Bacterial interaction forces in adhesion dynamics
Boks, Niels Peter

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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CHAPTER 5

BOND-STRENGTHENING IN STAPHYLOCOCCAL ADHESION TO HYDROPHILIC AND HYDROPHOBIC SURFACES USING AFM

Abstract

Time-dependent bacterial adhesion forces of four strains of *Staphylococcus epidermidis* to hydrophobic and hydrophilic surfaces were investigated. Initial adhesion forces differed significantly between the two surfaces and hovered around -0.4 nN. No unambiguous effect of substratum surface hydrophobicity on initial adhesion forces for the four different *S. epidermidis* strains was observed. Over time, strengthening of the adhesion forces was virtually absent on hydrophobic dimethyldichlorosilane (DDS)-coated glass, although in a few cases multiple adhesion peaks developed in the retract curves. Bond-strengthening on hydrophilic glass occurred within 5 to 35 s to maximum adhesion forces of -1.9 ± 0.7 nN, and was concurrent with the development of multiple adhesion peaks upon retract. Poisson analysis of the multiple adhesion peaks allowed to separate contributions of hydrogen bonding from other non-specific interaction forces and revealed a force contribution of -0.8 nN for hydrogen bonding and +0.3 nN for other non-specific interaction forces. Time-dependent bacterial adhesion forces were comparable for all four staphylococcal strains. It is concluded that on DDS-coated glass, the hydrophobic effect causes instantaneous adhesion, while strengthening of the bonds on hydrophilic glass is dominated by non-instantaneous hydrogen bond formation.
Introduction

Bacterial adhesion to surfaces is a crucial step in biofilm formation and associated problems, such as in biomaterials implant surgery. *Staphylococcus epidermidis* is one of the most often isolated bacterial pathogens in biomaterials-implant related infections [1,2]. One of the first steps in biofilm formation is transport of the bacterial cells towards the surface, which may be governed by sedimentation, convection and diffusion [3,4]. Once brought within the range of the interaction forces, bacteria can come in close contact with a substratum surface and adhere. Initially, adhesion is reversible but over time adhesion becomes irreversible [5,6], although the exact mechanism of this transition remains poorly understood.

Flow displacements systems have been extensively used to determine the close-range affinity of bacteria for a substratum surface [7]. One of the possibilities in such systems is to determine the hydrodynamic forces to prevent adhesion or to detach adhering bacteria. Usually, the forces to prevent adhesion are smaller than the forces needed to establish detachment, suggesting that the adhesion bond strengthens over time [8-10]. As a disadvantage, however, flow displacement systems only provide an indirect measure of the actual adhesion force, without a clear view on time dependence of strengthening of the bond between individual bacteria and the substratum surface. Atomic force microscopy (AFM) is a promising technique to directly measure the interaction forces between bacteria and substratum surfaces.

For example, Cao et al. [11] found adhesion forces upon retract between 5 and 24 nN for *Escherichia coli* and various hydrophilic and hydrophobic surfaces, while Sheng et al. [12] found adhesion forces upon retract of 0.5 to 5.6 nN for *Pseudomonas aeruginosa* on various metal surfaces. Others showed that
the presence of surface structures influence bacterial adhesion properties substantially, sometimes already in the approach curve [5,6,13].

Measuring bond-strengthening with AFM requires accurate control of the z-displacement during contact of the probe with a substratum surface. Previously, AFM systems were not fully capable of achieving this. Nowadays, precise feedback in z-displacement, ensures that the probe can remain at the same distance from a surface within a force-curve bandwidth of 0.1 nm root mean square. Bond-strengthening effects have been reported for eukaryotic cells with mica and silica [14,15], or polymer coated model colloids interacting with bovine serum albumin, lysozyme and dextran [16]. Furthermore, Vadillo-Rodriguez et al. [17] probed whole bacterial cells with silicon nitride tips at two different pH values and found bond-strengthening to occur within 60 s, independent of pH.

However, no study has been undertaken hitherto to demonstrate a potential role of substratum hydrophobicity on bond-strengthening between bacteria and substratum surfaces. Therefore, the aim of this chapter is to compare bond-strengthening of four Staphylococcus epidermidis strains on a hydrophobic (dimethyldichlorosilane-coated glass) and a hydrophilic (glass) substratum.

