Chapter 4

Poisson analysis of streptococcal bond strengthening on stainless steel with and without a salivary conditioning film

This chapter has been published in: Mei L, Van der Mei HC, Ren Y, Norde W, Busscher HJ. Langmuir 2009; 25:6227-6231. Reprinted with permission of the American Chemical Society.
Abstract

Poisson analysis of retract force-distance curves in atomic force microscopy (AFM) has yielded a new dimension to the decoupling of individual bond forces into a hydrogen bonding and nonspecific force component. Accordingly, bacterial adhesion forces have been decoupled into a hydrogen bonding and nonspecific Lifshitz-Van der Waals contribution. Due to the forced nature of AFM contact, the nonspecific force contribution has hitherto turned out to be repulsive in the analysis of bacterial adhesion forces on nonconducting surfaces. In this study, we present the results of a Poisson analysis of adhesion forces for streptococci adhering to a conducting surface. Adhesion forces measured between stainless steel, both in the absence and presence of an adsorbed salivary conditioning film, increased with increasing contact time between the streptococcal AFM probe and the surface. Concurrent with the increase in adhesion force, there was an increase in the number of minor force peaks in the retract force-distance curves. Poisson analyses of the adhesion forces indicated repulsive nonspecific Lifshitz-Van der Waals forces for streptococci adhering to saliva-coated stainless steel, but interestingly and for the first time, attractive nonspecific forces were revealed on stainless steel in the absence of a salivary conditioning film. We tentatively attribute this to attraction between the negatively charged streptococci and their positive image charges in the conducting material, which cannot develop in a nonconducting material or in the presence of a nonconductive protein layer on the stainless steel surface.
Introduction

Bacterial adhesion to surfaces is the first step in biofilm formation and is mediated by attractive Lifshitz-Van der Waals forces, hydrogen bonding forces, and attractive or repulsive electrostatic forces\(^1_2\). The forces involved in bacterial adhesion to surfaces can be measured using atomic force microscopy (AFM), either by immobilizing bacteria on a substratum surface and probing the cell surface with the AFM tip, or by attaching bacteria to a cantilever to constitute a bacterial probe to examine interaction with the substratum surface. The adhesion forces become evident from retract force-distance curves, often as a major peak, accompanied by a number of minor peaks at different interaction distances. Poisson analysis of these adhesion peaks in force-distance curves in AFM has yielded a new dimension to the decoupling of individual bond forces into a hydrogen bonding and nonspecific force components (i.e., Lifshitz-Van der Waals forces)\(^3\). Due to the forced nature of AFM contact, the nonspecific force contribution has hitherto turned out repulsive in the analysis of bacterial adhesion forces for nonconducting surfaces such as silicon nitride and glass\(^4_5\), but nothing is known about the nature of bacterial adhesion forces on conducting surfaces such as stainless steel, despite the fact that bacterial adhesion and biofilm formation form an equally big problem on conducting and nonconducting surfaces\(^6_8\).

Electron transfer has been implicated in bacterial adhesion to conducting surfaces\(^6_8\), which, consequently, affects the adhesion mechanism. Initial bacterial adhesion was found to be accompanied by a change in electric potential of the substratum surface with no measurable change in capacitance. It was calculated that, on average, a charge of about \(10^{-14}\) C per bacterium was exchanged during initial adhesion, that could either be to or from the bacterial cell surface, dependent on the bacterial strain involved and the ionic strength of the medium\(^8\).

In various applications, bacterial adhesion and biofilm formation occur on stainless steel, for instance, in orthodontics. Therefore, the aim of this study is to determine the nature of the adhesion forces responsible for streptococcal bond
strengthening to stainless steel with and without a salivary conditioning film, by Poisson analysis of adhesion force distributions measured using AFM.

Materials and Methods

Bacterial cultures

Streptococcus sanguinis ATCC 10556 and Streptococcus mutans ATCC700610 were precultured in Todd-Hewitt broth (Oxoid, Basingstoke, UK) for 24 h and inoculated into a main culture for 16 h at 37 °C in ambient air. Bacteria were harvested by centrifugation (5 min, 5000 g, 10 °C) and washed twice with demineralized water. Finally, bacteria were suspended in demineralized water and sonicated to break bacterial aggregates in an ice/water bath for 3 × 10 s at 30 W.

Stainless steel surfaces

Stainless steel 316 (Stryker Corp, Kiel, Germany), used for orthodontic brackets, was machined into 1 cm diameter discs. Subsequently, surfaces were polished with a diamond polishing paste of decreasing particle size from 14 to 0.05 μm. After polishing, stainless steel samples were cleaned by 2 min ultrasonication in a 35 kHz ultrasonic bath (Transsonic TP 690-A, Elma, Germany) and thoroughly rinsed with demineralized water.

