

Bacterial factors influencing adhesion of *Pseudomonas aeruginosa* strains to a poly(ethylene oxide) brush

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Most bacterial strains adhere poorly to poly(ethylene oxide) (PEO)-brush coatings, with the exception of a *Pseudomonas aeruginosa* strain. The aim of this study was to find factors determining whether *P. aeruginosa* strains do or do not adhere to a PEO-brush coating in a parallel plate flow chamber. On the basis of their adhesion, 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 released surfactive extracellular substances, which may be engaged in attractive interactions with the PEO chains. Atomic force microscopy showed that the adhesion energy, measured from the retract curves of a bacterial-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 (Derjaguin, Landau, Verwey, Overbeek) analyses, these stronger adhesion energies could be attributed to acid–base interactions. However, the energies of adhesion of all strains to a brush coating were small when compared with their energies of adhesion to 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. In conclusion, cell surface hydrophobicity and surfactant release are the main factors involved in adhesion of *P. aeruginosa* strains to PEO-brush coatings.

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INTRODUCTION

Bacteria tend to adhere to different kinds of surfaces, ranging from surfaces in the human body, and plants and clays, to plastics and metals. Once bacteria are attached to a surface, a multi-step process starts, resulting in a complex adhering microbial community called a 'biofilm' (Escher & Characklis, 1990). Biofilms can be beneficial, such as in wastewater treatment (Nicolella *et al.*, 2000), but they may also have hazardous consequences. For instance, in water-distribution systems, they may cause contamination of drinking water with pathogens such as *Legionella* spp. and *Pseudomonas aeruginosa* (Szewzyk *et al.*, 2000). Biofilm formation in food-processing equipment is known to cause contamination, resulting in spoilage or disease (Kumar & Anand, 1998), 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 (Yebara *et al.*, 2004), but these have recently been banned internationally, requiring the development of non-toxic antifouling surfaces. In the medical field, the formation of biofilms on devices such as catheters and orthopaedic implants frequently constitutes a reason for device failure and removal (Gristina, 1987).

Bacterial adhesion is influenced by properties of both the bacterial and the substratum surface. Bacterial characteristics known to influence adhesion are hydrophobicity, zeta potential (Bos *et al.*, 1999), motility (Kogure *et al.*, 1998), and release of extracellular substances, such as polysaccharides (Azeredo *et al.*, 1999), proteins (Dufrene *et al.*, 1996) and biosurfactants (Van Hoogmoed *et al.*, 2000). Relevant properties of the substratum surface are hydrophobicity, zeta potential (Bos *et al.*, 1999), and surface texture (Desai *et al.*, 1992; Holland *et al.*, 1998). The influence of the surface free energies of the substratum and the bacterium can be modelled using a thermodynamic approach (Bos *et al.*,

Abbreviations: AFM, atomic force microscopy; DLVO, Derjaguin, Landau, Verwey, Overbeek; PEO, poly(ethylene oxide).

1999). 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 (Van Oss *et al.*, 1986). The mechanistic knowledge of bacterial adhesion obtained from the extended-DLVO theory 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 a sufficiently high grafting density, the polymer chains stretch into the surrounding medium. Since the PEO chains are highly mobile (Harris, 1992), and attain extremely large exclusion volumes (Ryle, 1965), they make the surface difficult to approach by proteins or bacteria. Penetration or compression of the brush by incoming bacteria leads to an increase in the local concentration of PEO, which, in turn, leads to a repulsive osmotic interaction. Therewith, a PEO brush forms a physical barrier preventing close approach, thus keeping the particle at a distance at which 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 (Roosjen *et al.*, 2003).

The aim of this research was to identify the properties that determined whether or not a *P. aeruginosa* strain adhered 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, and using force–distance curves, measured by atomic force microscopy (AFM), between bacterial-coated cantilevers and brush-coated surfaces.

METHODS

Bacterial strains and growth conditions. The *P. aeruginosa* strains 6487, ATCC 19582, KEI 1025, D1, 6354 and # 3 were used in this study. All strains were taken from a frozen stock, and grown overnight at 37 °C on a blood agar plate, which was then kept at 4 °C for no longer than 1 week. Several colonies were used to inoculate 10 ml tryptone soya broth (TSB; Oxoid). This preculture was incubated aerobically at 37 °C for 24 h, and used to inoculate a second culture of 200 ml, which was then 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. Bacteria were suspended in PBS (10 mM potassium phosphate, 150 mM NaCl, pH 6.8) to the concentration needed for the assay. For contact-angle measurements, bacteria were suspended in 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.

