Factors influencing bacterial adhesion to a PEO brush

Poly(ethylene oxide) (PEO) brush coatings can almost fully prevent adhesion of biological particles and may reduce environmental problems related to biofilm buildup. Generally, bacteria hardly adhere to PEO brush coatings, but some strains do. The aim of this study was to find factors determining whether a strain does or does not adhere to a PEO brush coating. Bacterial adhesion was assessed in a parallel plate flow chamber. The bacterial adhesion results indicated that a distinction could be made between three adhesive and three non-adhesive strains of *P. aeruginosa*, while bacterial motilities and zeta potentials were comparable for all six strains. However, water contact angles indicated that the adhesive strains were much more hydrophobic than the non-adhesive strains. Furthermore, only adhesive strains produced surfactive extracellular substances, that may be engaged in attractive interactions with the PEO chains. Atomic force microscopy (AFM) showed that the adhesion energy, measured from the retract curves of a bacteria coated cantilever from a brush coating, was significantly more negative for adhesive strains than for non-adhesive strains (*p* < 0.001). Through surface thermodynamic and extended-DLVO analyses these stronger adhesion energies could be attributed to acid-base interactions. However, the energies of adhesion of all strains with a brush coating were small when compared with their energies of adhesion with a glass surface. Accordingly, even the adhesive *P. aeruginosa* strains could be easily removed from a PEO brush coating by the passage of a liquid-air interface.

Introduction

Bacteria tend to adhere to different kinds of surfaces, ranging from surfaces in the human body, plants and clays to plastics and metals [1]. Once bacteria are attached to a surface, a multi-step process starts resulting in a complex, adhering microbial community called a “biofilm” [2]. Biofilms can be beneficial, like in wastewater treatment [3], but may also have hazardous consequences. For instance, in water distribution systems they may cause contamination of drinking water with pathogens like *Legionella* spp. and *Pseudomonas aeruginosa* [4]. Biofilm formation in food processing equipment is known to cause contamination, causing spoilage or disease [5], while on ship hulls biofilms are responsible for increased fuel consumption. To avoid the formation of marine biofilms environmentally harmful antimicrobial paints have been used [6] and recently banned internationally requiring the development of non-toxic antifouling surfaces. In the medical field, the formation of a biofilm on devices like catheters and orthopedic implants frequently constitute a reason for device failure and removal [7].

Bacterial adhesion is influenced by properties of both the bacterial and substratum surface. Bacterial characteristics known to influence adhesion are hydrophobicity, zeta potential [8], motility [9] and release of extracellular substances, like polysaccharides [10], proteins [11] and biosurfactants [12]. Relevant properties of the substratum surface are hydrophobicity, zeta potential [8], and also surface texture [13,14]. The influence of the surface free energies of the substratum and the bacterium can be modeled using a thermodynamic approach [8]. The extended-DLVO (Derjaguin, Landau, Verwey, Overbeek) theory accounts for Lifshitz-Van der Waals, electrostatic and short range acid-base interaction energies between the surface and the bacterium as a function of their separation distance [15]. The mechanistic knowledge on bacterial adhesion obtained provides guidelines for the development of surface coatings exhibiting minimal adhesion of bacteria.

Poly(ethylene oxide) (PEO) brushes are composed of PEO chains end-grafted to a surface. At sufficiently high grafting density, the polymer chains stretch into the surrounding medium. As the PEO chains are highly mobile [16] and attain extremely large exclusion volumes [17], they make the surface difficult to approach by particles, like proteins or...
bacteria. Penetration or compression of the brush by incoming particles lead to an increase in the local concentration of PEO, which, in turn, would lead to a repulsive osmotic interaction. Therewith, a PEO brush forms a physical barrier preventing close approach, thus keeping the particle at a distance where the attractive Lifshitz-Van der Waals interaction is relatively weak. PEO brushes have been shown to effectively suppress adhesion of a great variety of bacterial strains and species, with the exception, however, of a *P. aeruginosa* strain [18].

The aim of this research is to determine the properties that determine whether or not a bacterial strain adheres to a PEO brush. Adhesion and retention of six *P. aeruginosa* strains were evaluated in a parallel plate flow chamber. Bacterial cell surface hydrophobicity, zeta potential, motility and surfactive extracellular substance release were determined. Interaction energies were calculated using the thermodynamic and extended-DLVO approach, as well as from force-distance curves measured by atomic force microscopy (AFM) between bacterial coated cantilevers and brush coated surfaces.