Formation of salivary conditioning films

Human whole saliva from 20 healthy volunteers of both sexes was collected into ice-chilled erlenmeyer flasks after stimulation by chewing Parafilm®. After the saliva was pooled and centrifuged twice (10,000 g, 15 min, 4°C), phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM as a protease inhibitor. Afterward, the solution was centrifuged again, dialyzed (24 h, 4°C) against demineralized water, and freeze-dried for storage. All volunteers gave their
informed consent to saliva donation, in agreement with the rules set out by the Ethics Committee at the University Medical Center Groningen. For each experiment, the lyophilized saliva was dissolved in adhesion buffer (2 mM potassium phosphate, 50 mM potassium chloride, 1 mM calcium chloride; pH 6.8) at a concentration of 1.5 g/L. The experimental stainless steel discs were immersed into the reconstituted saliva for 16 h to create a salivary conditioning film. After 16 h, all saliva conditioning film coated discs were dipped three times in demineralized water and immediately used for AFM experiments.

**AFM measurements**

Streptococci from suspension were immobilized onto tipless cantilevers (Ultrasharp, µ-Masch, Estonia). Cantilevers were first immersed in a drop of 0.01% (w/v) poly-L-lysine (Sigma, U.K.) for 1 min, dried for 2 min in air, and then dipped into a drop of bacterial suspension for 1 min to allow bacterial attachment. Each thus prepared bacterial AFM probe was used immediately for further measurement. All AFM measurements were performed using a Nanoscope IV (Digital instruments, Woodbury, NY) in the contact mode at room temperature in adhesion buffer, using a scan rate of 0.5 Hz, ramp size of 1.5 μm, and trigger threshold of 1V. Retraction of the bacterial probe from a saliva-coated stainless steel surface was done after different surface delays, ranging from 0 to 120 s. Forty-five force curves, measured with nine streptococcal probes prepared out of three separate bacterial cultures, were collected for each surface delay on randomly selected positions on the stainless steel surface. In order to check the integrity of the bacterial probe and the streptococcal cell surface as well as the absence of cell surface contamination by salivary proteins, two control experiments were conducted: 1. Scanning electron micrographs were regularly taken to confirm the integrity of the bacterial probe after measurements. It never happened that force-distance curves had to be discarded due to visual damage to the bacterial probe. 2. Adhesion forces with 0 s surface delay were measured at the onset of each measurement series (0-120 s surface
delays) and, as a control, again after completion of a series. Whenever the maximum adhesion force in the initial and control measurement did not coincide within 1 nN, data from that measurement cycle were discarded and a new streptococcal probe prepared.

**Calculation of Adhesion Forces and Poisson Analysis**

Adhesion forces were calculated after each surface delay time from the AFM deflection data using:

\[ F = K_{sp} D \]  

(1)

in which \( K_{sp} \) is the spring constant and \( D \) is the deflection of the cantilever. The spring constant of each cantilever was for each experiment determined experimentally using the thermal method\(^9\). The maximal adhesion force in each force-distance curve \( F_{adh}(t) \), in which \( t \) represents the surface delay time, was fitted using an exponential rise to maximum function

\[ F_{adh}(t) = F_{adh,0} + (F_{adh,\infty} - F_{adh,0}) \left( 1 - \exp \left( -\frac{t}{\tau} \right) \right) \]  

(2)

Where \( F_{adh,0} \) and \( F_{adh,\infty} \) are the maximum adhesion forces after 0 s surface delay and after bond strengthening (see also Fig. 1 and Fig. 2), respectively, and \( \tau \) is the characteristic time needed for strengthening. In addition, the number of minor force peaks after each surface delay were enumerated (see Fig. 3) and analyzed analogously to an exponential rise to maximum, as described above. Since the adhesion forces measured obey a Poisson distribution, the adhesion force can be expressed as

\[ P(F) = (F_{av})^n \frac{\exp(-F_{av})}{n!} \]  

(3)

with \( P(F) \) being the probability that an adhesion event involving force \( (F) \) will occur, \( F_{av} \) being the average of all adhesion forces, and \( n \) being the number of adhesion forces included. The total adhesion force comprises a main peak due to an
invariant nonspecific contribution and a variable number of minor peaks $n$, 
constituted by hydrogen bonds, according to

$$F = n_{H\text{-bond}} F_{H\text{-bond}} + F_{\text{Nonspecific}}$$

(4)

where $F_{H\text{-bond}}$ and $F_{\text{Nonspecific}}$ represent the contributions of hydrogen bonding and nonspecific interaction forces \(i.e\.,\) Lifshitz-Van der Waals and electrostatic forces to the total adhesion force, respectively.

