhesion were calculated and reported in a previous publication [36].

Figure 6. Correlation between mean adhesion forces and the percentage of non-viable bacteria that were retained on the 9 substrata.
Figure 1
Figure 2

A) Retraction Distance (nm)

B) Retraction Distance (nm)

C) Retraction Distance (nm)
Figure 3
Figure 4

![Graph showing mean number of cells retained per square millimeter against adhesion force in nanonewtons. The graph includes data points for various conditions: Gold, IPA, IAG, FBS + Gold, FBS + IPA, FBS + IAG, FN + Gold, FN + IPA, FN + IAG. The regression line equation is given as Y = 2174.50X - 324.12 with R² = 0.96.](image-url)
Figure 5

Gibbs Free Energy Change $\Delta G_{\text{adh}}$ (mJ/m$^2$)

Adhesion Force (nN)

$Y = -106.80X + 42.99$

$R^2 = 0.86$

- Bare Substrata
- FBS-deposited Substrata
- FN-deposited Substrata

Linear fit for bare and FBS-deposited Substrata
Figure 6

\[ Y = 69.57X - 0.74 \]
\[ R^2 = 0.93 \]
References


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Part IV: Common Techniques and Issues in Biological Interactions Study
Chapter 10: Atomic Force Microscopy Analysis of Bacterial Surface Morphology Before and After Cell Washing

Abstract

Microbial studies in laboratories are carried out with bacteria grown in standard culture media, followed by preparations for specific tests. In these preparation protocols there is not a consensus about the proper number of washing steps in order to “clean” the cells from the culture medium and other molecules excreted by the cells during their growth. For this reason, the main goal of this work was to characterize the surface of *Escherichia coli* before and after a certain number of washing steps, with the help of atomic force microscopy. Bacteria without washing were covered by a smooth granular-like matrix which impeded the observation of their rod-shape, their roughness, and provided unusual values of the bacterial length, width and height (5.0±2 µm, 4.0±2 µm and 1.2±0.5 µm, respectively). In addition, unwashed bacteria were protected from dehydration, suggested by irregular behaviour of the water contact angle with time. The washing process led to the appearance of the “real” morphology of bacteria. Three washing steps showed typical dimensions of bacteria (length = 1.9±0.4 µm, width = 0.7±0.4 µm and height = 0.5±0.1 µm) and an external surface rougher than that without wash (18±7 nm versus 7±3 nm). However, a greater number of washing steps (seven washing steps, in this case) could impart negative effects on the cell surface morphology, reflected in the loss of the typical cell shape and stiffness. Despite the evidence of the influence of the washing process on cell morphology, we cannot conclusively recommend one specific washing protocol that will be universal to all bacteria. However, the lack of complete knowledge of bacterial interactions with the surrounding...
environment obliges one to be consistent with the washing protocols followed for a specific research project.

**Introduction**

The atomic force microscope (AFM) has been used in the last few decades as a potent tool for analyzing different types of surfaces (7, 27, 46). Initially, the apparatus was designed for studying inert surfaces (16, 25) but the application of this technology to biological samples has helped to enrich the knowledge of microbial surfaces and the mechanisms of interaction between microorganisms and host surfaces (11, 14, 15, 29, 41, 48). For example, in relation to the bioadhesion process, the AFM can provide information about relevant microbial surface features such as the presence of adhesins or fibrils and even their distribution and lengths (1, 11, 24, 44). The interaction of microorganisms with the AFM tip has been also used to provide the magnitude of interaction forces (1, 2, 11, 30, 31, 33) as well as qualitative and quantitative analysis of physicochemical surface parameters such as hydrophobicity (22, 42). Moreover, AFM is able to examine the microbial surface through characterizing morphology, stiffness, roughness or location and size of relevant features (6, 35, 45).

In relation to micro-roughness, there is some controversy in defining the level of participation of roughness in microbial adhesion. Some authors find no correlation (4, 18, 32) while others indicate a direct link between initial bacterial adhesion and surface roughness (20, 28, 47). As far as we know, these conclusions are made taking into account the roughness of one of the surfaces involved in the bioadhesion, the substrate, but the roughness and topographical features of microbial cells have not been considered. The determination of the surface roughness of bacteria can be strongly affected by the
radius of curvature of the cells and for this reason only fine scale scanning areas can provide a reliable value of cellular surface roughness, not affected by bacterial curvature. Surface roughness features of the substrate can act as niches where bacteria are protected against erosion and external agents and, consequently, their values can be crucial in the formation of biofilms. However, at roughness values less than the microorganism size, the cell roughness, and more concretely the microbial surface topography, may contribute in an important way to the cell anchorage and further biofilm development. Detailed structural studies of the bacterial surface help us to understand molecular mechanisms of the biofilm formation and functioning (6).

Further, all microbial surface properties are affected by the molecules and substances present on such a surface. Since bacteria are prepared in research laboratories, the protocols followed for obtaining the samples are extremely relevant for the expressed surface properties. For example, the temperature and the components of the culture medium are crucial in determining the behaviour of microbial cells (34, 36, 39) and so, physicochemical properties of cells must be always related to the characteristics of the growth medium. Although no media is able to exactly reproduce the conditions “in vivo”, the components should be carefully selected in order to obtain the most accurate results for a particular application.

Another important aspect, usually treated only casually, is the washing process after microbial growth. In order to determine if 0.4% chlorhexidine used as a one-time vaginal wash can reduce the incidence of intraamniotic infection or endometritis caused by different bacteria such as *E. coli* in labouring pregnant women, either 20 ml of 0.4% chlorhexidine (n=481) or 20 ml of sterile water (n=466) were randomized tested on
pregnant women (39). In this work, a one-time 0.4% chlorhexidine vaginal wash did not mitigate vaginal infections, compared with the use of sterile water. However, the liquid viscosity, liquid exposure-time and volume employed could each affect the efficacy of the washing protocol.

It is also known that the ionic strength of the wash solution leads to the cells presenting different physicochemical characteristics (43). However, in general, authors do not pay specific attention to the washing liquid type for bacteria (usually phosphate buffered saline) or the number of washing times, and there is no universally accepted cleaning criterion, as there is for inert surfaces (12). A bibliographic survey shows that experiments dealing with biological processes, including bacterial adhesion experiments, are carried out with microorganisms without wash (13, 15, 17, 28, 32), after one washing step (18, 26, 38), two washing steps (3, 20, 42) or three steps (3, 8, 45) and similar variability in protocols is also seen in the study of mammalian cells (9, 37, 40).

In the washing process, the cells, after a certain period of incubation, are harvested with the aim of separating them from the culture medium, which implies the removal of the growth materials and other molecules “excreted” by the cells during their growth. This process is mainly carried out by centrifuging the bacterial suspension and resuspending the pellet in a buffer liquid which simulates the environment of interest. The subsequent centrifugation and resuspension of cells in the buffer liquid are interpreted as the first wash. Second and third washes imply the repetition of the last step.

Since bacteria interact with substrates through their surfaces, the washing process should be critical for determining which property is exposed to the surrounding environment. We seek to understand whether the cleaning process affects the
composition of the microbial surface and whether the “real” surface of bacteria can be seen after a certain number of washing steps.

Therefore, the main objective of this research was to evaluate the morphological state of bacterial cells before and after washing. We used AFM imaging to help characterize how the nature of the surface changed as a function of the washing protocol employed. To this end, a well-characterized strain of Escherichia coli was employed, grown in Tryptic Soy Broth (TSB) and washed zero, two, three, seven and fifteen times with Phosphate Buffered Saline (PBS). As an additional marker to describe surface modifications, we considered the cell surface hydrophobicity, quantified in terms of the water contact angle, for the case of unwashed bacteria and bacteria with three wash steps.

Materials and Methods

Microorganisms and growth conditions

The strain E. coli HB101 was obtained from the American Type Culture Collection (ATCC 33694). It is a plasmid-less, non-fimbriated bacterium (23). Bacteria were long-term stored at −80°C in glycerol solution. From the frozen stock, single bacteria colonies were inoculated on Tryptic Soy Agar (TSA) plates for short-term storage in the refrigerator at ~2-8 °C. Then single bacteria colonies were incubated in 30-50 ml of Tryptic Soy Broth (30 g/L TSB; Sigma, St. Louis, MO, USA) at 37 °C. Each culture was maintained at 37 °C in a water shaker bath. After 4-8 h, mid-exponential growth phase bacteria were harvested for further experiments. The measurement of the absorbance with a spectrophotometer (Thermo Genesys 20, Thermo Electron Corp., Waltham, MA, USA) at 600 nm was crucial for the selection of the appropriate growth state. Cells were harvested when the absorbance was 0.7 - 0.8, measured with a
spectrophotometer zeroed by pure TSB solution. Bacteria without wash were taken without any further treatment.

In those cases requiring a washing process, bacteria were harvested by centrifugation for 5 min at 1400 g (Fisher Scientific Model 225 Benchtop Centrifuge, USA). The supernatant was discarded and then the cells were washed with PBS (pH 7.4, NaCl 0.138 M, KCl 0.0027 M, K$_2$HPO$_4$ 0.005 M, KH$_2$PO$_4$ 0.005 M). Then, the cells were again centrifuged, the supernatant discarded, and resuspended in PBS. To increase the number of washing steps the last wash step was repeated as many times as desired. Several washing times were considered in this project but we concentrated on analyzing the results from 2, 3, 7 and 15 washing steps.

Sample Preparation for AFM Imaging

Prior to AFM analysis, bacteria were gently sonicated for 3 min in an ultrasonic bath (40 KHz, 130 W) (Branson, Model 2510, USA) in order to help us image isolated cells, rather than aggregates. Then a micro-droplet of bacterial suspension was deposited on freshly cleaved mica and dried in atmospheric air, at room temperature (~22 °C), for ≤ 1 h.