**Materials and methods**

**Bacterial strains and growth conditions.** The bacterial strains *P. aeruginosa* 6487, *P. aeruginosa* ATCC 19582, *P. aeruginosa* KEI 1025, *P. aeruginosa* D1, *P. aeruginosa* 6354 and *P. aeruginosa* # 3 were used in this study. All strains were first grown overnight at 37°C on an agar plate from a frozen stock, which was kept at 4°C, never longer than one week. Several colonies were used to inoculate 10 ml of tryptone soya broth (TSB, OXOID, Basingstoke, England). This preculture was incubated at 37°C in ambient air for 24 h and used to inoculate a second culture of 200 ml that was grown for 16 h. The bacteria from the second culture were harvested by centrifugation for 5 min at 5000 g and washed twice with demineralized water. All bacteria were rod shaped with a length of 2.8 μm and a width of 1.0 μm as determined from microscopic images. For calculations using the extended-DLVO theory a radius of 0.7 μm was used, which is the radius of a spherical particle of equal volume.
Chapter 3

Preparation of PEO brush coated glass. Methacryl-terminated PEO with a molar mass of 9800 (Polymer Source Inc., Dorval, Quebec, Canada) were grafted to microscope glass slides (Menzel-Gläser, Emergo, Landsmeer, The Netherlands). Grafting was done by reaction in a polymer melt, as described by Maas et al. [19]. In this reaction, surface silanol groups, such as on glass, react with vinyl terminated polymers. Previous research has shown that this leads to high surface grafting densities, where the PEO chains extend in a brush conformation in aqueous medium [18]. Glass slides (76 × 26 × 1 mm) were first sonicated in 2% RBS 35 detergent (Omnilabo International BV, Breda, The Netherlands), rinsed in demineralized water, sonicated in methanol and rinsed in demineralized water again, to remove oil contaminations and fingerprints. Next, possible metallic oxides on the surfaces were removed by submersing the slides in hot (95°C) nitric acid (65%, Merck, Darmstadt, Germany) for 60 min. Finally, the surfaces were extensively rinsed with demineralized water and Millipore-Q water and dried in a heat box at 80°C for 5 h. Surfaces were covered with a solution of the methacryl-terminated PEO in chloroform (0.4 mM). The solvent was evaporated in a stream of nitrogen, after which surfaces were annealed overnight in vacuum at 145°C. Prior to experiments, excess material was removed by washing with demineralized water. Only part of a glass slide was grafted with PEO chains, which allowed studying the bare glass surface and the PEO brush coated surface in one and the same experiment.

Bacterial adhesion and retention. The parallel plate flow chamber and image analysis system have been previously described [20]. The dimensions of the channel were 175 × 17 × 0.75 mm. Images were taken from the bottom plate, which consisted of a partly brush coated glass slide. The top plate of the chamber was made of glass. Deposition was observed with a CCD-MXRI camera (High Technology, Eindhoven, The Netherlands) mounted on a phase contrast microscope (Olympus BH-2) equipped with a 40 × ultra long working distance objective (Olympus ULWD-CD Plan 40 PL). The camera was coupled to an image analyzer (TEA, Difa, Breda, The Netherlands). Each image (512 × 512 pixels with 8 bit resolution) was obtained after summation of 15 consecutive images (time interval 1 s) in order to enhance the signal to noise ratio and to eliminate moving bacteria from the analysis. The surface area covered by an image is 0.017 mm².
Prior to each experiment, all tubes and the flow chamber were filled with PBS (10 mM potassium phosphate, 150 mM NaCl, pH 6.8), while care was taken to remove air bubbles from the system. Flasks, containing bacterial suspension (3 × 10⁸ ml⁻¹) and buffer, were positioned at the same height with respect to the chamber to ensure that immediately after the flows were started, all fluids would circulate through the chamber at the desired shear rate of 15.7 s⁻¹ (0.025 ml s⁻¹), which yields a laminar flow (Reynolds number 1.4). PBS was circulated through the system for 30 min followed by bacterial suspension for 4 h and images were obtained alternately from the glass and from the brush coated part.

The initial increase in the number of adhering bacteria with time, was expressed in a so-called initial deposition rate j₀ (cm⁻² s⁻¹), i.e. the number of adhering bacteria per unit area and time. The number of bacteria adhering after 4h, n₄h, was taken as an estimate of microbial adhesion in a more advanced state of the process. Finally, an air bubble was passed through the chamber, involving a relatively high removal force exerted by the air-liquid interface (around 1 × 10⁻⁷ N) [21]. Retention was measured by determining the number of bacteria remaining on the surface.

All values given in this chapter are averages of experiments on three separately prepared partly brush coated surfaces, and were carried out with separately grown bacteria.

**Bacterial characterization.** Bacterial motility in PBS was observed in a 3 × 10⁸ ml⁻¹ suspension in a Bürker-Türk counting chamber (Marienfeld, Germany, depth 0.01 mm). Motility analysis consisted of the observation of 40 bacteria and enumerating the number of moving and non-moving bacteria, thus yielding the % of motile bacteria in a suspension. Furthermore, six motile bacteria were timed while they traveled over a distance of 50 µm, yielding their average velocity.

Bacterial electrophoretic mobilities at 25°C in PBS were measured with a Lazer Zee Meter 501 (PenKem, Bedford Hills, NY, USA) equipped with an image analysis option for tracking and zeta sizing. The electrophoretic mobilities were converted to zeta potentials using Smoluchowski’s theory [22].