Based on eqs 3 and 4, the relationship between the force average ($\mu_F$) and variance ($\sigma_F^2$) of all adhesion events can be expressed as

$$\mu = \mu_{F_{H\text{-bond}}} + F_{\text{Nonspecific}}$$

(5)

$$\sigma_F^2 = \mu_{F_{H\text{-bond}}} - F_{H\text{-bond}} F_{\text{Nonspecific}}$$

(6)

According to eq 6, a plot of the variance ($\sigma_F^2$) versus the force average ($\mu_F$) yields a straight line (see Fig. 4 for an example). Linear regression of $\sigma_F^2$ versus $\mu_F$ directly decouples the adhesion force into a hydrogen bonding force $F_{H\text{-bond}}$ (from the slope) and the nonspecific adhesion force $F_{\text{Nonspecific}}$ (from the intercept).

**Results**

Fig. 1 presents an example of force-distance curve for *S. sanguinis* ATCC10556. The adhesion forces clearly increase with increasing surface delay times (see also Fig. 2) and extend over a distance of between 400 and 600 nm. In the absence of a salivary conditioning film on the stainless steel surface, forces appear to be stronger and to extend over a longer distance than in the presence of an adsorbed protein film, while in addition the number of minor peaks is larger (especially during the shorter surface delay times, as can be seen in Fig. 3). It is interesting to note that in the presence of an adsorbed salivary conditioning film the approach and retract force-distance curves at close distances (20-40 nm) overlap, but in the absence of a nonconducting adsorbed protein film the approach and retract curves deviate considerably.
Figure 1. Example of force-distance curves between S. sanguinis ATCC10556 and stainless steel surfaces in the absence (panel A, top) and presence (panel B, bottom) of a salivary conditioning film after different surface delay times.
Figure 2. The maximum adhesion forces between *S. sanguinis* ATCC10556 and stainless steel with and without a salivary conditioning film as a function of the surface delay time in a given experiment.

Figure 3. The number of minor adhesion force peaks between *S. sanguinis* ATCC10556 and stainless steel with and without a salivary conditioning film as a function of the surface delay time in a given experiment.
Table 1. The maximum adhesion forces and number of peaks for 0 s surface delay time ($F_0$ and $N_0$) and after bond-strengthening ($F_-$) and peaks increasing ($N_-$), with the characteristic time constant, $\tau$, of the strengthening/increasing process.$^a$

<table>
<thead>
<tr>
<th>Strain</th>
<th>$F_0$ (nN)</th>
<th>$F_-$ (nN)</th>
<th>$\tau$ (s)</th>
<th>$N_0$</th>
<th>$N_-$</th>
<th>$\tau$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. sanguinis ATCC10556</td>
<td>-8.6 ± 1.6</td>
<td>-31.5 ± 3.7</td>
<td>28 ± 8</td>
<td>3.0 ± 0.2</td>
<td>4.0 ± 0.3</td>
<td>20 ± 11</td>
</tr>
<tr>
<td>S. mutans ATCC700610</td>
<td>-2.5 ± 0.9</td>
<td>-9.9 ± 2.1</td>
<td>32 ± 16</td>
<td>2.6 ± 0.1</td>
<td>4.1 ± 0.1</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>S. sanguinis ATCC10556</td>
<td>-0.8 ± 0.2</td>
<td>-5.5 ± 0.8</td>
<td>59 ± 19</td>
<td>1.6 ± 0.3</td>
<td>4.1 ± 0.8</td>
<td>24 ± 13</td>
</tr>
<tr>
<td>S. mutans ATCC700610</td>
<td>-0.6 ± 0.3</td>
<td>-4.0 ± 0.6</td>
<td>16 ± 5</td>
<td>2.1 ± 0.3</td>
<td>4.4 ± 0.8</td>
<td>38 ± 23</td>
</tr>
</tbody>
</table>

$^a$ ± represents the standard deviation over 45 force distance curves from three different bacterial cultures. $^b$ negative sign denotes attractive while positive sign means repulsive.

A summary of the bond aging parameters is given in Table 1. On average, both strains show a similar behavior, and in the absence of a salivary conditioning film, the adhesion forces increase by a factor of 4 after initial contact to over 30 nN after 120 s surface delay. In the presence of a salivary conditioning film, adhesion forces are confined to less than 1 nN upon initial contact and increase to only 4-5 nN upon bond strengthening. Bond strengthening occurs in a time period of around 30 s in the absence and between 20 and 60 s in the presence of a salivary conditioning film. The number of minor peaks in the retract force-distance curves increase in a time period of between 10 and 40 s from approximately 2 to 4 peaks in one force-distance curve, regardless of the absence or presence of a salivary conditioning film.