**Materials and Methods**

**Staphylococcal strains and culture conditions.** Staphylococcus epidermidis strains ATCC 35983, 3399, HBH2 3 and HBH2 169 were cultured aerobically from blood agar plates in 10 ml Tryptone Soy Broth (OXOID, Basingstoke, England) for 24 h at 37ºC (Note that the latter three strains are clinical isolates). After 24 h, precultures were used to inoculate 200 ml main cultures, which were grown for 16 h under similar conditions as the precultures. Bacteria were harvested by centrifugation for 5 min at 5000 x g, washed twice with 10 mM potassium
phosphate buffer at pH 7 and resuspended in the same buffer. To break bacterial aggregates, 3 times 10 s sonication at 30 W (Vibra Cell model 375, Sonics and Materials Inc., Danbury, CT, USA) was carried out while cooling the suspension in a water/ice bath.

**Substratum surfaces.** Glass slides were sonicated during 3 min in 2% RBS35 (Omnilabo International BV, The Netherlands) followed by thorough rinsing with tap water, demineralised water, methanol, tap water and finally demineralized water again to obtain a hydrophilic surface (water contact angle 28 ± 8 degrees). After washing, the slides were either directly used or dried for 4 h at 80ºC prior to applying of a hydrophobic coating.

To obtain a hydrophobic surface (water contact angle 101 ± 2 degrees), the dried glass slides were submerged during 15 min in a solution of dimethyldichlorosilane (DDS, Merck, Germany) in trichloroethylene (0.05 w/v%) and washed with trichloroethylene, methanol and ultrapure water. Prepared slides were stored for no longer than 3 days at room temperature and rinsed with 10 mM potassium phosphate buffer before use.

**Bacterial probe preparation.** Staphylococci were immobilized to tipless “V”-shaped cantilevers (VEECO, DNP-0) by means of electrostatic attraction with positively charged poly-L-lysine. To this end, cantilevers were mounted in a micromanipulator under microscopic observation to allow only the tip of the cantilever to be coated. A droplet of poly-L-lysine solution was placed on a glass slide and the tip of the cantilever was dipped in the droplet for 1 min. After air drying the cantilever for 2 min, it was dipped in bacterial suspension for 1 min. Bacterial probes were freshly prepared for each experiment and checked regularly during an experiment for staphylococcal presence.
**Atomic Force Microscopy.** AFM experiments were carried out at room temperature in 10 mM potassium phosphate buffer (pH 7) using an optical lever microscope (Nanoscope IV Digital instruments). For each probe, force curves were measured for different surface delay times on the same, randomly chosen, spot on a hydrophobic or hydrophilic substratum surface. Interaction forces were measured after 0, 10, 30, 45, 60, 90 and 120 s of contact time ($\Delta t$) between the bacterial probe and the substratum surface with z-scan rates of less than 1 Hz. To ensure that no staphylococci detached from the cantilever during the experiment, 5 control force-distance curves were made with 0 s contact time after each measurement with a certain surface delay, as schematically outlined in Figure 1. Whenever the “0 s contact time” forces measured were out of range, a bacterial probe was replaced. For each combination of a bacterial strain and substratum surface, six probes were employed on average and the number of staphylococcal probes used depended on the outcome of the control measurements. Calibration of bacterial probes was done using the thermal tuning method (Nanoscope V6.13r1), yielding spring constants of $0.044 \pm 0.008 \text{ Nm}^{-1}$.

Subsequently, for each staphylococcal probe the maximum adhesion forces were plotted as a function of the surface delay time and fitted to:

$$F(\Delta t) = F_{0s} + (F_{\infty} - F_{0s}) \left(1 - \exp\left(-\frac{\Delta t}{\tau}\right)\right)$$

(1)

with $F_{0s}$ the maximum adhesion force at 0 s contact time, $F_{\infty}$ the maximum adhesion force after bond-strengthening and $\tau$ the characteristic time needed for the adhesion force to strengthen.
Figure 1. Schematics of the experimental time line, including go/no points after control measurements with a staphylococcal probe to determine the need to prepare a new bacterial probe. In the absence of the need for new probe preparation, the same probe was used for a new contact time \( i \) (30, 45, 60, 90 and 120 s).

Statistical Analysis. Data was analyzed with the Statistical Package for the Social Sciences (version 14.0, SPSS, Chicago Illinois, USA). A Wilcoxon signed rank test was used to analyze adhesion forces measured within a probe. Differences between the above bond-strengthening parameters on the two substratum surfaces were analyzed using the Mann-Whitney U test; this test was also used to analyze the total ageing of the adhesion force. The level of significance was set at \( p < 0.05 \).