With the aim of comparing the structure of the layer covering the bacteria to the structure of the supernatant itself, we also prepared samples of cell-free supernatant material. For the bacteria that were not washed, 9 mL of bacterial suspension, after growth, were centrifuged and ~2 mL of the supernatant were filtered using a 0.2 μm pore size syringe filter in order to remove any microbial cells and only analyze the fluid around the bacteria after growth. A droplet of this liquid was deposited on freshly cleaved
mica and dried before AFM analysis (in the same way as bacteria). A similar protocol was followed with the supernatant from the bacterial suspension after three washing steps. **AFM Imaging**

Bacterial morphology was studied with an AFM (Digital Instruments Dimension 3100 with Nanoscope IIIa controller, Veeco Instruments Inc., Woodbury, NY, USA), having a 100 μm scanner (x-y), with a maximum of 7 μm in the z direction. An optical microscope (resolution ~1800X) mounted with the AFM and connected to the computer monitor helped to locate the region of interest on the mica. The images were acquired using rectangular cantilevers (MikroMasch, Wilsonville, OR, USA, NSC36/AIBS, Cantilever type C) with conical silicon tips, having a nominal spring constant of 0.6 N m⁻¹ and a typical probe radius of curvature of 10 nm, taken from the manufacturer’s specifications. The maximum applied force was ~1.5 nN. Amplitude and height images were captured in tapping mode. The scan rate varied from 0.5 - 3 Hz and the images were recorded at 512×512 pixel resolution. Height images were flattened by using a second order filter in order to remove the background slope and non-linear scanner effects.

Bacterial dimensions (length, width and height) were obtained by cross-section analysis of individual cells, using the AFM software. The root-mean-square roughness (R_{rms}) was obtained from scan areas of different sizes (from (0.2×0.2) μm to (5×5) μm), taking into account the area of interest of the images and using the Nanoscope Version 5.12r5 software. Although the surface roughness values obtained are highly dependent on the type of measurement technique that is used (25), relative comparisons of roughness can be made for measurements that use the same technique.
Contact angle measurements

The contact angle was measured for water droplets on bacterial lawns, using ultrapure water (18.2 MΩ·cm resistivity and <10 ppb total organic carbon, Millipore Milli-Q Plus, Billerica, MA, USA), using the sessile drop technique (10). Bacteria without wash or after three washing steps were deposited onto 0.45 μm pore size filters (CAS# 9004-70-0 Millipore Corp.) using negative pressure. Filters were left to air dry at room temperature and micro-droplets of water were deposited on bacterial lawns as a function of time with the help of a goniometer (Ramé-Hart, Netcong, NJ. USA). The deposition of each droplet was recorded in a video and then analyzed in order to obtain the water contact angle.

Statistical analysis

Specific measurements such as bacterial length, width and height were repeated for each sample at least 10 times. The number of samples examined was related to the ability to locate single cells on the substrate. Treatments for 7 and 15 washing steps resulted in a lower number of repetitions because of the difficulties in obtaining individual cells in the images, as will be discussed. The average sizes are reported with the standard deviations for all cases.

In the measurement of the surface roughness, the statistical analysis was made for the results of each washing step, respectively. Comparisons between samples were done by using an unpaired Student’s t-test. The confidence interval selected was 95%. Therefore, differences were considered statistically significant if P<0.05.
Results and Discussion

Without wash

Figure 1 shows an AFM image of bacteria grown in TSB, without wash, on a cleaved mica substrate. The (40×40) µm image (Figure 1A) reveals that the substrate is intermittently covered by bacteria which are, in some cases, grouped. The bacteria seem to be covered by thick amorphous layers, with the presence of round-shaped clusters. Round clusters are also located on the substrate surface, especially in those areas close to the bacteria. This behavior is better appreciated in the (20×20) and (10×10) µm images (Figures 1B and 1C). The quantification of the roughness on those areas of the substratum covered by this granular-like film (not considering the granules) indicated that the average $R_{\text{rms}}$ was 9±4 nm. This value remained constant for several scan areas ranging from (0.2×0.2) µm to (2×2) µm.

Roughness was also analyzed on the bacteria-like structures at scan areas ranging from (0.2×0.2) µm to (1×1) µm. Henke et al. (25) noted that the size of the area over which the height variation is calculated and the sampling distance between data points could affect $R_{\text{rms}}$. Therefore, comparisons were made for similarly sized areas only. Roughness increased with the area scale of the surface analyzed, and this was mainly due to the curvature of bacteria (typical length of 2.7±0.7 µm and width of 1.1±0.2 µm). At the lowest scan area of (0.2×0.2) µm, $R_{\text{rms}}$ was 7±3 nm, (Table 1) most similar to that of the substrate (P>0.05); while scan areas of (0.5×0.5) µm gave $R_{\text{rms}}$ values of 30±8 nm, and $R_{\text{rms}}$ even reached as high as 163 nm for scan areas > (0.5×0.5) µm. The similarity between $R_{\text{rms}}$ of the bacteria and substrate at the lowest scan sizes quantifies what one can observe: bacteria are covered by a smooth-granular layer that is also present on the
substratum.

The (3×3) µm image shows two bacterial cells (Figure 1D). The layer covering the cells is clearly observed and it is so thick that we are not able to define the line of contact between bacterium and substrate. The bacterial size and length/width ratio were not as expected for *E. coli*, with larger and more spherical cells being observed. A systematic cross-length profile analysis showed that the average length for bacteria was 5.0±2.0 µm, with widths of 4.0±2.0 µm and heights of 1.2±0.5 µm (Table 1). These values appear larger than the typical dimensions of *E. coli*, suggesting that the coating is masking the real structure of the cells.

Since bacteria were not washed with PBS, the layer covering the bacteria can only be due to the components of the culture medium, or possibly molecules excreted by the cells during their growth. We also imaged the cell-free supernatant from this solution (Figure 2). In the (20×20) µm image, a thick matrix was observed to be completely covering the substrate. Its structure is better appreciated in the (5×5) µm image. This granular-like film is very similar to the one observed on the bacteria, especially when considering the areas with bacterial aggregates (i.e. Figure 1A). The granular-like matrix of Figure 2B is also similar to the clusters on the substrate observed in Figure 1C. The roughness on the substrate, obtained on areas avoiding the irregular granules, was 8±3 nm, similar to that obtained on cell-free areas for the bacterial preparation and clearly different from the roughness of freshly cleaved mica (R<sub>rms</sub> = 0.34±0.02 nm).

Two washing steps

The coverage of the substrate, as well as the appearance of the bacteria, is different for bacteria after two washing steps, compared to unwashed cells (Figure 3). A
matrix is fully covering the surface of the mica and it contains bacteria-like structures that resemble leaves in a tree (see especially Figures 3A and 3B) with bacteria randomly distributed (see arrows in Figures 3A and 3B). The surface texture of the bacteria appears to be different from the unwashed cells. Figure 3C shows a typical single bacterium “emerging” from a zone where the accumulation of extracellular materials is high. This bacterium has some smooth areas on its surface, as well as some patches showing accumulations of other substances, probably coming from the culture medium. The structured materials on the substrate are likely the result of crystallization of molecules and ions from the culture medium, TSB, and the washing liquid, PBS.

In order to check whether such material comes from the media, the filtered supernatant was also examined. The cell-free TSB+PBS materials (Figure 4) exhibit similar structures as those seen on areas near the bacteria. We conclude that the bacteria were surrounded by a mixture of crystals coming from the ions and other molecules presented in TSB and PBS, which can be also responsible for the material accumulations located on the bacterial surface and not removed after two washing steps. In addition, the materials observed and their conformation must be from TSB or due to the mixture of PBS with TSB. PBS alone is not able to crystallize in this way, and shows only very small features on the mica surface (Figure 5).

We compared the sizes of the bacteria after two washing steps to those of unwashed cells. The average dimensions for bacteria after two washing steps, obtained by analyzing several cells, gave lengths of 2.6±0.5 µm, widths of 1.5±0.6 µm and heights of 0.6±0.2 µm (Table 1), which are smaller than the dimensions of bacteria without wash (P<0.05 for length and width).
The roughness values of the bacterial surface showed high dispersion; for example in values for scan areas of (0.2×0.2) µm, the standard deviation of the R_{rms} was very close to the magnitude: \( R_{rms} = 11 \pm 9 \) nm (Table 1). In the case of the (0.5×0.5) µm area, the R_{rms} reached values as high as 60 nm. In this case, the average roughness of the substrate obtained at the lowest sized box-areas (20±10 nm) tended to be higher than the average R_{rms} roughness on the bacterial surfaces (11±9 nm); however we should be prudent with this relationship because of the high dispersions obtained. The high standard deviations reflect the specific distribution of the crystals on the mica and bacterial surfaces, which led to a scale-dependence in the roughness on the surface area selected, especially for small scan sizes. In the case of mica covered only by TSB+PBS, the R_{rms} was nearly constant (\( R_{rms} = 225 \pm 5 \) nm), up to a scan area of (60×60) µm.

Three washing steps

As many standard laboratory protocols involve three wash steps to remove components from the growth medium, we next examined the bacterial morphology for cells that were washed three times (Figure 6). In the (40×40) µm image (Figure 6A), the substrate is fairly clean. Bacteria are randomly located and clearly surrounded by a structure similar to the one covering the mica in Figure 3. Crystallization of the ions of PBS and some molecules of TSB produces a conformation on mica similar to the ones observed with less washing steps but these materials are more difficult to discern, probably because the TSB has been almost completely removed. One of the most interesting things to note is that the crystallized ions are confined to the areas surrounding the bacteria, and they mimic the shape of the bacteria. This finding suggests that the washing procedure is removing most of the remaining components of TSB and that the
ions-crystals are located mainly around the bacteria due to the evaporation of residual water.

In order to clearly study the state of the bacterial surface in this case, we zoomed in and scanned certain areas of the images where a single bacterium was present, as shown in Figures 6B and 6C. Here we can see that bacteria are surrounded by a matrix-like substance which may have been “secreted” by the cells during their growth. In addition, the surface of the bacterium seems to be “cleaner” and we can appreciate their rod shapes and also their typical roughness. Average dimensions were: length = 1.9±0.4 µm, width = 0.7±0.4 µm and height = 0.5±0.1 µm (Table 1). The length and width were lower than any of the values obtained previously for the fewer number of washing steps (P<0.05), which could mean that bacteria are apparently becoming smaller with continued washing steps. The height is also smaller than for unwashed bacteria and slightly smaller than the values for cells washed twice. The mixture of TSB+PBS that was on top of the bacterium was loosely associated with the cell, and after the three wash steps, this material seemed to have fallen off of the cell due to gravity. Therefore, the height also decreased.

