Importance of Molecular Details in Predicting Bacterial Adhesion to Hydrophobic Surfaces

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Electrostatic and hydrophobic forces are generally recognized as important in bacterial adhesion. Current continuum models for these forces often wrongly predict measurements of bacterial adhesion forces. The hypothesis tested here is that even qualitative guides to bacterial adhesion often require more than continuum information about hydrophobic forces; they require knowledge about molecular details of the bacteria and substrate surface. In this study, four different strains of bacteria were adsorbed to silica surfaces hydrophobilized with alkylsilanes. The thickness of the lipopolysaccharide layers varied on the different bacteria, and the lengths of the alkylsilane molecules were varied from experiment to experiment. Bacterial adhesion was assessed using column experiments and atomic force microscopy (AFM) experiments. Results show that hydrophobilized surfaces have higher bacterial sticking coefficients and stronger adhesion forces than bare silica surfaces, as expected. However, adhesion decreased as the solution Debye length became longer than the alkylsilane, perhaps since the silane molecules could not “reach” the bacterial surface. Similarly, those bacteria with a long O-antigen layer had decreased adhesion, perhaps since the silane molecules could not reach surface-bound proteins on the bacteria. This study reveals that macroscopic measurements such as contact angle are not able to fully describe bacterial adhesion; rather, additional details such as the molecular length are required to predict adhesion.

Introduction

Bacterial adhesion impacts a wide variety of fields, including bioremediation,1,2 medical implants, and filtration efficiency.3 As a result, bacterial adhesion has been studied using many techniques, which have revealed the importance on adhesion of many factors: bacterial growth conditions,4 solution ionic strength,5,6 substrate physical properties,7 bacterial surface molecular structure,8 and local hydrodynamic shear fields.9 These factors affect one or more of several forces that are important in bacterial adhesion, including electrostatics, van der Waals, hydrophobic, solvation, depletion, and biospecific.10

Electrostatic forces11 arise due to charge groups on bacteria (e.g., phosphates or carboxyls on proteins or lipopolysaccharides) or the substrate.12 These forces can be either attractive or repulsive,13 depending on the sign of charge of the substrate and bacteria. They are usually considered in conjunction with attractive van der Waals forces by using classical Derjaguin–Landau–Verwey–Overbeek (DLVO) theory. The DLVO model assumes perfect smooth and uniform surfaces,14 and it neglects at least three considerations: (1) rough surfaces15,16 due to molecular or nanoscale bacterial or substrate surface features, (2) charge or chemical nonuniformity over the bacterial surface, which can be dominant,17,18 and (3) other forces, such as the hydrophobic force, solvation force, receptor–ligand force, or depletion force. Hydrophobic forces19,20 and the continuum models used to describe them are usually based on macroscopic measurements such as contact angles.21 DLVO-AB theory22 (also known as extended DLVO theory), which includes acid–base interactions in addition to the standard electrostatic and van der Waals forces, has been used along with other models, with slightly more success in predicting bacterial adhesion.23–25 The fact that hydrophobic forces

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are important is a bit surprising since contact angle data of bacterial lawns show that most bacteria are in fact hydrophilic.\(^{29,30}\) However, X-ray photoelectron spectroscopy (XPS) experiments show that surface proteins contribute to the hydrophobic force.\(^{31}\) The experiments concerning proteins have backing from continuum modeling.\(^{32–34}\) However, little work has been done to examine the modeling from a molecular viewpoint using techniques such as molecular dynamics or Monte Carlo.\(^{35}\) Such molecular modeling might also reveal new insights and heuristics into how hydrophobicity affects bacterial adhesion.

Studies demonstrating the importance of bacterial surface molecules have generally fallen into two types. The first is receptor—ligand (specific) interactions.\(^{36–38}\) When present, these forces often play a dominant role in adhesion to organic surfaces.\(^{39,40}\) The second type of molecular study involves examination of adhesion as a function of the characteristics of the lipopolysaccharide (LPS) layer on the outer membrane of gram negative bacteria.\(^{41,42}\) It has been debated, with data to support both sides of the issue,\(^{43–45}\) whether the LPS molecules on the surface bacteria, such as \textit{Escherichia coli} and \textit{Burkholderia cepacia}, are important in their attachment to surfaces.\(^{46}\) Since some previous research has shown that, for a given strain, the length of the LPS molecules can affect adhesion—although not in a universal way—\(^{47}\) the effect of LPS length remains an important question in understanding adhesion at the molecular scale.