Results

As an example, Figure 2 presents force-distance curves obtained for \( S. epidermidis \) ATCC 35983 on hydrophobic DDS-coated and on hydrophilic glass. A downward peak in the retract curves indicates attractive forces between the bacterium and surface and the maximum adhesion force corresponds to the
largest peak. From Figure 2A it is clear that there is hardly any strengthening of the maximum adhesion force on hydrophobic DDS-coated glass, although upon longer contact times (i.e. 120 s) a few multiple adhesion peaks may be observed. On hydrophilic glass (Figure 2B), the maximum adhesion force increases strongly with increasing surface delay time and multiple adhesion peaks, that may already be observed at 0 s contact time, strongly develop during prolonged contact between bacterial cell and substratum surface. Note that the long-range detachment events at several hundred nanometers in the retract curves represent stretching of staphylococcal cell surface structures.

Figures 3 and 4 show the maximum adhesion forces as a function of contact time for the four staphylococcal strains involved in this study for both DDS-coated glass (Figure 3) and bare glass (Figure 4). In both figures grey regions represent the force window of the control curves at 0 s contact time. All control curves had similar appearances and within each set of measurements no significant changes in their adhesion force was observed. On a hydrophobic substratum (Figure 3), adhesion forces hardly increase upon increasing the contact time between the staphylococcal probe and the substratum surface. On hydrophilic glass, however, adhesion forces increase significantly with longer contact times (Figure 4). Adhesion forces already significantly strengthen within 10 s of contact for all strains and reach stable values within approximately 60 s. Table 1 summarizes the bond-strengthening parameters, as can be determined by using Eq. (1) for all four *S. epidermidis* strains investigated. There is no unambiguous influence of the substratum on the initial maximal adhesion forces ($F_{0s}$) as can be seen from Table 1. However, in line with the qualitative features of Figures 3 and 4, the increases in maximum adhesion force on hydrophobic DDS-coated glass are limited to 0.3 nN and only significant for strains 3399 and HBH2 3. Conversely, on the hydrophilic glass the maximum adhesion forces increase significantly for all strains with increments ranging between 0.7 nN and
Figure 2. Examples of force-distance curves for *S. epidermidis* ATCC 35983 on hydrophobic DDS-coated glass (A) and hydrophilic glass (B) with retract curves after 0, 10, 60 and 120 s. Maximum adhesion forces ($F_{\text{max}}$) are defined as the force associated with the largest adhesion peak.
1.5 nN. The characteristic time $\tau$ needed for the adhesion force to strengthen is maximally 32 s (see Table 1).

**Table 1.** Bond-strengthening parameters for four *S. epidermidis* strains on DDS-coated glass (hydrophobic) and glass (hydrophilic). Values represent the averages and standard deviations of 6 staphylococcal probes, each used to measure 5 force distance curves.

<table>
<thead>
<tr>
<th>Strain</th>
<th>DDS-coated glass</th>
<th></th>
<th></th>
<th>Glass</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_0$ (nN)</td>
<td>$F_\infty$ (nN)</td>
<td>$\tau$ (s)</td>
<td>$F_0$ (nN)</td>
<td>$F_\infty$ (nN)</td>
<td>$\tau$ (s)</td>
</tr>
<tr>
<td>3399</td>
<td>$-0.4 \pm 0.3$</td>
<td>$-0.6 \pm 0.4$</td>
<td>$17 \pm 21$</td>
<td>$-0.2 \pm 0.2$</td>
<td>$-0.9 \pm 0.2$</td>
<td>$17 \pm 13$</td>
</tr>
<tr>
<td>ATCC 35983</td>
<td>$-0.4 \pm 0.3$</td>
<td>$-0.6 \pm 0.5$</td>
<td>$16 \pm 14$</td>
<td>$-0.5 \pm 0.2$</td>
<td>$-1.9 \pm 0.7$</td>
<td>$17 \pm 15$</td>
</tr>
<tr>
<td>HBH2 3</td>
<td>$-0.3 \pm 0.1$</td>
<td>$-0.6 \pm 0.3$</td>
<td>$8 \pm 6$</td>
<td>$-0.3 \pm 0.1$</td>
<td>$-1.8 \pm 0.9$</td>
<td>$32 \pm 27$</td>
</tr>
<tr>
<td>HBH2 169</td>
<td>$-0.1 \pm 0.1$</td>
<td>$-0.1 \pm 0.1$</td>
<td>$*-$</td>
<td>$-0.6 \pm 0.3$</td>
<td>$-1.8 \pm 0.4$</td>
<td>$3 \pm 1$</td>
</tr>
</tbody>
</table>