Strains were examined for release of surfactive extracellular substances, like biosurfactants, using axisymmetric drop shape analysis by profile (ADSA-P) [23], recording
the effect of substance release on the surface tension of bacterial suspensions. 100 µl droplets containing 1 × 10⁹ bacteria ml⁻¹ suspended in PBS, were placed on a fluoroethylenepropylene surface (Fluorplast Nederland BV, Raamsdonksveer, The Netherlands) and the circumference of the suspension droplet was monitored during 2 h in an enclosed chamber with 100% humidity at room temperature. Droplet circumferences were recorded twice with a minimal time interval (< 0.5 s) between measurements and fitted to the Laplace equation of capillarity yielding the surface tension of the bacterial suspensions.

To determine bacterial cell surface hydrophobicity, water contact angles (θₘ) on lawns of the P. aeruginosa strains were measured using the sessile drop technique. Briefly, bacterial cells were layered from demineralized water onto 0.45 µm pore size filters (Millipore Corporation, Bedford, MA, USA) using negative pressure. The filters were left to dry in ambient air until so-called “plateau contact angles” could be measured. To allow surface thermodynamic analyses [24], contact angles were measured with water, 1-bromonaphthalene, diiodomethane and formamide.

Bacterial characterization experiments were carried out in triplicate with separately grown bacteria.

**The thermodynamic approach.** In the thermodynamic approach [8] the contact angle between a liquid and a substratum surface or bacterial lawn is related to their surface free energies according to Young’s equation

\[ \gamma_{lv} \cos \theta = \gamma_{lv} - \gamma_{sl} \]  

where “l” stands for the liquid, “v” for the surrounding vapor and “s” for the solid, which can either be the substratum surface or a bacterial surface in which case the subscript “b” is used.

To solve Equation 1, the surface free energies can be considered as a summation of their Lifshitz-Van der Waals (\( \gamma^{LW} \)) and an acid-base components (\( \gamma^{AB} \)). Furthermore the acid-base component is expressed in an electron donor (\( \gamma^- \)) and electron acceptor (\( \gamma^+ \)) parameter. This leads to Young’s equation in the following form

\[ \cos \theta = -1 + \frac{2 \sqrt{\gamma_{sv}^{LW} \gamma_{lv}^{LW}}}{\gamma_{lv}} + \frac{2 \sqrt{\gamma_{sv}^{+} \gamma_{lv}^{+}}}{\gamma_{lv}} + \frac{2 \sqrt{\gamma_{sv}^{-} \gamma_{lv}^{-}}}{\gamma_{lv}} \]  

which in the absence of polar interactions reduces to
Factors influencing bacterial adhesion on PEO brushes

$$\cos \theta = -1 + 2\sqrt{\frac{\gamma_{lw}^{lw}}{\gamma_{lv}}}$$  \hspace{1cm} (3)

Contact angles on bacterial lawns and substratum surfaces with the a-polar liquids 1-bromonaphthalene and diiodomethane were inserted in Equation 3 to yield the Lifshitz-Van der Waals component of the surface free energy of the substratum ($\gamma_{lw}^{lw}$) and bacterial surface ($\gamma_{bw}^{lw}$), after which contact angles with water and formamide were used in Equation 2 to give the acid-base component ($\gamma_{sv}^{ab}$) and its parameters ($\gamma_{sv}^{+} \gamma_{sv}^{-}$), according to

$$\gamma_{sv}^{ab} = 2\sqrt{\gamma_{sv}^{+} \gamma_{sv}^{-}}$$  \hspace{1cm} (4)

Note that at the sl interface, the brush will probably be in a swollen state, whereas at the sv interface it is most likely non-swollen. Subsequently, the Lifshitz-Van der Waals component of the free energy of adhesion can be calculated according to

$$\Delta G_{slb}^{lw} = -2\left(\sqrt{\gamma_{bw}^{lw}} - \sqrt{\gamma_{lv}^{lw}}\right) \left(\sqrt{\gamma_{sv}^{lw}} - \sqrt{\gamma_{lv}^{lw}}\right)$$  \hspace{1cm} (5)

while the acid-base component of the free energy of adhesion at contact follows from

$$\Delta G_{slb}^{ab} = 2 \left[\left(\sqrt{\gamma_{bw}^{+}} - \sqrt{\gamma_{sv}^{+}}\right)\left(\sqrt{\gamma_{bw}^{-}} - \sqrt{\gamma_{sv}^{-}}\right) - \left(\sqrt{\gamma_{bw}^{+}} - \sqrt{\gamma_{sv}^{-}}\right)\left(\sqrt{\gamma_{bw}^{-}} - \sqrt{\gamma_{sv}^{+}}\right)\right]$$  \hspace{1cm} (6)

The free energy of adhesion at contact ($\Delta G_{slb}$) according to the thermodynamic approach is the summation of these two components. The thermodynamically derived free energies of adhesion do not account for a distance dependence of the interaction energy.

On adhesion, a bacterium may undergo a small deformation to give a contact area of $\pi a^2$, with “a” being the radius of the circular contact area. The maximal radius of this circular contact area is the radius of the bacterium ($r$) and to obtain an upper limit for free energy of adhesion per bacterium, values derived from Equation 5 and Equation 6 were multiplied by $\pi r^2$.