An example of Poisson decoupling of the streptococcal adhesion forces to stainless steel in the absence and presence of a salivary conditioning film is given in Fig. 4, and the derived hydrogen bonding and nonspecific force contributions are summarized in Table 2. Hydrogen bonding forces are attractive for both strains, irrespective of the absence or presence of a salivary conditioning film, and they range between 0.5 and 0.9 nN. The nonspecific forces are repulsive (0.3-0.5 nN).
on the stainless steel surface covered with the salivary protein film, but they are relatively strongly attractive (1.2 and 8.1 nN, depending on the bacterial strain) for the bare stainless steel surface.

Figure 4. Example of Poisson analysis of the retract forces between *S. sanguinis* ATCC10556 and stainless steel at 120 s in the absence (panel A, top) and presence (panel B, bottom) of a salivary conditioning film. Linear correlation coefficients $R^2$ are 0.82 and 0.73 in the absence and presence, respectively, of a salivary conditioning film.
Table 2. Hydrogen bonding ($F_{\text{H-bond}}$) and non-specific forces ($F_{\text{Non-specific}}$) of two bacterial strains to stainless steel from Poisson analysis of retract curves at 120 s surface delay. $F_{\text{H-bond}}$ are all attractive (negative values denote attractive), while $F_{\text{Non-specific}}$ for saliva-coated stainless steel surfaces are repulsive (positive values), while for without salivary conditioning film stainless steel surfaces are attractive (negative values).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>$F_{\text{H-bond}}$ (nN)</th>
<th>$F_{\text{Non-specific}}$ (nN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without saliva</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. sanguinis ATCC10556</td>
<td>-0.6 ± 0.2</td>
<td>-8.1 ± 2.1</td>
</tr>
<tr>
<td>S. mutans ATCC700610</td>
<td>-0.5 ± 0.1</td>
<td>-1.2 ± 0.3</td>
</tr>
<tr>
<td>With saliva</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. sanguinis ATCC10556</td>
<td>-0.9 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>S. mutans ATCC700610</td>
<td>-0.7 ± 0.1</td>
<td>0.5 ± 0.2</td>
</tr>
</tbody>
</table>

* $\pm$ represents the standard deviation over nine bacteria probes from three different bacterial cultures.

Figure 5. Charge transfer and image charge formation in streptococcal adhesion to conducting stainless steel. Note that, like nearly all bacterial strains, streptococci are negatively charged like most surfaces in nature. (A) Bacterial approach to the surface. (B) At closer approach, the image charge forms at an equal distance from the surface as the approaching charged particle. (C) Upon contact, the attractive force between the negatively charged particle and its image charge is maximal, and in addition charge transfer is possible. (D) Interaction with its image charge causes an additional attraction with the negatively charged bacterium while moving away from the stainless steel surface.
Discussion

Poisson analysis of retract force-distance curves in AFM has yielded a new dimension to the decoupling of individual bond forces into a hydrogen bonding and a nonspecific force component. Hitherto, Poisson analyses of bacterial adhesion forces have only revealed repulsive nonspecific force contributions\textsuperscript{4,5}, because the forced nature of the contact in AFM pushes the contact beyond the minimum of the interaction free energy, that is, to a separation distance where the dispersion force between the bacterium and the substratum is repulsive. This study is the first to reveal attractive nonspecific bacterial adhesion forces by Poisson analysis, which we attribute to the fact that we used conducting stainless steel as a substratum. This argument is enforced by the observation that the presence of a nonconducting film of salivary proteins yields a repulsive nonspecific force, similar to what has been derived for other nonconducting materials.

The mechanism of bacterial adhesion on conducting surfaces differs from the one forwarded for nonconducting surfaces in the sense that conducting surfaces may facilitate charge transfer\textsuperscript{8}. Charge transfer between the conducting stainless steel surface and the bacterial cell surface may be responsible for the deviating retract and approach force-distance curves at short separation distance (20-40 nm) between the two surfaces (Fig. 1). Such deviations are usually not observed for nonconducting surfaces and, accordingly, are absent in our study when the stainless steel surface is coated with a salivary protein coating.

It is interesting to speculate about the reason why the nonspecific force derived from the Poisson analysis is attractive on conducting surfaces. Upon approach of a charged particle toward a charge-conducting material, a so-called image charge develops in the conducting material (Fig. 5). The image charge is of opposite sign than the charge of the approaching particle and forms or disappears by charge rearrangement in the conducting material upon approaches or retracts of the charged particle from the surface, respectively. Since the interaction between a charged particle and its image charge is not disturbed by the forced nature of the
AFM contact, we suggest that the attractive nonspecific force contribution revealed here for streptococcal adhesion to stainless steel is due to attractive electrostatic interactions between the image charge and the negatively charged streptococcal cell surfaces\textsuperscript{10}.

In summary, this is the first time that attractive nonspecific forces between negatively charged bacteria and their image charges formed in a conducting material have been directly revealed by Poisson analysis of retract force-distance curves measured using AFM. Development of these attractive forces does not occur when the stainless steel surface is covered by a layer of adsorbed salivary proteins.
References