* No bond-strengthening found

**Discussion**

In this chapter, we investigated the time-dependent adhesion forces between four *S. epidermidis* strains and hydrophilic and hydrophobic substratum surfaces. Hydrophobicity was created by DDS-coating of glass surfaces, and thus had little effect on the roughness of the substratum surface. Both glass as well as DDS-coated glass are negatively charged with comparable zeta potentials [18]. Therefore, hydrophobicity is considered to be the main difference between the two surfaces. Strengthening of the bond was virtually absent on hydrophobic DDS-coated glass, but on hydrophilic glass strengthening of the bond by factors up to 6 occurred within a few tens of seconds, concurrent with the consistent development of multiple adhesion peaks in the retract force-distance curves. All four staphylococcal strains roughly exhibited a similar behaviour with respect to bond-strengthening. In order to rule out artefacts due
to bacterial detachment from the probe and/or due to bacterial footprints on the substratum surface [19], force-distance curves with 0 s contact time were recorded after each measurement with a given surface delay time. Control experiments indicated that staphylococci did not detach from the probe and that there were no bacterial footprints left on the substratum surface after measurement of a force-distance curve, with a measurable influence on the 0 s force-distance curves.

**Figure 3.** Adhesion forces of four *S. epidermidis* strains on DDS-coated glass as a function of the surface delay time. Grey regions denote the force window of the control curves at 0 s contact time, based on the average and standard deviation of 180 measurements. Each point represents the average and standard deviation of 30 measurements divided over 6 bacterial probes.

Bond-strengthening was significantly different on hydrophobic DDS-coated glass than on hydrophilic glass. The hydrophobicity of DDS-coated glass is
caused by the presence of apolar CH$_3$-groups. Water molecules adjacent to the substratum surface are not able to form hydrogen bonds with the apolar surface, and therefore they will do so as much as possible with other water molecules at the solution side of the surface [20]. This phenomenon is known as the hydrophobic effect. As a result the water molecules near the surface are restricted in their rotational freedom. Consequently, bacterial adhesion to a DDS-coated substratum is driven by an entropically favourable release of DDS-associated water molecules, giving relatively weak adhesion forces between bacteria and substratum surface. This release of water molecules from the surface region is a

![Figure 4. Adhesion forces of four *S. epidermidis* strains on glass as a function of the surface delay time. Grey regions denote the force window of the control curves at 0 second contact time, based on the average and standard deviation of 180 measurements. Each point represents the average and standard deviation of 30 measurements divided over 6 bacterial probes.](image-url)
fast process, and from the current data can be expected to be completed within the first 10 s of contact.

Alternatively, hydrophilic glass offers numerous sites for hydrogen bonding and the hydrophobic effect is not likely to play any significant role in bond-strengthening between staphylococci and glass. Similar to the glass surface, also the staphylococcal strains involved are negatively charged, hydrophilic and able to form hydrogen bonds [21]. Upon approach of the bacteria towards the hydrophilic surface, the outer cell surface first forms hydrogen bonds with the substratum, therewith expelling surface-associated water molecules. This implies a gain in entropy and a relatively fast rise in adhesion force. However, upon prolonged contact times, more extensive rearrangements of bacterial surface structures may occur to create additional bonds and cause the adhesion force to strengthen further. This is reflected in Figure 2, by the development of multiple adhesion peaks upon prolonged contact times. Interestingly, Abu-Lail and Camesano [22] recently performed a Poisson analysis of these multiple adhesion peaks in the interaction of *E. coli* with silicon nitride AFM tips and associated these peaks with multiple hydrogen bonds with an individual force value of -0.13 nN.