**Extended-DLVO theory.** The energy of interaction, as a function of distance between the bacterial cell surface and the bare or brush coated glass substratum was assessed according to the extended-DLVO theory for colloidal stability, assuming a sphere-plane geometry [26].
Lifshitz-Van der Waals interaction energy as a function of separation distance was calculated using [26]

\[
\Delta G_{LW}^l(l) = -\frac{A}{6} \left[ \frac{2r(l + r)}{l(l + 2r)} - \ln\left(\frac{l + 2r}{l}\right) \right]
\]  

(7)

where \(l\) is the separation distance, \(r\) the radius of the bacterium and \(A\) the Hamaker constant as determined from [26]

\[
\Delta G_{\text{slb}}^LW = \frac{A}{12\pi l_0^2}
\]  

(8)

where \(\Delta G_{\text{slb}}^LW\) is obtained as described above from surface thermodynamic analyses, i.e. Equation 5 and \(l_0\) is the minimum separation distance (0.157 nm [27]).

Electrostatic interaction energy as a function of separation distance was calculated for the sphere-plane geometry as well, using [28]

\[
\Delta G_{EL}^l(l) = \frac{8\pi rcz^2 F^2 \Phi_{sl} \Phi_{bl}}{\kappa^2 RT} \ln[1 + \exp(-\kappa l)]
\]  

(9)

where \(z\) is the valency of the ions, \(c\) is the ion concentration, \(F\) the Faraday constant, \(R\) the universal gas constant, \(\kappa\) the reciprocal Debye length and \(\Phi_{sl}\) and \(\Phi_{bl}\) are surface potentials of the bacterium and substratum surface immersed in the surrounding liquid, respectively. Surface potentials were approximated by zeta potentials. For the substrata, zeta potentials were derived in PBS from measured streaming potentials in a home-made parallel plate flow chamber [29], yielding -46 mV and -15 mV for bare glass and PEO brush coated glass, respectively [20].

Acid-base interaction energy as a function of separation distance was calculated for the sphere-plane geometry using [26]

\[
\Delta G_{AB}^l(l) = 2\Delta G_{\text{slb}}^{AB} \pi r\lambda \exp\left(\frac{l_0 - l}{\lambda}\right)
\]  

(10)

where \(\Delta G_{\text{slb}}^{AB}\) was determined using Equation 6, \(\lambda\) is the correlation length of molecules in the liquid medium (estimated 0.6 nm for hydrophilic bacteria and 13 nm for hydrophobic bacteria [26]).

The total interaction energy (\(\Delta G(l)\)) was obtained by summation of Lifshitz-Van der Waals, electrostatic and acid-base interaction energies.
**Factors influencing bacterial adhesion on PEO brushes**

**Atomic force microscopy.** V-shaped silicon nitride tipless cantilevers from Park Scientific Instruments (Mountain View, CA, USA) with a spring constant of 0.06 Nm⁻¹ were pre-coated by placing the extremity of the cantilever for 10 s in 0.01% poly-L-lysine solution followed by drying in ambient air for at least 15 min. Next, cantilevers were coated by bacteria by placing the extremity of the cantilever for 60 s in a concentrated suspension of a *P. aeruginosa* strain, followed by drying in ambient air for at least 30 min. For control, three cantilevers were prepared with only a poly-L-lysine coating. Cantilevers were used for AFM measurement within 6 h of preparation. For each bacterium, bonding to the poly-L-lysine cantilever was checked by scanning electron microscopy.

A Nanoscope III AFM (Digital Instruments, Santa Barbara, CA, USA) operating in the contact mode was used to measure interaction forces. Measurements were done at room temperature in a PBS solution. Individual force curves with z-displacements up to 2000 nm were collected at z-scan rates of 1.99 Hz. Integral and proportional gains of the feedback loop were about 2 and 3, respectively. The slopes of the retraction force curves in the region where probe and sample are in contact were used to convert the voltage into cantilever deflection. Conversion of deflection into force was carried out as previously described [30]. The approach part of a force-distance curve was fitted to a negative exponential

\[
F(l) = F_0 \exp \left( \frac{-l}{\Lambda} \right)
\]

in which \(F\) is the measured force at separation distance \(l\), \(F_0\) is the force at zero separation, and \(\Lambda\) is the characteristic decay length (separation distance over which \(F\) decays from \(F_0\) to \(F_0/e\)). All curves displayed repulsion on approach, and integration of the approach curve represents a repulsive energy \(E_{\text{repulsive}}\) to be overcome to establish close contact. The retract curve regularly indicated an adhesive force, where its integration yields an adhesive energy \(E_{\text{adhesive}}\). Furthermore, the percentage occurrence of adhesive interactions (%adhesion) was registered.