After three washing steps, bacteria seem to expose their “real” surfaces. Therefore, we considered it interesting not only to provide the bacterial roughness but also to show a line profile of a small area on the bacterial surface. Figure 7 presents the profile of one (500×500) nm area on a bacterium. The bacterial surface shows granular-like structures that varied in size from 50 to 150 nm. The average $R_{\text{rms}}$ was 18±7 nm (Table 1). Despite the high dispersion in $R_{\text{rms}}$, we can appreciate, if compared with unwashed bacteria, that the bacterial surface is becoming rougher after some washing is
performed to clean the surface (P<0.05).

Two or three washing times are the typical number of times that different authors use to wash their microorganisms prior to their experiments (3, 8, 20, 42, 45). We determined that bacteria seem to lose their coating film, coming from the culture medium, when they are washed three times. To determine if we could get a “cleaner” surface if further washing was performed, some samples were exposed to a higher number of washing steps.

Seven washing steps

Figure 8 shows *E. coli* after seven washing steps. Bacteria appear on the substrate surrounded by granular-like features. We speculate that the TSB has disappeared and we are left with mainly very small features, typical of the molecules observed when pure PBS was deposited on the mica (see Figure 5). In Figure 8A, numerous interferences or noisy scan lines were seen, especially when scanning the top part of the highest zones, which can imply that the sample was becoming softer and/or was less tightly attached to the substrate than for the previous samples. It was very difficult to obtain bacterial images at any scan length and scan speed because the material did not seem tightly bound to the mica. Several attempts were needed to provide images similar to the ones presented. Figure 8B shows how the materials deposited on mica were very vulnerable to the tip scanning. For example, the arrow shows how materials were moved by the tip sweeping the surface. Figure 8C exhibits one of the few bacterial cells found (white arrow) completely encased by a soft matrix. Since most of the TSB components have been removed, such a matrix should be the result of loosely bound materials from the cell surface. The washing process, after such a high number of washing steps, could have
damaged the cellular wall, leading to the release of the amorphous materials observed in Figure 8B or marked with black arrows in Figure 8C. The bacterial dimensions were: length=1.7±0.2 µm, width=0.8±0.2 µm, and height=0.6±0.1 µm (Table 1). The roughness could not be accurately measured due to the interferences and scan lines in the images, caused by the movement of deposited materials by the tip.

Fifteen washing steps

The last case we considered was that employing fifteen washing steps. Figure 9 shows how the bacteria are again mixed with materials covering the substrate. Similar to the previous case, this matrix is soft and sticky to the AFM tip. In those areas where neither bacteria nor soft matrix were observed the substrate appeared mostly clean. In the top part of Figure 9A, some single cells can be observed. We zoomed in on this area in Figure 9B. The bacteria, in this case, seem to have an irregular surface. There were areas covered by a granular-like biomaterial, similar to the one appreciated in Figure 6C, and other areas were smoother (see the central part of the cell). This behaviour visually confirmed our previous supposition that the external part of the cell, after such a high number of washing steps, became damaged. Bacteria may have lost intrinsic components of their wall in the washing process, such as materials or molecules important for defining their structural conformation. This seems likely because several scan areas examined revealed no bacterial cells. Bacterial adhesive behaviour also apparently changed, due to their loose attachment to mica. It is also possible that some bacterial cells lysed, and the cumulus of soft materials around bacteria can be components of dead bacterial cells. Although the number of bacterial cells observed was low, the measured dimensions of the cells that could be imaged were 1.8±0.3 µm (length), 0.5±0.2 µm...
These results demonstrate the importance of the washing protocol in the characterisation and evaluation of microbial surface properties. Although the bacterial surface seems to appear cleaner with continued washing, we think that a high number of washes damaged the bacterial surface. If a greater centrifugal force was used during the centrifugation step, the cellular damage may have appeared sooner and/or to a greater degree (5). Nevertheless, despite clear evidence that the washing process affects the bacterial surface, we cannot conclusively recommend one specific washing protocol. When bacteria are “in vivo” they must be covered by a matrix similar to that of the zero washing time, so, accurate replication in the laboratory should avoid any washing process for these types of experiments.

However, if bacteria in their environment are able to exhibit components and properties of their intrinsic bacterial surface (and this has been indirectly checked by the different bacterial reactions in various bioadhesion process (1, 11, 24, 44)), accurate reproducibility in the laboratory should include two or three washing steps, but no more. In any case and based on the results of this work, the lack of a complete knowledge of the bacterial interaction mechanisms obliges researchers to be consistent with the washing protocols employed in a specific research project.

These observations can be crucial when trying to relate initial bacterial adhesion with the formation of biofilms. During the formation of biofilms bacteria must be immersed in their growth medium, but for studying initial bacterial adhesion, bacteria are usually washed in PBS buffer. Flemming et al. noted that trends in initial bacterial adhesion do not necessarily correspond to similar trends in long-term colonization (17).
and the discrepancy can be due in part to the properties of the bacterial surface in each case.

Furthermore, special attention must be paid when performing local elasticity measurements for stiffness determination by AFM (15, 35), because it is expected that the results obtained will depend on the cell washing protocol. We think that this study could be the initial step for further physicochemical surface tests or specific microbiological assays in order to better understand the effects of the washing process on bacterial surface properties.

Bacterial cell surface hydrophobicity

As a first approach to further studies, we evaluated one of the most relevant microbial surface properties in relation to the bioadhesion process, the hydrophobicity. The degree of hydrophobicity is crucial in the approach of bacteria to host surfaces and the subsequent attachment. Different tests have been described in order to quantify the hydrophobicity, but one of the most important and reliable tests is the water contact angle measurement (10, 19). The validity of the water contact angle to characterize hydrophobicity is strictly related to the accurate determination of the sample drying time (22). Therefore, we studied whether the drying time was altered by the number of washing steps of the sample. We characterized the drying process for bacteria suspended in TSB, without wash, and after three washing steps (Figure 10).

To ideally dry a bacterial sample, one supposes that residual water remains among cells for a certain period of time and then when this water evaporates, only the water belonging to bacterial constituents (hydration water) stays internally. This last state is the one that provides information about the bacterial surface. Any schematic representation
of water contact angle as a function of time should show a low constant value at early
times and then a jump to higher water contact angle values, which represents the ideal
state to measure the contact angle (21).

A perfect example of evaporation of the residual water was observed for cells
washed three times (Figure 10B), while for unwashed cells (Figure 10A), the bacterial
film seemed to maintain its residual water for a longer period of time, as evidenced by the
fluctuations in the measurements until 80 min. For bacteria washed three times, 70 min
was sufficient for the measurement to reach a stable value, while > 80 min were needed
to reach a stationary-like state for bacteria without wash. The water contact angle for
unwashed cells was $60\pm 5^\circ$ and for bacteria washed three times it was $68\pm 5^\circ$, which
indicates a tendency of washed bacteria to be less internally hydrated. The matrix
covering bacteria without wash (observed in Figure 1) can be responsible for increasing
the hydration of the bacterial cells, as observed not only in the difficulty of eliminating
the residual water but also in the lower water contact angle when compared with bacteria
washed three times. Another point of view is that, in fact, this matrix is the only material
that the water droplet “sees”, so we could also say that in the case without wash, we are
not measuring the properties of the bacterial cells but the properties of the matrix
covering the cells.

**Conclusions**

This work revealed that the number of washing steps, during the cleaning process
after bacterial growth, directly affected the morphology of *E. coli* and their hydration
state. Non-washed bacteria exhibited a smooth granular-like layer on their surface which
made the definition of their shape, dimensions, and roughness difficult to observe, and
preserved the cells from dehydration. After washing, the cells were able to present their externally rough surface and to eliminate residual water more easily. However, a high number of washing steps imparted negative effects on the cell surface morphology, reflected in the loss of the typical cell shape and stiffness. Although we cannot conclusively recommend a specific number of washing steps for all bacteria, the results of this study suggest that researchers need to be consistent with the washing protocols for comparison of results from different series of experiments or among laboratories.

Acknowledgements

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References


Table 1. Root-mean-squared roughness ($R_{\text{rms}}$) for the surface of *E. coli* HB101 obtained with scan areas of (0.2x0.2) μm, as well as length, width and height of the bacteria after different washing steps. Data are expressed with standard deviations.

<table>
<thead>
<tr>
<th>Washing Steps</th>
<th>$R_{\text{rms}}$ (nm)</th>
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<th>Width (μm)</th>
<th>Height (μm)</th>
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<td>5.0±2</td>
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<tr>
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<tr>
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<tr>
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<td>-^a</td>
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</tr>
<tr>
<td>15</td>
<td>-</td>
<td>1.8±0.3</td>
<td>0.5±0.2</td>
<td>0.5±0.1</td>
</tr>
</tbody>
</table>

^aNot Determined
Figure Captions

Figure 1. Amplitude AFM images of *E. coli* HB101, without wash, on freshly cleaved mica.

Figure 2. Amplitude AFM images of cell-free TSB solution, after bacterial growth, deposited on freshly cleaved mica.

Figure 3. Amplitude AFM images of *E. coli* HB101 after two washing times on freshly cleaved mica. Arrows mark bacteria randomly distributed.

Figure 4. Amplitude AFM images of the cell-free solution of TSB+PBS, coming from the first bacterial wash, deposited on freshly cleaved mica.

Figure 5. Amplitude AFM images of pure PBS on freshly cleaved mica.

Figure 6. Amplitude AFM images of *E. coli* HB101, after three washing times, on freshly cleaved mica.

Figure 7. Example of one section profile analysis of the surface “granules” of *E. coli* HB101 after three washing times (left image) obtained from the height image (right).