In the Poisson analysis of multiple adhesion forces, it is assumed that the average force of all adhesion peaks ($\mu_F$) is related to the variance ($\sigma_F^2$) of the adhesion force according to:

$$\sigma_F^2 = \mu_F F_{H\text{-bond}} - F_{H\text{-bond}} F_{\text{Non-specific}}$$

(2)

in which $F_{H\text{-bond}}$ and $F_{\text{Non-specific}}$ represent the contributions of hydrogen bonding and other non-specific interaction forces (*i.e.* contributions of Lifshitz-Van der Waals-, electrostatic- and steric interactions) to the adhesion force, respectively. Thus, in a plot of $\sigma_F^2$ versus $\mu_F$, the slope of a linear fit will yield $F_{H\text{-bond}}$, while $F_{\text{Non-specific}}$ can be calculated from the intercept.
Figure 5. Example of a Poisson analysis of the multiple adhesion peaks appearing after 120 s contact between *S. epidermidis* ATCC 35983 and hydrophilic glass. The linear dependency of the average adhesion force of all peaks (μF) versus the variance (σF²) of the adhesion force is denoted by the solid line (r² = 0.86). The slope of the regression yields \( F_{H-bond} \), while from the intercept \( F_{Non-specific} \) is calculated.

Poisson analysis of our retract curves of staphylococci from glass obtained after 120 s contact time were completely in line with the observations by Abu-Lail and Camesano [22] as can be seen in Figure 5 for a selected example. From the straight line dependencies as in Figure 5, contributions of H-bonding and non-specific interaction forces to the total adhesion forces between staphylococci and glass after bond-strengthening could be calculated, as summarized in Table 2.
Table 2. Poisson analysis on the multiple adhesion peaks as observed for four *S. epidermidis* strains on glass after a contact time of 120 s. Adhesion forces were separated in a hydrogen bonding component \((F_{\text{H-bond}})\) and a component for non-specific interactions \((F_{\text{Non-specific}})\).

<table>
<thead>
<tr>
<th>Strain</th>
<th>(F_{\text{H-bond}}) (nN)</th>
<th>(F_{\text{Non-specific}}) (nN)</th>
<th>((F_{\text{H-bond}} + F_{\text{Non-specific}}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>3399</td>
<td>-0.24</td>
<td>+0.07</td>
<td>-0.17</td>
</tr>
<tr>
<td>ATCC 35983</td>
<td>-0.79</td>
<td>+0.33</td>
<td>-0.46</td>
</tr>
<tr>
<td>HBH(_2) 3</td>
<td>-1.02</td>
<td>+0.58</td>
<td>-0.45</td>
</tr>
<tr>
<td>HBH(_2) 169</td>
<td>-0.75</td>
<td>+0.41</td>
<td>-0.34</td>
</tr>
</tbody>
</table>

All \(F_{\text{Non-specific}}\) values are positive, indicating that non-specific interactions have a net repulsive contribution due to the forced nature of the contact in AFM. Whereas under non-forced conditions, it is difficult to establish contact closer than in the secondary minimum of the DLVO interaction curve [23]. It is clear from the current results that in AFM close contact beyond the secondary minimum is imposed, causing the non-specific forces, attractive at long range, to become repulsive at short range. \(F_{\text{H-bond}}\) is negative for all four staphylococcal strains involved and amounts to -0.70 nN on average. Abu-Lail *et al.* [22] measured a force contribution \(F_{\text{H-bond}}\) of -0.13 nN, but their measurements involved a smaller contact area, *i.e.* a native AFM tip *versus* a bacterial cell surface, whereas we used a bacterial probe *versus* a macroscopic glass surface. Note that the net interaction force (*i.e.* \(F_{\text{H-bond}} + F_{\text{Non-specific}}\)) is attractive in all cases, but less than the maximum adhesion forces after bond-strengthening measured, because the Poisson analysis is based on average forces and not on the maximum forces observed.
Conclusions

Staphylococcal bond-strengthening between hydrophobic DDS-coated and hydrophilic glass proceeds according to different mechanisms, as revealed here by using atomic force microscopy. On hydrophobic DDS-coated glass bond-strengthening was fast (less than 10 s), limited to a minor increase in adhesion force and likely governed by hydrophobic interaction. On hydrophilic glass, bond strength increased during contact time and is ascribed to progressive formation of hydrogen bonds made possible by ongoing rearrangements of outer cell surface structures over a time scale of, typically, a few tens of seconds. As a consequence, adhesion forces strengthened considerably more on hydrophilic glass than on hydrophobic DDS-coated glass, as confirmed by Poisson analysis of the multiple adhesion peaks upon retract of the staphylococci from hydrophilic glass surfaces.

References