For each bacterial strain, six cantilevers were prepared, equally divided over three separate bacterial cultures. 15 force measurements were performed with one cantilever at three locations on bare glass and brush coated glass, leading to a total of 90 force measurements per bacterium-substratum combination.
Results

Bacterial adhesion and retention. Adhesion and retention of the six *P. aeruginosa* strains are listed in Table 1. Initial deposition rates of the bacteria on glass were between 5 and \(18 \times 10^2\) cm\(^2\) s\(^{-1}\) and adhesion after 4 h between 2.5 and \(9.6 \times 10^6\) cm\(^2\). Three bacterial strains show average reductions by the brush coating as compared to glass of 95 and 96% in \(j_0\) and \(n_{4h}\), respectively. These strains will be denoted as “non-adhesive”. The three other strains show average reductions of 50 and 64% and will be denoted “adhesive”. Both non-adhesive and adhesive strains show relatively low average removal percentages from glass by an air bubble (17 and 23%) and relatively high average removal percentages from brush coated glass (89 and 86%).

Table 1. Initial deposition rates \((j_0)\) and adhesion after 4 h \((n_{4h})\) on glass, combined with reduction by a PEO brush coating as compared to glass, together with the % detachment in the number of adhering bacteria stimulated by a passing air bubble as a measure for their retention on glass and PEO brush coated glass. Bacterial adhesion and retention were determined in a parallel plate flow chamber.*

<table>
<thead>
<tr>
<th>Non-adhesive strains</th>
<th>Glass</th>
<th>PEO brush coated glass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(j_0) (10(^2) cm(^2) s(^{-1}))</td>
<td>(n_{4h}) (10(^6) cm(^2))</td>
</tr>
<tr>
<td>6487</td>
<td>18 ± 4</td>
<td>7.9 ± 3.2</td>
</tr>
<tr>
<td>ATCC 19582</td>
<td>10 ± 3</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td>KEI 1025</td>
<td>18 ± 5</td>
<td>9.6 ± 4.4</td>
</tr>
<tr>
<td>Average</td>
<td>15</td>
<td>7.4</td>
</tr>
<tr>
<td>Adhesive strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>13 ± 6</td>
<td>3.6 ± 1.7</td>
</tr>
<tr>
<td>6354</td>
<td>6 ± 2</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td># 3</td>
<td>5 ± 3</td>
<td>2.5 ± 1.5</td>
</tr>
<tr>
<td>Average</td>
<td>8</td>
<td>2.9</td>
</tr>
</tbody>
</table>

*± denotes standard deviations over three measurements.
Factors influencing bacterial adhesion on PEO brushes

**Bacterial characteristics.** Table 2 summarizes surface characteristics of all *P. aeruginosa* strains. For both the non-adhesive and the adhesive strains on average 14% were motile, with an average speed of around 11 µm s⁻¹. The zeta potential was also not distinctive with respect to a strain being adhesive or non-adhesive. However, the non-adhesive strains were found to be hydrophilic (average θ<sub>W</sub> 22 degrees), while the adhesive strains were very hydrophobic (average θ<sub>W</sub> 132 degrees). Furthermore, adhesive strains reduced the surface tension of an aqueous suspension by 19 mJ m⁻² on average, indicating release of surface active extracellular substances, while the non-adhesive strains released a minimal amount of substances affecting the surface tension of their suspension liquid.

**Table 2.** Percentage motile bacteria, motility, zeta potential (ζ) and water contact angle (θ<sub>W</sub>) together with the change in surface tension of a *P. aeruginosa* suspension (Δγ<sub>lv</sub>).*

<table>
<thead>
<tr>
<th></th>
<th>Non-adhesive strains</th>
<th>Adhesive strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% motile</td>
<td>Motility (µm s⁻¹)</td>
</tr>
<tr>
<td>6487</td>
<td>18 ± 9</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>ATCC 19582</td>
<td>15 ± 8</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>KEI 1025</td>
<td>10 ± 5</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>D1</td>
<td>19 ± 4</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>6354</td>
<td>13 ± 3</td>
<td>11 ± 3</td>
</tr>
<tr>
<td># 3</td>
<td>9 ± 6</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>Average</td>
<td>14</td>
<td>11</td>
</tr>
</tbody>
</table>

*± denotes standard deviations over three measurements

**Thermodynamic approach.** Upper limits for the free energies of adhesion as determined from the thermodynamic approach between glass or brush coated glass and the *P. aeruginosa* strains are collected in Table 3. All strains are attracted towards glass with an average free energy of adhesion (ΔG<sub>slb</sub>) of -47 × 10⁻¹⁶ J for the non-adhesive strains and -767 × 10⁻¹⁶ J for the adhesive strains. For the brush coating, however, a repulsive interaction was found for the non-adhesive strains, whereas the adhesive strains showed an attractive interaction with brush
coated glass. These differences were attributable to the acid-base contribution of the free energy of adhesion, as the Lifshitz-Van der Waals interaction energies are comparable for all strains.

Table 3. Upper limits for the free energy of adhesion $(10^{16}$ J) according to a thermodynamic approach between glass or the PEO brush coated glass and the six P. aeruginosa strains used in this study.