Figure 8. Amplitude AFM images of *E. coli* HB101 after seven washing times on freshly cleaved mica. In (B) arrow marks the movement of deposited materials by the tip sweeping the surface. In (C) white arrow marks one single bacterium and black arrows mark accumulations of amorphous materials.

Figure 9. Amplitude AFM images of *E. coli* HB101 after fifteen washing times on freshly cleaved mica.

Figure 10. Water contact angles on bacterial lawns versus time for *E. coli* HB101 without wash (A) and after three washing times (B).
Figure 1
Figure 3

(A)

(B)

(C)
Figure 4
Figure 6

(A)

(B)

(C)
Figure 7
Figure 8
Figure 9
Figure 10

(A) Water contact angle (°) vs. Drying time (min)

(B) Water contact angle (°) vs. Drying time (min)
Abstract

The atomic force microscope (AFM) is a powerful microbiological tool that allows for high resolution imaging or force measurements on intact microbes in liquid. In order to take advantage of this benefit, the microbes must be immobilized with minimal alterations to their surface properties. This study investigates the feasibility and application of three immobilization methods, namely bacterial attachment through covalent bonding, electrostatic forces, and mechanical trapping. Special focus is given to the zero-length cross-link covalent bonding reaction commonly used for protein and bacterial immobilization. Based on comparing AFM images obtained with the three immobilization methods, experimental conditions were optimized through modifications in cell washing, sonication, and substrate selection, to achieve the easiest, most reproducible, and artifact-free cell immobilization. In addition, the strengths of the immobilization methods were evaluated.

Introduction

Overview of Microscopy Techniques used in Microbiology

Life scientists have long been trying to understand whether function determines form or function follows form. Regardless, direct observation of morphology can usually greatly promote the understanding of the function. An excellent example is the discovery of the three-dimensional structure of the DNA double helix, which created a revolution in biology and created whole new branches of genetic science and engineering.
Microbial research as an important life science branch has been important since the earliest microscopy studies to observe living cells in the late 17\textsuperscript{th} century. Better understanding of function creates new questions to be answered in microbiology, which then require better imaging techniques that can reach to molecular and sub-molecular levels. Fluorescence microscopy can be combined with stains that target DNA or can be used to determine cellular viability, or fluorescent tags for proteins, thus extending the power of optical microscopy \((1)\). Confocal microscopy, which improves the fluorescence microscope by exclusively collecting the fluorescence from the focus point, can be used to construct 3-D images of microbes or biological samples by scanning many continuous thin sections of the sample with the help of a computer. This technique has been quite useful for imaging microbial biofilm structures \((2)\).

Based on the same theory as the optical microscope, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) utilize electrons as the “light source” which have much shorter wavelengths, thus allowing for high resolution imaging at the nanoscale, since resolution is proportional to wavelength. However, the complex sample preparation, operation in vacuum and requirement of dried samples somewhat limit the application of TEM and SEM in microbiology. Cryo-TEM attempts to image “liquid samples” by freezing the sample rapidly to form an amorphous solid. This method produces detailed images of surface morphology and even internal structures, with careful application \((3)\).

Based on an entirely different principle of operation, atomic force microscopy (AFM) derives topographic images by probing the surface with an ultrasharp tip, at the end of a cantilever. The subtle deformation of the cantilever is amplified and monitored
by a laser lever collected by a photodetector. The piezo sensor is used to manipulate and record the AFM tip motion in three dimensions. AFM can image samples with nanometer resolution in the X-Y dimensions, with forces ranging from several picoNewtons to hundreds of nanoNewtons, a range that is well suited for imaging microbes without damage. A key advantage for microbiological studies is that the AFM can image samples both in air and in liquid, enabling the observation of microbes in their natural environment. Another unique advantage of AFM is that it can be used to directly measure the interaction forces between bare or functionalized probes and the substrates, such as biomaterials, proteins or other cells, under conditions very similar to their real environments. In addition to gaining surface structure information, one can quantify the adhesion forces between microbes and uroepithelial cells, proteins, receptors, etc. as reviewed in Ref. (4).

Preparing Bacteria for AFM Imaging and Force Measurements

When microbes are imaged in air, immobilization of samples on substrata (usually glass slide, membrane filters or mica) can be spontaneously accomplished with adsorption of the microbes to the surface. An issue that must be addressed under this circumstance is how to wash the microbes properly to remove residue of the culture medium and products secreted by the microbes, all without damaging the native bacterial surfaces.

Examining bacteria under liquid solutions usually requires further steps in the preparation process due to the hydrophilicity of most microbes and the forces exerted on the cells during the AFM probing. Several techniques have been applied in this context.
One commonly used technique is to employ covalent bonding between bacterial cells and molecules attached to a glass slide. For several Gram-negative bacterial strains, the EDC/NHS zero-length crossing-linking reaction has been applied to couple carboxylic groups on the bacterial surfaces with amino groups on 3-aminopropyl-trimethoxysilane treated glass slides (5-7). This covalent immobilization method has also been used for small biologically active molecules such as DNA, proteins and enzymes, etc. (8). However, for some microbes with fewer carboxylic groups or with strong hydrophilicity such as *Pseudomonas aeruginosa* PAO1, the covalent bonds may be not strong enough to withstand the hydrophilic forces between microbes and the liquid. A modification of this method is needed. In this study, we demonstrate how to reversibly apply the EDC/NHS zero-length crossing-link reactions and successfully immobilize microbes that cannot be bonded through the original reaction scheme, broadening the application of this method.

A second commonly used methodology is to bond bacteria to a substrate based on electrostatic interactions. Poly-L-Lysine (PLL) and polyethylene imide (PEI) are agents frequently used to create a positively charged surface on glass (9, 10), mica (11, 12) or tapered fiber (13), in order to physically adsorb negatively charged microbes to the substrates. One study reported that gelatin solution was superior to PLL in immobilizing bacterial cells grown on Luria broth (LB) agar plates, in terms of the number of absorbed cells and the affinity of immobilization (12). Due to non-specific interactions, electrostatic forces can be used to immobilize many cells or biomolecules at once. The strength of the physical bonding compared to the covalent attachment method has not been evaluated.
A third method we have evaluated is that of mechanically trapping bacterial cells. Most mechanical trap experiments are done via an isopore filter membrane, although one study immobilized bacteria in 40-45° molten agar. For example, yeast cells *Saccharomyces cerevisiae* were immobilized in agar for AFM imaging that would allow the observation of cell growth and division over a 6-7 hour period (14).

Kasas et. al. first reported the anchoring of round-shaped cells via filter membranes with comparable pore size with bacteria dimension (15). This simple method has been mostly applied to round bacteria i.e. Gram-positive cells such as *Lactococcus lactis* (16), *Staphylococcus aureus* (17) and *Streptococcus salivarius* (18), usually with an 0.8-μm isopore polycarbonate (Millipore) filter membrane. However, trapping microbes by a filter membrane has not been successfully and widely applied to rod-shaped Gram-negative bacteria. An exception was the trapping of rod-shaped *Klebsiella terrigena* by a filter membrane (9). In the present study, we attempt to apply this method to more examples of rod-shaped Gram-negative bacteria and evaluate the effect of filtering on the morphology of the cells.

**Methods and Materials**

Bacteria Cultures and Harvesting

Three bacterial strains were used in this study. *Escherichia coli* HB101, a Gram-negative bacterium, was purchased from the American Type Culture Collection (ATCC 33694). It is a plasmid-free, non-fimbriated strain (19). *Pseudomonas aeruginosa* PAO1, a Gram-negative bacterium, was kindly provided by Professor Gerald Pier (Channing Laboratory, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA, USA). *Staphylococcus epidermidis*, a Gram-positive bacterium,
was a gift from Professor Stephen Heard (Department of Anesthesiology, University of Massachusetts Medical School, Worcester, MA, USA). Bacterial cells were precultured in 30 g/L Tryptic Soy Broth (TSB) (Sigma, St. Louis, MO) at 37 ºC overnight. Next, 0.5 ml bacteria solution from the preculture was added into ~30-50 ml fresh TSB and cultured in a water shaker bath at 37 ºC. Bacterial growth was monitored by the absorbance at 600 nm using a spectrophotometer. Bacterial cells were harvested in the middle exponential phase, corresponding to absorbance measurements of 0.7-0.8 for *E. coli* HB101, 0.8-0.9 for *P. aeruginosa* PAO1, and 0.5-0.6 for *S. epidermidis*. Cells were collected by centrifuging at 1400 g for 15 min (Fisher Scientific Model 225 Benchtop Centrifuge, Pittsburgh, PA, USA). Bacterial cells were washed three times with PBS buffer, unless otherwise specified, following a procedure described previously (20). In some cases, bacteria were not washed, so that the effect of not washing the cells on trapping and immobilization could be studied. Final bacterial concentrations were adjusted to $2 \times 10^7$ cells/ml in 0.1 M sodium phosphate (pH=7.1). Bacterial solutions were sonicated in an ultrasonic bath (40 KHz, 130 W) (Branson, Model 2510, USA) for ~5 min to break up any aggregates.

**Immobilization Methods**

(1) Covalent Bonding Immobilization

The covalent bonding reaction could be applied in two ways, depending on whether we wanted to target carboxylic groups or amine groups on the bacterial surfaces (Figure 1A-C).

To attach bacteria to substrates based on targeting the bacterial carboxyl groups, we followed a protocol developed previously (21), with some modifications (Figure 1A).
The substrate could be glass or mica. Glass slides were acid cleaned before use, as described in (5) followed by 15 min sonication in ultrapure water, and rinsing with copious amounts of pure ethanol and analytical grade methanol (Fisher Scientific, Fair Lawn, NJ, USA), to remove all residual water from the glass slides. For mica substrates, Ruby red mica sheets (Electron Microscopy Science, Washington, PA, USA) were freshly cleaved immediately prior to use, but were not cleaned with any chemical agents.

Glass slides or mica sheets were immersed in 10-50 v/v% 3-aminopropyltrimethoxysilane (Aldrich, Steinheim, Germany) in analytical grade methanol for 10-60 min. Slides were rinsed with excess methanol (>50 ml/slide) followed by ultrapure water (> 50ml/slide). After this step, amine groups have been attached to the glass or mica.

EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl) and Sulfo-NHS (N-Hydroxysulfosuccinimide) (Pierce, Rockford, IL, USA) were stored at -20°C in an airtight container to prevent contact with moisture. EDC and Sulfo-NHS were dissolved in 0.1 M sodium phosphate (pH=7.1) to form stock solutions with final concentrations at 0.5 M and 0.1 M, respectively, and stored at 4°C for short-term use (< 1 week).

EDC and Sulfo-NHS stock solutions were simultaneously added to bacterial solution (2 × 10^7 cells/ml), to reach final concentrations of 50 mM and 20 mM, respectively. The resulting solution was mixed on a rotator at ~70-125 rpm for 10-30 min. The solution was added to the prepared glass or mica substrates, followed by gentle shaking at ~125 rpm for 6-8 hours (21). During the entire reaction process, the system pH was around 7, since EDC, Sulfo-NHS and bacteria were all dissolved in 0.1 M sodium phosphate buffered solution. In order to bond bacteria covalently through a reaction that used the amine groups on their surfaces, the EDC/NHS procedure was again applied, but
with using modified glass slides whose surfaces expressed carboxyl group functionalities (Figures 1B and 1C). Glass slides with gold coating were then prepared with an isophthalic acid compound, in order to aid in attachment of bacteria to these surfaces. The preparation and characterization of IPA slides was described previously (22). Briefly, commercially available gold-coated slides (Evaporated Metal Films; Ithaca, NY) were acid cleaned in piranha solution (70% sulfuric acid and 30% hydrogen peroxide) for 10 min, followed by ultrapure water rinse, ethanol rinse, and drying under nitrogen. Slides were immersed in 1 mM IPA ethanolic solution for 24 hours to form self-assembled monolayers (SAMs) on the substrates. A second ultrapure water/ethanol rinse and drying procedure was applied.

EDC and Sulfo-NHS solutions were added to the IPA-slides to form final concentrations of 100 mM and 40 mM, respectively, and agitated for 30 min at 125 rpm. Next, bacterial solution was added, followed by gentle shaking for >2 hours at 125 rpm, to allow the zero-length cross-link reactions to complete. Incubation times of 4-6 hours yielded the best immobilizations.

After applying either of these covalent immobilization procedures, slides were removed from solution and rinsed with ultrapure water to remove any loosely attached cells or other chemicals.

(2) Bacterial Immobilization through Electrostatic Forces

Poly-L-Lysine (PLL) (MW: 150,000-300,000) solution (0.1% w/v, in water) (Sigma, St. Louis, MO, USA) was used to create a positively charged surface on glass and mica. Glass slides and mica sheets were cleaned or cleaved, as described above. Then the slides were coated with PLL solution in a covered petri dish. When the slides were
dry, a thin film of bacteria solution was deposited on top of the PLL-treated glass or mica. After 30-40 min, the slides were gently rinsed with ultrapure water.

During each of these immobilization processes, bacterial cells remained hydrated, even while briefly exposed to air. Bacteria can retain their residual moisture for several hours, as has been demonstrated through contact angle experiments (20).

(3) Mechanical Immobilization

Based on the size of *E. coli* HB101 and *P. aeruginosa* PAO1 cells, 0.22 and 0.6-μm isopore polycarbonate filter membranes were used for the mechanical trapping experiments (Millipore Corp., Billerica, MA, USA). In order to form a bacterial monolayer on the filter membrane, 100 μl bacteria solution (2 × 10^7 cells/ml) was diluted into 1-3 ml solution and was captured onto the filter at times ranging from 10 sec to 1 min, using a vacuum filtration pump. The membranes were attached to glass slides using double-sided tape.

AFM Imaging

All AFM images were acquired in ultrapure water at room temperature. AFM (Digital Instruments Dimension 3100 with Nanoscope IIIa controller) was operated in tapping mode unless specified. Rectangular silicon cantilevers (NSC36/AIBS, cantilever type C) (MikroMasch, Wilsonville, OR, USA) with a nominal tip curvature <10 nm and a nominal spring constant of 0.6 N/m were employed. The optical microscope was used to roughly select an area to engage the AFM tip. Images were acquired at a scan rate of 1.0 Hz, with 512 samples/line.

Atomic Force Microscopy Force Profiles
As a further way to characterize how the immobilization technique affected the bacterial surface and interfacial properties, AFM force profiles were obtained for some samples. After immobilization via covalent bonding or the electrostatic forces method, individual *E. coli* HB101 bacterial cells were probed by triangular AFM Si$_3$N$_4$ tip (DNPS, Digital Instruments). These tips have an average spring constant of 0.06 ± 0.03 N/m, calibrated according to the method of Cleveland et al (23, 24). Data from the approach and retraction portions of the AFM profiles was analyzed as described (6). Briefly, a steric model was employed to analyze approach profiles and characterize the surface polymers on the bacterial cell (25, 26). Two parameters can be generated from application of the steric model, the equilibrium length which corresponds to the outer membrane polymer and protein extension distance from the cell surface, and the density of outer membrane polymers and proteins. The adhesion forces were collected from the retraction data and statistical analyses and histograms were used to compile and compare the data. At least 5 bacterial cells were probed for force measurements under each condition and at least 8 force curves per cell were recorded.

**Results and Discussion**

For the chemical bonding and electrostatic immobilization of bacteria, the protocols were tested on all three bacterial strains. Mechanical trapping was not performed for *S. epidermidis*, since that technique is already well established for round-shaped Gram-positive bacteria and yeasts, but our aim was to extend its application to Gram-negative bacteria.

**Covalent Bonding EDC/NHS Protocol**
When the bacterial cells have enough carboxylic terminal groups, the EDC/NHS reaction can be used to attach bacteria, forming semi-stable amine reactive NHS-esters, followed by conjugation with the amine terminals on the substrate via firm amide bonds. This strategy worked well for *E. coli* HB101 (Figure 2A), *S. epidermidis* (Figure 2B), and has been successful in the past with other bacterial strains (5, 6, 21). However, if the carboxylic terminals are inadequate, this reaction does not work well, as we observed for *P. aeruginosa* PAO1 (no images could be obtained since the cells were not attached to the slides). Since PAO1 has many proteins present on its surface (27, 28), we instead chose to target the bacterial amine terminal groups, bonding them with carboxyl groups formed on IPA-coated slides. Thus, the reverse form of the EDC/NHS immobilization method (Figure 1C) was successfully applied for *P. aeruginosa* (Figure 2C). The advantage of using the IPA SAM is that a high density of carboxyl groups can be created, with all of the terminal molecules already in the correct orientation to be able to react with the bacterial amine groups.

In some previous studies using the EDC/NHS reaction, there was concern that the molecules to be immobilized would be altered through the reaction process. For example, Vermette and Meagher studied the impact of concentration of EDC/NHS on the coupling of poly(acrylic acid) (PAAC) onto thin films of n-heptylamine (8). Since PAAC contains many carboxylic groups in different orientations, the thickness of the films measured differed depending on the ratio and concentration of EDC/NHS used. With higher concentrations, more PAAC could bind to the films. The results show that the EDC/NHS reaction can yield different efficiencies under varying conditions. On the other hand, there are important differences to consider between how this set of reactions was
applied and the way we apply it for bacterial bonding. The bacterial binding to glass occurs on the underside of the bacteria, while the AFM can only probe the top of the cell surfaces. The remaining functional groups on the bacterial surface that did not participate in the EDC/NHS reaction will rapidly revert to their original carboxylate form if they cannot contact the amine groups on the substrate, because the intermediate compounds are too unstable to remain in this state (both the O-acylisourea intermediate and the semi-stable amine-reactive NHS-ester). Thus, the exposed surfaces of the immobilized bacterial cells retain their intact biological activity. The same logic is true for the reverse application of the EDC/NHS reaction, because the same unstable intermediates also form.

The hydrolysis rate constant is only 2-3 sec⁻¹ for the active ester at pH 4.7 (29). The rate constant should be fairly stable since the reaction activity does not differ significantly when pH is increased to 7.5.

The application of similar bonding methods has shown that the chemicals used for the EDC/NHS reaction do not alter biological activities (30, 31). Wissink et al. compared the effects of different crosslinking agents such as formaldehyde, glutaraldehyde and EDC/NHS on endothelial cell seeding. The first two agents can cause cytotoxic reactions, while EDC/NHS does not have such side effects. Furthermore, the EDC/NHS linking method significantly increased the proliferation of seeded endothelial cells without causing morphological changes or other abnormal biological activities (30).

Our results show that the covalent bonding procedure works well for Gram-negative and Gram-positive bacteria. It is simple to construct substrates that have desired functional groups, with either carboxyl or amine-group terminations. When applying this reaction scheme to different strains of bacteria, the method can be applied in one of two
ways, depending on whether carboxyl groups or amine groups dominate the bacterial surface. The only drawback of this method is that the bacterial cells can sometimes become aggregated due to the exposure to EDC and NHS. Therefore, it may be difficult to discern individual bacterial cells if sonication is not applied (discussed below).

Immobilization on Glass and Mica via Electrostatic Interactions

The chemical PLL was used to help bacteria attach to glass and mica. This method is practically universal, and worked well for *E. coli*, *S. epidermidis*, and *P. aeruginosa* (Figures 3A-C).

Glass slides and mica are the most commonly used substrates for microbe immobilization. Mica sheets are easier to prepare than glass slides, but for whole cell imaging or force measurements, there are no great differences between the two substrates. Due to the smoothness of mica, it can provide a better background when imaging fine structures such as the EPS and LPS associated with *P. aeruginosa* PAO1 (Figure 4). Although both Figure 3C and Figure 4 show strain PAO1 immobilized using the PLL method under identical conditions, the differences in the underlying substrate’s smoothness do affect the resolution of the features on the bacteria that can be observed. Fine molecules on the bacterial surface, which may include flagella, lipopolysaccharides, and polysaccharides, can be better appreciated for the bacteria attached to mica.