The LPS is composed of three main sections: the inner core, which is composed of a lipid A moiety and a ketodeoxyoctonate (KDO) group, the core polysaccharide, and the \(\alpha\)-antigen layer.\(^{48}\) The \(\alpha\)-antigen layer is composed of sugars, with as many as 50 repeats, thus extending up to 40 nm off the surface of the bacterium.\(^{49}\) Data has shown that the length of the LPS does not correlate with the macroscopic adhesion efficiency of the bacteria.\(^{50}\) It has also been shown through the use of an atomic force microscope (AFM) that there is no correlation between LPS length and the molecular-scale interactions with the AFM tip. In addition, AFM retraction curves show that for two types of \textit{E. coli} K12—both D21\(^{41}\) and D21P\(^{42}\) (the latter is a mutant strain of D21 that lacks any LPS layer)—increased attractive forces result when contacting an 18-silane-treated surface versus a bare silica surface.

In this study, we used three different lengths of alkylsilane molecules and conducted adhesion experiments at various salt concentrations in order to vary the Debye length \((\kappa^{-1})\). The hypothesis of this paper is that molecular details, such as the length \((L)\) of the molecules on the bacterial surface or the substrate, are important in considering the balance of electrostatic and hydrophobic forces.

Materials and Methods

Bacterial Cultures and Growth Procedure. Two strains of \textit{E. coli} K12 (JM109 and D21) and two strains of \textit{B. cepacia} (G4 and Env435) were used in this study. \textit{E. coli} K12 strains have been studied extensively in many fields,\(^{46,47}\) including bacterial adhesion.\(^{48}\) JM109 possesses a full LPS layer, complete with \(\alpha\)-antigen repeating units, while strain D21 has an LPS layer truncated at the core polysaccharides and therefore lacks an \(\alpha\)-antigen layer. D21 was obtained from the Genetic Stock Center (Department of Biology, Yale University, New Haven, CT). The \textit{JM109} strain was obtained from Shahriar Mobashery (Department of Chemistry at Wayne State University, Detroit, MI).

\textit{B. cepacia} is an environmentally relevant bacterium since it is a natural degrader of chlorinated hydrocarbons such as trichloroethylene and tetrachloroethylene.\(^{51}\) Env435 is a genetically modified strain of the G4 wild-type that has been isolated because of its nonadhesive properties. It has been shown by polyacrylamide gel electrophoresis that Env435 differs from G4 in its LPS structure and lacks an \(\alpha\)-antigen layer.\(^{44,45}\) G4 and Env435 were obtained from Mary DeFlaun at Envirogen Corp.

Bacteria were stored at \(-80^\circ C\) in a Luria Broth/glycerol solution (50% v/v), revived overnight in 5 mL of Luria broth (Miller’s) at 30 °C, and then transferred to fresh media (100 mL of LB). This solution was then grown at 30 °C until the mid-exponential growth phase was reached (~3 h for \textit{E. coli}, ~4 h for \textit{B. cepacia}). Bacteria were washed three times by centrifugation (2800 \(\times\) 20 °C, 10 min) in test solution before use in experiments. For the column tests, the bacteria were then radiolabeled with tritiated leucine for 3 h. The uptake was measured using a liquid scintillation counter (Wallac LKB, Turku, Finland). Bacterial suspensions were diluted to \(10^{-6}\) mL \(^{-1}\) before experiments, and acridine orange direct cell counts were used to verify the amount of radiolabel per cell.

Test Solutions. Cells were suspended in different background solutions for different experiments. The 1, 10, and 100 mM phosphate buffer solutions (PBS) used in many of the experiments in this study were made from KH\(_2\)PO\(_4\) (Fisher Scientific), and KH\(_2\)PO\(_4\) (Fisher Scientific) in deionized (DI) water (Millipore, Marlborough, MA). The 100 mM PBS contained 1.55 g of KH\(_2\)PO\(_4\) and 5.72 g of K\(_2\)HPO\(_4\) in 1 L of DI water.

Column Adhesion Tests. Sticking efficiency was measured for each experiment using a mini-column test, designated the Microbe and Radiolabel Kinesis (MARK) test, described in more detail elsewhere.\(^{56,57}\) The bacteria are radiolabeled as described above, then injected into the columns. The top portion of the column is sliced off and analyzed for radioactivity. From this, a column efficiency \((\epsilon)\) is obtained. Each column, 3 mL of radiolabeled bacteria (50% v/v) in a 3-mL plastic column (as described below) was added to the column as a wet slurry, for a final bed volume between 1.0 and 1.5 mL. The porous medium was agitated slightly with a spatula to release any entrapped air bubbles and then washed with 10 pore volumes of DI water.
followed by 10 pore volumes of test solution. The cell suspension was pulled by vacuum through the packed bed, followed immediately by a 6-mL rinse of test solution, to remove any loosely attached bacteria. The vacuum was left on for 15–30 s after the rinse to dry the packing. The top of the column was then extruded, sliced, and placed in a liquid scintillation vial for analysis of radioactivity. The fraction of retained bacteria was calculated from the mass of radiolabel present.