<table>
<thead>
<tr>
<th>Non-adhesive strains</th>
<th>Glass</th>
<th>PEO brush coated glass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta G_{\text{LW}}^{\text{slb}}$</td>
<td>$\Delta G_{\text{AB}}^{\text{slb}}$</td>
</tr>
<tr>
<td>6487</td>
<td>-32</td>
<td>-15</td>
</tr>
<tr>
<td>ATCC 19582</td>
<td>-30</td>
<td>-9</td>
</tr>
<tr>
<td>KEI 1025</td>
<td>-46</td>
<td>-26</td>
</tr>
<tr>
<td>Average</td>
<td>-36</td>
<td>-17</td>
</tr>
<tr>
<td>Adhesive strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>-39</td>
<td>-640</td>
</tr>
<tr>
<td>6354</td>
<td>-39</td>
<td>-763</td>
</tr>
<tr>
<td># 3</td>
<td>-30</td>
<td>-790</td>
</tr>
<tr>
<td>Average</td>
<td>-36</td>
<td>-731</td>
</tr>
</tbody>
</table>

Extended-DLVO theory. In contrast to the thermodynamic approach, the extended-DLVO theory provides the opportunity to incorporate the three dimensional nature of the swollen PEO brush in the calculation of the adhesion energies. This is, however, not an unambiguous task. The PEO brush is known to be highly hydrated and stretch into the aqueous surroundings to a thickness of 23.7 nm. Therefore, it was assumed that Lifshitz-Van der Waals interaction energy between the brush surface and the bacterium is negligibly small and arises solely from the underlying glass surface. Thus, the Lifshitz-Van der Waals interaction energy at closest approach to the brush is calculated as acting at a separation of 23.7 nm from the glass surface. Since it has been suggested that water is strongly immobilized inside a brush and zeta potentials reflect the potential at the outside of the brush [20], electrostatic interactions were calculated with respect to the outer brush surface. Similarly, also the short ranged [26] acid-base interactions were calculated with respect to the outer brush surface. However, the density of acid-base interacting groups is lower in a swollen state than during

58
Factors influencing bacterial adhesion on PEO brushes

contact angle measurements, which can be accounted for by calculating the volume density of PEO in the swollen brush. Based on the volume taken up by a PEO chain of 220 EO monomers in the swollen brush (118 nm$^3$ [31]) and the dry volume of a PEO chain (16 nm$^3$ [34]), it can be calculated that 14% of the total brush volume is taken up by PEO chains. Consequently, the acid-base interaction energies calculated from Equation 10 are multiplied by 0.14.

The average distance dependence of the interaction energies between non-adhesive or adhesive strains and glass or PEO brush coated glass, calculated as described above is shown in Figure 1. Interaction energies at the minimal separation distance are compiled in Table 4. For the non-adhesive strains a relatively small positive interaction energy with glass was found, indicating repulsive interaction. In contrast, for the adhesive strains a large acid-base component leads to relatively strong attraction to glass. The non-adhesive strains have a negligible Lifshitz-Van der Waals interaction energy with the PEO brush coated glass, leaving repulsive acid-base and electrostatic contributions and yielding a repulsive total interaction. In contrast, the interaction of the adhesive strains with the PEO brush coated glass is dominated by an attractive acid-base interaction, yielding an average attractive total interaction of $-1.1 \times 10^{-16}$ J.