The drawback of using mica is that the sheet is thin and provides poor mechanical strength, sometimes deteriorating the image quality. To avoid this, multiple layers of mica sheet are preferred.
Breakup of Bacterial Aggregates through Sonication

A potential complication of using chemicals to aid in bacterial bonding is that after treatment with PLL or EDC/NHS, the bacterial cells may become sticky and aggregate. Therefore, we have incorporated sonication into our methodology, to help break up bacterial aggregates. For example, all of the images shown in Figures 2 and 3 were of sonicated bacteria. When this step was not performed, bacteria were always in aggregates. This was true regardless of whether glass or mica was the substrate, and for either the EDC/NHS or PLL immobilization techniques. Examples are shown of cells that were not sonicated, for E. coli HB101 (Figure 5A) and P. aeruginosa PAO1 (Figure 5B). By comparison with Figure 2A and 3C for E. coli HB101 and P. aeruginosa PAO1, respectively, it is clear that sonicating the cells for 5 min improved the clarity of the images that could be obtained by decreasing aggregation.

Gram-positive bacteria behave differently and always tend to aggregate. Sonication was not able to break up the aggregates of S. epidermidis (Figures 2B and 3B), and images of cells that were not sonicated were identical to the sonicated ones (images not shown).

Sonication appears to provide a benefit for the imaging of bacterial morphology for Gram-negative bacteria. Previous studies have shown that the duration and dose of sonication used in this study disperses bacterial aggregates without affecting bacterial viability or morphology (20).

Effect of Bacterial Cell Washing on Immobilization

In previous work, we showed that proper washing steps are necessary to reveal the bacterial surface’s true morphology (20). This is especially important for
physicochemical characterizations of bacterial surfaces, such as measuring the contact angles to determine the surface energies, or probing with AFM. Crystallization of materials from the culture medium and residue of molecules secreted during bacterial growth can easily mask the true cell surface. Washing cells has not been considered necessary for investigations of bacteria in liquids since it was assumed that the soluble molecules from the culture medium would be dissolved in liquid and hence unable to cover the bacterial surface. However, the materials produced by the bacteria, such as extracellular polymeric substances (EPS) or ornithine-derived lipids produced by *P. aeruginosa* PAO1 under growth in rich medium (32), remain on the bacterial surface, especially adsorbing to surface appendages such as LPS, flagella, fimbriae, and polysaccharides. After introducing the immobilization agents such as EDC/NHS or PPL, these materials can cover the bacterial surfaces (Figures 6A and 6B). Excess EPS can interact with the AFM probe, which was especially problematic using PLL immobilization method. Figure 6B represents a poor image with many scan lines due to EPS remaining on the *P. aeruginosa* PAO1 cell surfaces. These types of artifacts were not observed when the cells were washed (i.e. Figures 2C and 3C), following a protocol discussed previously (20).

**Im mobilization Strength and Duration**

Generally, a researcher must spend several hours or longer to image bacteria or obtain force measurements with AFM. Therefore, the duration that the immobilization reaction endures must be investigated. Either form of the EDC/NHS reaction provides stable bacterial immobilization for at least five hours, without notable bacteria dissociation from the slides. However, immobilization via PLL started to break down after two hours, as
shown for *E. coli* HB101, although similar results were obtained with all bacteria investigated (Figure 7). Due to the weak nature of non-specific interactions (electrostatic forces) compared to covalent bonds, and the water solubility of PLL, this chemical was not able to immobilize bacteria for as long as the covalent bonding methods.

**Optimal Immobilization Conditions**

In some studies, a greater density of bacteria must be immobilized onto the substrates. For example, bonding of bacteria to a specific receptor can be investigated if the receptor is coated to an AFM tip. Modified tips may not provide very sharp images or may become altered by repeated scanning, so for certain types of studies, it is preferable to have a complete lawn of bacteria, ensuring that the tip will probe bacteria regardless of the location the tip makes contact with the surface. We discuss a few potential approaches to help increase the immobilization.

1. **Increase the Dose of EDC/NHS and of the Aminosilane Compound**

   Excess EDC or NHS does not boost the immobilization efficiency, since the amounts typically used are ten-fold molar excess over the amount of the target molecules such as proteins present (29). This amount should already be sufficient to react with the carboxylic functional groups on the bacterial surface.

   Therefore, the controlling step is the limitation in the number of equivalent amine groups on the substrate. We found that both using a higher silane concentration and leaving the aminosilane in contact with the slide for longer can help, but using a higher concentration had a more substantial effect. Sometimes, leaving the aminosilane solution on the glass slide for too long led to drying and crystallization of the molecules, which obscured the substrate in later images. After several optimization experiments, we found
that concentrations ≤ 50 v/v % aminosilane solution for one hour contact time was optimal.

(2) Lowering EDC/NHS Reaction System pH

The optimal reaction pH range for the EDC/NHS reaction is between 4.7 and 6, but research has suggested that the carbodiimide reaction remains effective up to pH 7.5 without significant loss of yield (29). Lowering the pH may not be possible when taking into account the integrity of the bacteria. Also, care must be taken to ensure that components in the buffer system do not interfere with the reaction. Previous research determined that 0.1 M MES [2-(N-morpholino) ethane sulfonic acid] can be used at low pH (pH 4.7-6.0) and 0.1 M phosphate buffer is suitable for neutral pH reactions (pH 7.3) (29). Therefore, no pH adjustments are needed during the course of reactions.

(3) Combination of EDC/NHS and PLL Methods

The immobilization methods of covalent bonding combined with electrostatic forces were applied simultaneously, to create a surface completely covered with bacteria, as demonstrated for *E. coli* HB101 on mica (Figure 8; note that similar results can be obtained on glass). The procedures used were the same as described above when the reactions were treated individually, with 50 v/v % aminosilane solution and a 1 hour incubation time. Although the mica surface was fully covered by *E. coli* HB101, we expect this surface to remain stable for only 2-3 hours due to the solubility of PLL and its relatively weaker ability to adhere bacteria to the substrate.

Another possibility we tested was to increase the bacterial concentration. This did not improve the number of cells attaching to any appreciable degree, because the limiting step in this reaction is the relatively weaker attractive forces between the bacterial cells
and the substrate. Simply increasing the bacterial concentration did not attract more cells to the substrate because the bacterial concentration was already high enough that availability of attachment sites was not the concern.

Mechanical Immobilization

Bacteria immobilized by mechanical trapping in a filter were the final group investigated. To mechanically trap bacterial cells into a pore, the pore size should be slightly smaller than the bacterial dimension. This method is usually limited to round-shaped bacteria (15-18). The pore size selection for rod-shaped bacteria should be based on the bacterial width. Figure 9 represents an attempt to trap *E. coli* HB101 with a 0.22-μm filter membrane, but no cells could be found even after examining multiple areas of the filter surface. While the average pore size should be 0.22 μm, in some areas, several pores merged together to form openings much larger than the average size, even reaching a size of 1.2 μm (near center of Figure 9). The width of this bacterium was previously shown to be 0.5 ± 0.2 μm (20). Filters were examined in both right side up and upside down orientations, and failed to display any bacterial cells.

The 0.6-μm filter membrane was found to be capable of trapping *E. coli* (Figure 10), but this was possible only through several trials. We could not consistently and easily trap rod-shaped Gram-negative bacteria in any of the filter membranes tested. For spherical Gram-positive bacterial cells, filter trapping may be a suitable method of immobilization. However, besides the experimental difficulties, there may be other reasons why mechanical trapping is not well-suited for rod shaped bacteria. Trapping of rod-shaped cells exposes only the ends for AFM investigation, while the middle part of the cells can have different physical and mechanical properties. It is generally the
“center” of rod-shaped bacteria that are probed with AFM, since previous research has shown that artifacts could be caused by measuring force profiles of *E. coli* K12 strains at the edges of the cells (33). Further, the charge properties of *E. coli* K12 strains were found to be different at the ends compared to the center (34). Using differential electrophoresis, Jones et al. showed that polystyrene preferentially adhered to the tips of *E. coli* compared to the middle portion, with the authors proposing the existence of nanodomains on the *E. coli* tips that could cause different charge properties.

**Role of Immobilization Method on AFM Force Profiles**

As a more quantitative method of comparing how the different immobilization methods affected bacterial properties, we captured force profiles on the bacteria and analyzed the approach and retraction portions of these cycles. Since only a few *E. coli* HB101 cells could be mechanically trapped with our protocol, we could not make force measurements for this condition.

The analysis of force profiles from the AFM data demonstrated that quite different force profiles were obtained depending on whether *E. coli* were immobilized by covalent bonding or through electrostatic forces (Figure 11). Figures 11A and B show the distributions of pull-off distances and adhesion forces acquired from the summation of all retraction curves. The PLL-immobilized cells had longer pull-off distances and greater adhesion forces with the bare Si$_3$N$_4$ tip compared to the EDC/NHS immobilized cells.

The application of the steric model to the approach curve data also demonstrated differences for the two methods. With the EDC/NHS method, the average equilibrium polymer length was $32 \pm 10$ nm and the average polymer density was $(5.27 \pm 1.62) \times 10^{16}$
m². With the PLL method of immobilization, the equilibrium length extended to 169 ± 47 nm and the polymer density decreased to (1.32 ± 0.26) × 10¹⁶ m⁻².

We believe the changes are caused by PLL adsorbing also to the top of the bacterial cells and altering some of their chemical structures. So when we probed the PLL-immobilized bacteria, we may have also collected small amounts of PLL on the AFM tip, leading to higher adhesion forces and longer pull-off distances. The presence of PLL could also have accounted for the longer equilibrium lengths we predicted based on the steric model. These results show that even for washed bacteria, some PLL can remain on top of the bacterial cells and can produce artifacts in the observed profiles. Although the PLL is water soluble, it appears that some residual PLL may remain on the bacterial surface when the force measurements are obtained. The force profiles for either condition are equally reproducible, so that may indicate that the small amount of PLL that does remain on the bacterial cells is difficult to remove and will always be present when prepared in the same way.

Vadillo-Rodriguez et al. were the first to note that bacteria immobilized by physical adsorption (using poly(ethylene)imide) produced different AFM force profiles than bacteria mechanically trapped in a filter, using Klebsiella terrigena as the test organism (9). The authors did not compare these force profiles with that of bacteria covalently bound to a substrate.