The sticking coefficient, $\alpha$, was calculated from the Rajagopalan and Tien filtration model, which results in the following equation:

$$\alpha = \frac{-4a_0 \ln(1 - R)}{3(1 - \theta)}$$

where $a_0$ is the radius of the beads (20 $\mu$m for this study), $R$ is the fraction of bacteria retained in the column, $\theta$ is the porosity (0.46 for SiO$_2$ beads), $\eta$ is the collision efficiency (typically around 0.025 for this system), and $L$ is the length of the slice of glass beads extruded from the column (between 0.5 and 1.0 cm). The mathematics of the application of this model have been detailed elsewhere.

**Column Packings.** Bare glass beads (soda lime, Polysciences Inc., Warrington, PA) were used as the column packing for some of the experiments. Beads were cleaned by agitation in an acid bath (10% sulfuric acid) for 3 hours and rinsed with DI water prior to use or chemical treatment.

Some beads were used bare, while others were treated with one of three different lengths of silane molecules (all from Fisher Scientific): the single-carbon methyltrichlorosilane (1-silane), the 8-carbon octyltrichlorosilane (8-silane), or the 18-carbon octadecyltrichlorosilane (18-silane). A 10% (w/w) solution of silane was prepared, and 1-silane (100 mM) was used. The 10% solution of 8-silane (Table 1) was treated with the AFM. The negatively charged bacteria are attracted to the PEI, and thus the bacteria adhere to the surface strongly enough to be imaged and probed with the AFM.

**Results**

**Contact Angle and $\zeta$ Potential Results.** The three silane-treated surfaces were all hydrophobic, with their water contact angles ranging from 72° for 1-silane to 86° for 8-silane (Table 1). The surface treated with 18-silane had an intermediate contact angle of 76°. These values are in general agreement with reported values in the literature, although since our values were a bit lower than those reported in the literature, use of the Cassie equation suggests that our surface coverage was less than 100% (perhaps around 60%). The SiO$_2$ was, as expected, the only hydrophilic surface. The $\zeta$ potentials of the surfaces ranged from −34 mV for 8-silane treated, to −58 mV for 18-silane treated.

**Column Adhesion Tests.** Bacterial adhesion was an order of magnitude greater for the silanized surfaces than for bare SiO$_2$ (Figure 1). The adhesion of strain D21 was not a simple function of contact angle, nor even of silane length. The 8-silane coated surface had a sticking coefficient that was significantly lower than that of the 1-silane coated surface ($p = 0.032$), and it also had the largest contact angle of all our surfaces. Experiments on 8-silane and 18-silane at lower ionic strength showed lower adhesion values than at 100 mM PBS. On 8-silane, the 10 mM solution gave an $\alpha$ of 0.029, while 1 mM gave an $\alpha$ of 0.0022. On 18-silane, the bacteria showed an $\alpha$ of 0.091 in 10 mM PBS, and 0.085 in 1 mM.
In 10 mM PBS, the Debye length $\kappa^{-1} = 3$ nm, slightly longer than the 18-silane chains (2.8 nm), whereas the 1 mM solution provided a $\kappa^{-1} = 10$ nm, much longer than the 18-silane chains.

For the two bacterial strains without o-antigen layers (D21 and Env435), the adhesion for the short chain lengths (1-silane and 8-silane) was significantly less than the adhesion for the longest chain (Figure 2). The 8-silane is roughly 1.2 nm long. For both of these strains, 8-silane showed lower sticking coefficients than the 1-silane (0.054 vs 0.097 for D21, and 0.16 vs 0.24 for Env435). The difference for Env435, however, was not statistically significant.

For both bacterial strains with full LPS layers, the sticking coefficients to silanized surfaces follow the trend of 8-silane having the lowest, then 1-silane, then 18-silane. However, the difference between the three silanized surfaces is statistically insignificant. The reason for this possible dip between 1, 8, and 18 silanes is not clear to us. The magnitude of the dip is the most obvious difference between the bacteria with o-antigen layers and those without them. For G4 and JM109, it is important to note that the length of the silane chain did not impact adhesion significantly, whereas it did for D21 and ENV435. This is most likely due to the fact that the LPS layer is much longer than the silane chains, and therefore a relatively small change in silane chain length is not as important to adhesion.