**AFM.** Characteristics of the force-distance curves measured with AFM between the *P. aeruginosa* strains and bare or brush coated glass surfaces are compiled in Table 5. Effective coating of the AFM cantilever by bacteria was demonstrated in control experiments: the repulsive energy upon approach between a poly-L-lysine coated cantilever without bacteria and glass ($0.8 \pm 0.9 \times 10^{-16}$ J) is significantly lower than that between the different bacteria coated cantilevers and glass ($p < 0.001$, two-sided Student t-test). Furthermore, the adhesive energy upon retract ($-30 \pm 28 \times 10^{-16}$ J) is significantly higher ($p < 0.001$) for a poly-L-lysine coated cantilever in the absence of bacteria. Thus it is concluded that the cantilevers are effectively covered with bacteria. The adhesive strains have to overcome a stronger repulsion extending over a larger distance than the non-adhesive strains ($p < 0.001$), upon approach of both the glass and the brush coated glass surface, with little difference of whether glass or
Figure 1. Interaction energies, according to the extended-DLVO theory, between an average non-adhesive or adhesive *P. aeruginosa* bacterium and glass or PEO brush coated glass as a function of separation distance. The interaction energy is represented by a solid line, while the electrostatic, Lifshitz-Van der Waals and acid-base interaction energies are represented by the dashed-dotted, dashed and dotted line, respectively. The $\Delta G_{LW}^{\text{w}}(l)$ for PEO brush coated glass was assumed to originate from glass operating over a distance of 23.7 nm.
Table 4. Interaction energies (10^{-16} J) between glass or PEO brush coated glass and the six P. aeruginosa strains used in this study, according to the extended-DLVO theory at the minimal separation distance \( l_0 \) of 0.157 nm.*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glass ( \Delta G^E )</th>
<th>Glass ( \Delta G^L )</th>
<th>Glass ( \Delta G^{AB} )</th>
<th>Glass ( \Delta G )</th>
<th>PEO brush coated glass ( \Delta G^E )</th>
<th>PEO brush coated glass ( \Delta G^L )</th>
<th>PEO brush coated glass ( \Delta G^{AB} )</th>
<th>PEO brush coated glass ( \Delta G )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-adhesive strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6487</td>
<td>0.047</td>
<td>-0.014</td>
<td>-0.0037</td>
<td>0.029</td>
<td>0.015</td>
<td>-0.00011</td>
<td>0.084</td>
<td>0.099</td>
</tr>
<tr>
<td>ATCC 19582</td>
<td>0.050</td>
<td>-0.013</td>
<td>0.0021</td>
<td>0.035</td>
<td>0.016</td>
<td>-0.00010</td>
<td>0.084</td>
<td>0.10</td>
</tr>
<tr>
<td>KEI 1025</td>
<td>0.040</td>
<td>-0.021</td>
<td>-0.0062</td>
<td>0.013</td>
<td>0.013</td>
<td>-0.00015</td>
<td>0.089</td>
<td>0.10</td>
</tr>
<tr>
<td>Average</td>
<td>0.046</td>
<td>-0.016</td>
<td>-0.0040</td>
<td>0.026</td>
<td>0.015</td>
<td>-0.00012</td>
<td>0.086</td>
<td>0.10</td>
</tr>
<tr>
<td>Adhesive strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>0.040</td>
<td>-0.018</td>
<td>-3.3</td>
<td>-3.3</td>
<td>0.013</td>
<td>-0.00013</td>
<td>-0.84</td>
<td>-0.83</td>
</tr>
<tr>
<td>6354</td>
<td>0.060</td>
<td>-0.017</td>
<td>-4.0</td>
<td>-4.0</td>
<td>0.020</td>
<td>-0.00013</td>
<td>-1.2</td>
<td>-1.2</td>
</tr>
<tr>
<td># 3</td>
<td>0.037</td>
<td>-0.013</td>
<td>-4.1</td>
<td>-4.1</td>
<td>0.012</td>
<td>-0.00010</td>
<td>-1.3</td>
<td>-1.3</td>
</tr>
<tr>
<td>Average</td>
<td>0.046</td>
<td>-0.016</td>
<td>-3.8</td>
<td>-3.8</td>
<td>0.015</td>
<td>-0.00012</td>
<td>-1.1</td>
<td>-1.1</td>
</tr>
</tbody>
</table>

* The \( \Delta G^{LW}(l_0) \) for PEO brush coated glass was assumed to originate from glass operating over a distance of 23.7 nm.

brush coated glass is approached. However, upon retract of non-adhesive strains from a brush coated surface, a proportionally lower percentage of force-distance curves demonstrate adhesion than upon retract of the adhesive strains. In addition, if adhesion is observed upon retract, the non-adhesive strains experience a significantly (\( p < 0.001 \)) smaller adhesion energy upon retract from the brush coated glass than upon retract from glass, whereas the adhesive strains show roughly similar adhesion energies upon retract from glass as from brush coated glass. Also, upon retract from a brush coating the adhesive strains experience an average adhesion energy of \(-1.5 \times 10^{-16} \) J, which is significantly more negative than the non-adhesive strains (\( p < 0.001 \)).
Table 5. Characteristics of the force-distance curves between the P. aeruginosa strains used in this study on the cantilever and bare glass or PEO brush coated glass, as determined by AFM.*

<table>
<thead>
<tr>
<th></th>
<th>Glass Approach curve</th>
<th>Glass Retract curve</th>
<th>Brush coated glass Approach curve</th>
<th>Brush coated glass Retract curve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E\text{_\text{repulsion}} (10^{-16} J)</td>
<td>(\Lambda) (nm)</td>
<td>E\text{_\text{adhesion}} (10^{-16} J)</td>
<td>%\text{adhesion}</td>
</tr>
<tr>
<td>Non-adhesive strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6487</td>
<td>2.7 ± 3.2</td>
<td>40 ± 35</td>
<td>-1.4 ± 1.4</td>
<td>100</td>
</tr>
<tr>
<td>ATCC 19582</td>
<td>7.5 ± 12</td>
<td>56 ± 73</td>
<td>-9.7 ± 9.7</td>
<td>71</td>
</tr>
<tr>
<td>KEI 1025</td>
<td>1.4 ± 0.8</td>
<td>40 ± 45</td>
<td>-0.9 ± 1.1</td>
<td>100</td>
</tr>
<tr>
<td>Average</td>
<td>3.9</td>
<td>45</td>
<td>-4.0</td>
<td>90</td>
</tr>
<tr>
<td>Adhesive strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>5.2 ± 7.0</td>
<td>146 ± 211</td>
<td>-3.9 ± 5.4</td>
<td>75</td>
</tr>
<tr>
<td>6354</td>
<td>19 ± 29</td>
<td>301 ± 375</td>
<td>-1.1 ± 1.3</td>
<td>33</td>
</tr>
<tr>
<td># 3</td>
<td>69 ± 65</td>
<td>565 ± 260</td>
<td>-0.5 ± 1.0</td>
<td>17</td>
</tr>
<tr>
<td>Average</td>
<td>31</td>
<td>337</td>
<td>-1.8</td>
<td>42</td>
</tr>
</tbody>
</table>