Conclusions

Three commonly used microbial immobilization methods were introduced and compared. The zero-length cross-link technique was broadened to utilize the amine groups present on bacterial surfaces instead of carboxylic groups, extending the number
of bacterial strains that can be immobilized. Due to the instability of the intermediates during the carbodiimide reaction, the intermediates on the surface of the cells which do not contact with the substrate will revert back to the original carboxylic groups or amine groups after undergoing a swift hydrolysis reaction.

Overall, the covalent bonding method can provide highly efficient immobilization with minimal alteration to the bacterial cell surfaces. This method appears to be well-suited to immobilizing a range of types of bacterial cells for AFM imaging and force measurements in liquid.

References

Figure Captions

Figure 1A. Schematic of reaction used to immobilize bacterial cells via EDC/NHS covalent bonding, shown for –COOH rich E. coli HB101 and S. epidermidis immobilized on glass slides treated with aminosilane.

Figure 1B. Isophthalic acid attached to gold-coated glass slide to impart carboxyl functionality to surface

Figure 1C. Schematic of reaction to immobilize –NH2 rich Pseudomonas aeruginosa PAO1 on EDC/NHS-treated IPA slide.

Figure 2. Examples of bacterial immobilization using the covalent bonding (EDC/NHS) reaction pathway.
A) E. coli HB101 immobilized on glass slide using method shown in Figure 1A, where bacterial carboxyl groups are coupled with amine groups from an aminosilane compound;
B) S. epidermidis immobilized on glass slide using same method as in 2A;
C) P. aeruginosa PAO1 immobilized on glass slide that was treated with IPA to impart carboxyl functionality, for coupling with bacterial amine groups using method described in Figure 1C. All images in ultrapure water.

Figure 3. Bacteria immobilized on PLL-coated glass slides.
A) E. coli HB101;
B) S. epidermidis;
C) P. aeruginosa PAO1
All images acquired in ultrapure water.
Figure 4. *P. aeruginosa* PAO1 immobilized on mica slide via PLL method, imaged in ultrapure water. (In comparison with Figure 3C, fine LPS structures can be appreciated on mica slide.)

Figure 5. Representative examples of bacterial cells that were not sonicated in solution to break up aggregates.

A) *E. coli* HB101 immobilized on glass slide via EDC/NHS;

B) *P. aeruginosa* PAO1 immobilized on glass via electrostatic forces (PLL). All images obtained in ultrapure water.

Figure 6. Necessity of washing bacterial cells, even for AFM investigations in liquids. Some representative examples are shown of bacteria that were not washed.

A) *P. aeruginosa* PAO1 immobilized on IPA-coated glass slide via EDC/NHS, without washing;

B) *P. aeruginosa* PAO1 immobilized on PLL-coated glass slide, without washing. All images obtained in ultrapure water.

Figure 7. *E. coli* HB101 immobilized on glass slide via PLL method, imaged for two hours. After the two hour period, weakly immobilized bacterial cells could be displaced by the friction forces of the AFM cantilever. All images obtained in ultrapure water.

Figure 8. *E. coli* HB101 immobilized on mica via both EDC/NHS reactions and PLL method applied simultaneously, imaged under ultrapure water.

Figure 9. Isopore polycarbonate membrane filter (pore size of 0.22 μm), where we had attempted to immobilize *E. coli* HB101, imaged in ultrapure water.

Figure 10. Isopore polycarbonate membrane filter (pore size of 0.60 μm), a few *E. coli* HB101 cells have been immobilized. Image obtained in ultrapure water.
Figure 11. Force measurements data analysis and comparison between covalent bonding method and electrostatic forces method based on *E. coli* HB101

A) Histogram of pull-off distances distribution;

B) Histogram of adhesion forces distribution.
Figure 1A. Schematic of reaction used to immobilize bacterial cells via EDC/NHS covalent bonding, shown for –COOH rich *E. coli* HB101 and *S. epidermidis* immobilized on glass slides treated with aminosilane.
Figure 1B. Isophthalic acid attached to gold-coated glass slide to impart carboxyl functionality to surface
Figure 1C. Schematic of reaction to immobilize $\text{–NH}_2$ rich *Pseudomonas aeruginosa* PAO1 on EDC/NHS-treated IPA slide.
Figure 2. Examples of bacterial immobilization using the covalent bonding (EDC/NHS) reaction pathway. A) *E. coli* HB101 immobilized on glass slide using method shown in Figure 1A, where bacterial carboxyl groups are coupled with amine groups from an aminosilane compound; B) *S. epidermidis* immobilized on glass slide using same method as in 2A; C) *P. aeruginosa* PAO1 immobilized on glass slide that was treated with IPA to impart carboxyl functionality, for coupling with bacterial amine groups using method described in Figure 1C. All images in ultrapure water.
Figure 3. Bacteria immobilized on PLL-coated glass slides.

A) *E. coli HB101*; B) *S. epidermidis*; C) *P. aeruginosa PAO1*

All images acquired in ultrapure water.
Figure 4. *P. aeruginosa PAO1* immobilized on mica slide via PLL method, imaged in ultrapure water. (In comparison with Figure 3C, fine LPS structures can be appreciated on mica slide.)
A) *E. coli* HB101 immobilized on glass slide via EDC/NHS;

B) *P. aeruginosa* PAO1 immobilized on glass via electrostatic forces (PLL). All images obtained in ultrapure water.

Figure 5. Representative examples of bacterial cells that were not sonicated in solution to break up aggregates.
A) *P. aeruginosa* PAO1 immobilized on IPA-coated glass slide via EDC/NHS, without washing:

B) *P. aeruginosa* PAO1 immobilized on PLL-coated glass slide, without washing. All images obtained in ultrapure water.

Figure 6. Necessity of washing bacterial cells, even for AFM investigations in liquids. Some representative examples are shown of bacteria that were not washed.
Figure 7. *E. coli HB101* immobilized on glass slide via PLL method, imaged for two hours. After the two hour period, weakly immobilized bacterial cells could be displaced by the friction forces of the AFM cantilever. All images obtained in ultrapure water.
Figure 8. *E. coli* HB101 immobilized on mica via both EDC/NHS reactions and PLL method applied simultaneously, imaged under ultrapure water.
Figure 9. Isopore polycarbonate membrane filter (pore size of 0.22 μm), where we had attempted to immobilize *E. coli* HB101, imaged in ultrapure water.
Figure 10. Isopore polycarbonate membrane filter (pore size of 0.60 μm), a few *E. coli* HB101 cells have been immobilized. Image obtained in ultrapure water.
A) Histogram of pull-off distances distribution;

B) Histogram of adhesion forces distribution.

Figure 11. Force measurements data analysis and comparison between covalent bonding method and electrostatic forces method based on *E. coli* HB101
Chapter 12: Influence of Common AFM Settings on the Roughness Measurements

Introduction

Surface roughness is an important value to characterize the substrate including all kinds of materials and biological samples such as cell surface and protein absorption. As the most commonly used parameter of surface characterization, roughness especially $R_{\text{rms}}$ (root-mean-square roughness) can be easily measured with AFM compared to other optical or mechanical methods. $R_{\text{rms}}$ has been used in thin film industry such as SAMs, LCD and semi-conduct fields, health care industry such as hair, and biological samples such as bacteria and proteins. Besides purely used to characterize the surfaces, $R_{\text{rms}}$ is also used to correlate and predict roughness and surface energy or protein adsorption \(^1\), roughness and wetting properties of thin films \(^2\), roughness and adhesion forces between a particle and surface with nanoscale roughness \(^3\). Light scattering technique such as light profilers can be used to measure the surface roughness. However lots of disadvantages associate with that method limit its accuracy and application. TEM and SEM are also used to measure surface roughness by generating a topographic map of the surface. However sample preparation and surface conductivity requirements hamper their wide application. As a probe-microscope, atomic force microscope (AFM) can be used to obtain accurate surface topography easily based on relatively vast sample points without damaging the surface. This promotes an efficient and accurate technique to quantify the surface roughness in terms of Rms or power spectrum. Researches on roughness measured via AFM have increased with the prevalence of AFM. However, before we enjoy the convenience on roughness measurements brought by AFM, some experimental
issues need to be answered. For example, Morrow, J. B. et. al. found that AFM probe hydrophobicity/geometry and imaging fluid can affect the substratum RMS roughness and feature heights\(^4\). Increased interaction of the hydrophilic Si\(_3\)N\(_4\) AFM probe with hydrophobic OTS (octadecyltrichlorosilane)-coated glass substratum in organic imaging fluids resulted in increased RMS roughness measurements, which was attributed to polymer conformation\(^4\). With various AFM tip and cantilever selections, combined with different scan settings such as contact/tapping mode, scan size and scan rate, these issues should be given enough attention towards their influence on the nano scale \(R_{\text{rms}}\) measurements.

According to the literature search, the scan parameters were not given or only partially given in most studies. Considering the various system and different preference of researchers, those scan parameters such as the scan rate can be assured quite different with one another. Should those scan parameter settings affect the roughness measurement? There are couples of important scan parameters (Table 1), which deserve some quantitative descriptions.

Scan time is how long it takes to complete one image scan, which ranges from around 2 seconds up to approximately one and half hours depending on the AFM models. It is a function of scan rate, samples/line and aspect ratio, but not affected by scan size. Scan rate (in Hz) is defined based on how many fast scan lines (x direction, or from left to right on the monitor) are completed per second.

Tip velocity (in \(\mu m/s\)) is defined based on how fast the AFM tip moves in the slow scan direction (y direction, or from top to bottom on the monitor, vice versa). This value is determined by scan rate and scan size.
Aspect ratio is calculated as width scan size (x direction) divided by length scan size (y direction). The square scan is most widely used, where the aspect ratio equals one. We only discuss this scenario in this chapter.

Samples/line parameter determines the number of sample data points per scan line. There are usually three options: 128, 256 and 512. Bigger samples/line value produces higher resolution at the expense of longer scan time. Generally, 512 samples/line is adopted in most researches. To summarize the above scan parameters, they are listed in Table 1.