**Atomic Force Microscopy Results.** To try to understand the differences in the adhesion of the bacteria to the different surfaces, we probed these surfaces using colloidal probe AFM. The AFM was used to probe bare SiO$_2$ with treated and bare colloid probes. Experiments were done in both methanol and water. The results shown in Figure 3 indicate that, in methanol, probes treated with silane had much stronger attraction forces than untreated probes. The attraction triangle in the retraction curve translates to a pull-off force for a 1-silane-treated bead of 14 nN, as opposed to only 0.3 nN for a bare silica colloid. The same force is shown to occur in water in Figure 4: The pull-off force is 5 nN for a 1-silane-treated bead, but only 2.1 nN for a bare silica colloid. Interestingly, there is significant “snap-in” when the treated bead is about 11 nm away from the silica surface. This shows a large long-range attractive force between the surfaces. The origin of this effect is most likely hydrophobicity. The silane-treated surface is so hydrophobic that it is attracted to any other surface near it in an aqueous environment.

Experiments were also run to show how a bacterium behaves in contact with colloidal probes. The data from these experiments (not shown) were not reproducible, and the shape of the force curves varied significantly from run to run. This is mostly likely due to contamination on the colloidal probes.\(^{58-60}\) This contamination is difficult to control in our system, as the area must be imaged first in order to locate a bacterium before acquiring force curve data.

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\(^{61}\) The Debye length, $\kappa^{-1}$, is defined from: $\kappa^2 = (2\pi Z e^2) / (kT)$. Details appear in Hunter, R. Zeta Potential; Academic Press: New York, 1981.
During this imaging step, the colloid probe’s large surface area (compared to a normal AFM tip) most likely picks up biomass and other contaminants, which then induce nonreproducible results for subsequent force curves.

**Discussion and Conclusions**

For both strains of bacteria lacking an o-antigen layer, the 18-silane-treated surface provided greater adhesion than the shorter chain lengths. The large values of adhesion for 18-silane are most likely a result of the hydrophobic hydrocarbon chains being able to interact significantly with the hydrophobic moieties on the bacterial surface since the 18-silane molecules are longer (2.8 nm) than a couple Debye lengths ($\kappa^{-1} = 1$ nm for 100 mM PBS). To test this further, experiments were run at lower ionic strength, such that $\kappa^{-1}$ was greater than the length ($L$) of the 18-silane chains. At both 1 and 10 mM PBS, D21 behaved almost identically on 18-silane, but both were significantly less adherent than at 100 mM on the same surface. This reveals a possible mechanism in the balancing of hydrophobic and electrostatic forces, based on the value of $\kappa L$ (chain length divided by Debye length).

A key question is why the sticking coefficient ($\alpha$) is not monotonic with silane chain length. No conclusive hypothesis has been developed. Nevertheless, the fact that the trend is not monotonic emphasizes that contact angle alone is not sufficient to characterize hydrophobicity; molecular details must be considered. Since the 8-silane surface is not only the least negative surface tested but it also has the highest contact angle—and these data remain a puzzling point to us—its lower surface tested but also has the highest contact angle—and these data remain a puzzling point to us—its lower adhesion values show that the molecular details are indeed important.

For strains with an o-antigen, the difference in silane length does not affect the adhesion as strongly due to o-antigen chains being much longer than the silane chains.

The AFM gave irreproducible results when probing bacteria with colloid probes. The increased surface area of a 4-μm colloid probe versus a typical AFM tip is beneficial in some ways, as it provides more interaction area with the surface of interest, as well as the colloid having a well-defined geometry. However, because of the added surface area, the probe also is more prone to becoming contaminated, especially in a biological system. When a colloid was used by the AFM to image an area in order to locate a bacterium suitable for experimentation, it likely picked up and accumulated biomass from the cells. Since bacteria often produce extracellular polymeric substances (EPS, which can contain polysaccharides, proteins, lipids, and nucleic acids) when stressed, this EPS is possibly contaminating the colloid probe. This would explain the long-range repulsion we saw in many such experiments.

In conclusion, we have found that a measurement of contact angle is not sufficient to characterize the hydrophobic attraction of bacteria to silanized glass surfaces. Molecular details, including the length of the hydrophobic molecule, are also important. In addition, on the basis of the fact that adhesion did not always increase monotonically with silane length, it seems that even further analysis is warranted of hydrophobicity at the molecular level.

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