*\pm\ denotes standard deviations over 90 force-distance curves.
**Discussion**

PEO brush coatings are supposed to create a barrier between the surface and any approaching particle, thus providing a generic anti-adhesive effect [33]. In this research it has been shown that several, here called “non-adhesive”, *P. aeruginosa* strains comply with this general rule, as do many other strains and species [18]. However, adhesion of other, “adhesive” *P. aeruginosa* strains was much less suppressed by the brush.

Kogure *et al.* [9] showed that motility increased adhesion to a bare glass substratum. This was attributed to the increase in collisions with the substratum surface [34]. Also a decrease in zeta potential is known to increase adhesion to a bare substratum, caused by reduced electrostatic repulsion [35]. In this study, motilities and zeta potentials were not distinctive for adhesive and non-adhesive strains. However, here it was also shown that only adhesive strains released surfactive substances. Indeed *P. aeruginosa* strains are known to produce a biosurfactant called rhamnolipid [36], with a potential role in their adhesion to substratum surfaces. Also in another study [37], adherence of *Pseudomonas* sp. to a PEO coating was found and attributed to the release of surfactive substances as well. Furthermore, AFM showed a relatively long average decay lengths (337 nm) on glass for the adhesive strains. Decay lengths of interaction between a bacterium and a bare surface between 10 and 33 nm [38] are known, which are in the same range as the non-adhesive strains in this study (45 nm) and are attributed to steric repulsion upon approach. Thus, it is anticipated that the adhesive bacteria are covered by a relatively thick layer of extracellular substances that may cause increased attractive interaction with the PEO brush as the substances of which this layer consists can penetrate the PEO chains.

Results from the thermodynamic approach and extended-DLVO theory, both indicate attractive interaction of the adhesive bacteria with the brush coated glass, but the values differs by two orders of magnitude, i.e. (-211 - -295) $\times 10^{-16}$ J and (-0.8 - -1.3) $\times 10^{-16}$ J, respectively, as can be seen by comparison of Tables 3 and 4. The thermodynamic approach assumes direct contact between the particle and the substratum, with the formation of a new particle-substratum interface. However, because appendages and hydrated polymers are often present at biological interfaces direct contact is a difficult concept, as distance is hard to
define in such cases. Furthermore, the contact area may be smaller than the maximum contact area as used here. The extended-DLVO values include the electrostatic contributions as well as reduction of Lifshitz-Van der Waals interactions and acid-base interactions induced by the swollen brush. Hence, these values are considered the most reliable ones. Indeed, the values for the interaction energy calculated using the extended-DLVO theory are in the same range as the adhesive energies derived from direct measurements using AFM.

The thermodynamic approach and the extended-DLVO theory both indicate that the attractive interaction between the adhesive bacteria and the brush coated glass is governed by acid-base interactions. When considering that hydrogen bridges are one of the most important acid-base interactions, this attractive interaction may be interpreted in terms of hydrophobic interaction [39]. Introduction of an apolar or hydrophobic solute in water is known to reduce the entropy. The reason for this is that at the hydrophobic surface water molecules are not able to form hydrogen bridges in all four directions, thus restricting their rotational freedom. In our system, adhesion of a hydrophobic bacterium reduces the hydrophobic water-accessible surface area leading to an entropically favorable release of water, and, hence, to a total attractive interaction free energy. Indeed, the adhesive bacteria used in this research have a much more hydrophobic character than the non-adhesive bacteria. Also hydrophobic proteins have been suggested to adsorb to PEO brushes by their hydrophobic moieties [40], and attraction of PEO to non-polar surfaces has also been demonstrated [41].

For future practical applications it should be mentioned that most bacteria are hydrophilic in nature (θ_W < 60 degrees) [42] and not all bacteria produce substantial amounts of extracellular substances [43]. Furthermore, AFM indicated that for non-adhesive strains adhesive interaction is reduced by the application of a PEO brush coating, as was also found in a study by Razatos et al. [44]. For the adhesive bacteria adhesion is reduced by 33 to 63%, which is comparable to other surface modification techniques [45,46]. Moreover, the energies of adhesion as calculated using the thermodynamic and extended-DLVO analyses of all strains with a brush coating were small when compared with their energies of adhesion with a glass surface. Accordingly, both the adhesive and the non-adhesive strains can be readily removed by an passing air bubble. In conclusion, PEO coatings can be considered very promising to prevent, or at least reduce, environmental problems related to bacterial adhesion.
Acknowledgements

The authors gratefully acknowledge Carol Lakkis for providing *P. aeruginosa* strains. Furthermore we would like to thank Ietse Stokroos for taking scanning electron micrographs.

References