There are interesting and useful quantitative equations governing the relationship among the aforementioned scan parameters. The symbols used in the equations are also listed in Table 1.

\[ M = \frac{SL}{AR} = SL \]  
\[ t = \frac{M}{R} \]  
\[ t = \frac{S}{v} \]  
\[ d = \frac{S}{M} = \frac{S}{SL} \]  

Combine (2) and (3), we have:

\[ \frac{M}{R} = \frac{S}{v} \Rightarrow v = \frac{1}{M} \cdot S \cdot R \]  

In most control panels, aseptic ratio (constantly equals one in square scan), scan size and scan rate are independent variables, while scan time and tip velocity are dependent variables. A well calibrated AFM can reproduce the quantitative relations experimentally as shown in Figure 1.
Our present study is aimed to evaluate the impact of different scan parameter settings on roughness measurements. Common scenarios were considered and several factors were selected including: scan rate, tip radius, samples/line and scan mode (contact mode vs. tapping mode).

**Materials and Methods**

Atomic Force Microscope Imaging

Atomic force microscope (Digital Instruments Dimension 3100 with Nanoscope IIIa controller, Veeco Instruments Inc. USA) was used to acquire all AFM images. Four commercially available and most commonly used AFM tips, Silicon nitride triangle AFM tip (DNPS, Digital Instruments), silicon rectangular one (NSC 36/AIBS, type C, MikroMasch, Wilsonville, OR), two silicon rectangular cantilevers (FORT, NanoScience Instruments, phoenix, AZ) were used. The nominal tip radii and spring constant values are list in Table 2.

Samples

There are four samples were selected as the substrata. *E. coli* HB101 bacteria were prepared for AFM after three times wash according to the procedure described previously. Fifty percent fetal bovine serum solution was deposited on clean gold surface. Petri dish was chosen due to its macro-scale reproducibility. Clean ion stage was also chosen to broaden the sample differentiations.

Reproducibility

When studying the effects of scan rate on roughness measurements, a same area was used from around 15 repetitive scans with different scan rates. At the end of the experiments, several images were acquired with the same scan parameters used at the
beginning. Then roughness measurements were performed to compare the difference caused by the repetitive scans.

Roughness analysis

The AFM software, Nanoscope version 5.12r5, was used to calculate the root-mean-square ($R_{rms}$) roughness values at different scan scales specified individually.

Results and discussion

Influence of scan rate on surface roughness

Twelve different scan rates were set to obtain corresponding AFM images on a clean Petri Dish surface in contact mode using Si$_3$N$_4$. Two roughness measurements were applied based on the whole image (100 μm scan size) and the same selected area (25μm × 25μm) on all the AFM images as shown in Figure 1.

With the increase of scan rate, roughness values decrease. Lower scan rate enables lower tip velocity, thus fine structures can well mapped by AFM probe. As a result, roughness increases. But when the scan rate is lower than certain value (depending on different system), contribution of scan rate to roughness becomes subtle. In some cases, low tip velocity caused by low scan rate may alter some soft surface and yield fake roughness values. Nevertheless, it is hard to determine which scan rate should be chosen if only based on the resolution of the images. In this case, all the images present very similar appearance when the scan rate is lower than 2 Hz. To eliminate the roughness difference caused by scan rate when comparing the roughness results or modeling roughness with other parameters, consistency and caution should be taken in choosing an appropriate scan rate. For hard surface, usually high scan rate (greater than 3) can be applied. For most biological system, in order to reveal and to avoid to damage the fine
structures, low scan rate (around 0.5~2) is preferred. As a trade-off, low scan rate requires long scan time to complete the image. However, scan rate is not the lower the better especially in air. Low scan rate is more vulnerable to capillary force effects due to the moisture on the surface, which hence deteriorates the resolution.

Comparison of tapping mode and contact mode on surface roughness analysis

Both tapping mode and contact mode were applied to image the same area of Petri dish surface. Figure 2 shows the roughness measurements under both tapping mode and contact mode. Tapping mode always gives higher roughness values than contact mode. As aforementioned, contact mode can produce the same topography given appropriate AFM tips and scan settings. The resolution wise, it is hard to determine the disparity. In contact mode, AFM tip interacts with the substrate in repulsive force region. When the image is carried out in air, the ambient humidity forms a thin hydrated film on the substrate which causes attractive capillary forces. In tapping mode, AFM cantilever is tuned to oscillate at its resonant frequency. At comparable setpoint and well adjusted feedback control, AFM tip can better track the topography change on the substrate.

Tapping mode eliminates the lateral forces and frictions existing in contact mode and hence avoids damaging samples by vibrating the AFM tip above the sample instead of maintaining contact interaction forces. As a trade-off, AFM tip can not map the surface as close as that in contact mode. Technically, tapping mode gives a contour right above the surface. The roughness values in tapping mode are always slightly higher than contact mode.
Influence of tip radius on surface roughness measurement

Table 2 and Figure 3 show the surface roughness measurements on the clean Petri dish at 100 μm scan size. The roughness values given by varying AFM tips with different tip radius do not differ significantly, though the image sharpness improves with the decrease of the tip radius.

AFM rebuilds the topography through an interaction force map between the AFM tip and the substrate. Small tip radius reduces the interaction area, enabling smaller interactions being sensed. As a result, the image resolution and sharpness can be boosted. Thanks to the development of current AFM tip manufacture technique, the commonly used AFM tip radii are around 10 nm. Compared to most surface structures, the impact of tip radius on roughness measurements will not introduce errors. The other important parameter is the spring constant of the cantilever. Soft and low-resonance frequency cantilevers are more suitable for imaging in contact mode in liquid, while stiff and high-resonance frequency cantilevers are more appropriate for tapping mode in air.

Soft cantilevers are more suitable for biology samples, including imaging and force measurements. Stiff cantilevers also can be used to image biology samples such as bacterial cells or proteins provided that scan parameters are set appropriately in tapping mode. For FORT and ACT AFM tips, they have same nominal tip radius with entirely different spring constants. However, they gave similar roughness measurements. With suitable scan parameter settings in AFM imaging, both tip radius and spring constant have broad selection range without causing errors in surface roughness measurements.

Influence of samples/line and scan size on surface roughness analysis.
Figure 4 shows the effects on roughness measurements when different samples/line parameters were chosen. Usually 512 sample points are taken per scan line since it gives significantly higher image resolution. There are also two other options: 128 and 256 samples/line. Experimentally the three settings did not make significant difference in roughness measurements as shown on Figure 4.

Samples/line determines how many sample points will be counted. When the aspect ratio equals one, (512 × 512), (256 × 256) and (128 × 128) data points will be collected under 512, 256 and 128 samples/line settings respectively. The distance between two adjacent sample points is proportional to the scan size and inversely proportional to the samples/line as shown in equation (4). In the case of 512 samples/line, the distance changes from 1.95 nm to 195.31 nm when the scan size changes from 1 μm to 100 μm as shown in Figure 5 and Table 3. Similarly, the distance changes from 3.91 nm to 390.63 nm and from 7.81 nm to 781.25 nm when the samples/line is 256 and 128 respectively. Different samples/line setting will not affect roughness measurements if the surface is roughly homogenous at the scale smaller or similar to the distance between two adjacent sample points. If it is not the case, the roughness measurements under different samples/line settings will give out distinct values simply due to different sample points are collected and calculated. Intuitively, more sample points will give more accurate values. However, the increase of scan size will counteract the increase of samples/line. At the highest samples/line setting 512, when the scan size is 100 μm, the distance between two adjacent sample points is around 200 nm. If the fine surface structures are less than that value, error in roughness measurements will be introduced. Under this circumstance, small scan size should be chosen instead of analyzing a small area from a large scan size.
image. Different scan size can yield varying roughness values. It is not only due to the heterogeneity of the surface structures, but also due to the sample points counted differently when scan size varies.
References


Figure Caption

Figure 1. Influence of scan rate on surface roughness measurement

Figure 2. Comparison between tapping mode and contact mode on surface roughness measurements

Figure 3. Influence of tip radius on surface roughness measurement

Figure 4. Influence of samples/line on surface roughness analysis

Figure 5. Influence of scan size on surface roughness measurement

Table 1. Important Scan Parameters

Table 2. Influence of Tip Radius on Surface Roughness

Table 3. Correlation between samples/line and scan size settings
Figure 1. Influence of scan rate on surface roughness measurement
Figure 2. Comparison between tapping mode and contact mode on surface roughness measurements
Figure 3. Influence of tip radius on surface roughness measurement
Figure 4. Influence of samples/line on surface roughness analysis
Figure 5. Influence of scan size on surface roughness measurement
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Scan Parameters</th>
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<tr>
<td>t</td>
<td>Scan time</td>
<td>Second</td>
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<tr>
<td>M</td>
<td>Number of scan lines per image</td>
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<tr>
<td>R</td>
<td>Scan rate</td>
<td>Hz</td>
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<tr>
<td>S</td>
<td>Scan size</td>
<td>μm</td>
</tr>
<tr>
<td>ν</td>
<td>Tip velocity</td>
<td>μm/s</td>
</tr>
<tr>
<td>SL</td>
<td>Samples/per line</td>
<td>(dimensionless)</td>
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<tr>
<td>d</td>
<td>Distance b/w two adjacent sample points</td>
<td>nm</td>
</tr>
<tr>
<td>AR</td>
<td>Aspect ratio (equals one here)</td>
<td>(dimensionless)</td>
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Table 2. Influence of Tip Radius on Surface Roughness

<table>
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<tr>
<th>AFM Tips</th>
<th>Nominal Tip Radius (nm)</th>
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<td>ACT</td>
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<td>40</td>
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<td>DNPS</td>
<td>40</td>
<td>0.06</td>
<td>9.551 ± 1.193</td>
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*: Roughness values were measured in tapping mode.
Table 3. Correlation between samples/line and scan size settings

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<tr>
<th>Scan Size (µm)</th>
<th>Distance between two adjacent sample points (nm)</th>
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