Preparation of PEO-brush-coated glass. Methacryl-terminated PEO, with a molar mass of 9800 Da (Polymer Source), was grafted

to microscope glass slides. Grafting was done by reaction in a polymer melt, as described by Maas *et al.* (2003). In this reaction, surface silanol groups, such as those on glass, react with vinyl-terminated polymers. Previous research has shown that this leads to high surface grafting densities, in which the PEO chains extend in a brush conformation in aqueous medium (Roosjen *et al.*, 2003). Glass slides (76 × 26 × 1 mm) were sonicated in 2% RBS 35 detergent (Omnilabo International), rinsed in demineralized water, sonicated in methanol, and rinsed in demineralized water again, to remove oil contamination and fingerprints. Next, possible metallic oxides on the surfaces were removed by submerging the slides in hot (95 °C) nitric acid (65%; Merck) 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 under 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 us to study the bare glass surface and the PEO-brush-coated surface in the same experiment.

Bacterial adhesion and retention. The parallel plate flow chamber and image analysis system have been described previously (Kaper *et al.*, 2003). The dimensions of the channel were 175 × 17 × 0.75 mm. Images were taken of adhering bacteria on the bottom plate, which consisted of a partly brush-coated glass slide, positioned parallel to the direction of flow through the flow chamber. The top plate of the chamber was made of glass. Deposition was observed with a CCD-MXRi camera (High Technology) mounted on a phase-contrast microscope (Olympus BH-2) equipped with a ×40 ultralong working distance objective (Olympus ULWD-CD Plan 40 PL). The camera was coupled to an image analyser (TEA; Difa). 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 was 0.017 mm².

Prior to each experiment, all tubes and the flow chamber were filled with PBS, while care was taken to remove air bubbles from the system. Flasks containing bacterial suspension (3×10^8 bacteria 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 with a Reynolds number of 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 of the glass.

The initial increase in the number of adhering bacteria with time was expressed in a so-called initial deposition rate j_0 (cm⁻² s⁻¹), i.e. the number of adhering bacteria per unit area and time. The initial deposition rate was calculated over the first 20–30 min from linear regression analysis of the number of bacteria per unit area versus time. The number of bacteria adhering after 4 h, n_{4h} , 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) (Gomez-Suárez *et al.*, 1999). Retention was measured by determining the number of bacteria remaining on the surface.

All values given are means of experiments on three separately prepared partly brush-coated surfaces, and carried out with separately grown bacteria.

Bacterial characterization. Bacterial motility in PBS was observed using a suspension containing 3×10^8 bacteria ml^{-1} in a Bürker-Türk counting chamber (depth 0.01 mm). Motility analysis consisted of the observation of 40 bacteria, and enumeration of the number of moving and non-moving bacteria, thus yielding the percentage of motile bacteria in a suspension. Furthermore, six motile bacteria were timed while they travelled over a distance of 50 μm , yielding their mean velocity.

Bacterial electrophoretic mobilities at 25 °C in PBS were measured with a Lazer Zee Meter 501 (PenKem) equipped with an image analysis option for tracking and zeta sizing. The electrophoretic mobilities were converted to zeta potentials using Smoluchowski's theory (Hiemenz, 1991).

Strains were examined for release of surfactive extracellular substances, such as biosurfactants, using axisymmetric drop shape analysis by profile (Cheng & Neumann, 1992), recording the effect of substance release on the surface tension of bacterial suspensions. Droplets (100 μl), containing 1×10^9 bacteria ml^{-1} suspended in PBS, were placed on a fluoroethylenepropylene surface (Fluorplast), and the circumference of the droplet was monitored for 2 h in an enclosed chamber, with 100% humidity at room temperature. Droplet circumference was 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 (θ_w) 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) using negative pressure. The filters were left to dry in air until so-called 'plateau contact angles' could be measured; these are known to represent a state of cell surface hydration most closely resembling that occurring in suspension (Van Oss, 1994; Van der Mei *et al.*, 1998). To allow surface thermodynamic analyses (Van Oss, 1994), contact angles were measured with water, 1-bromonaphthalene, diiodomethane and formamide.

The thermodynamic approach. In the thermodynamic approach (Bos *et al.*, 1999), 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:

$$\gamma_{lv} \cos\theta = \gamma_{sv} - \gamma_{sl} \quad (1)$$

where l stands for the liquid, v stands for the surrounding vapour, and s stands for the solid, which can be either the substratum surface or the 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 (γ^{LW}) and an acid–base component (γ^{AB}). Furthermore, the acid–base component is expressed in an electron donor (γ^-) and electron acceptor (γ^+) parameter. This leads to Young's equation in the following form:

$$\cos\theta = -1 + \frac{2\sqrt{\gamma_{sv}^{LW}\gamma_{lv}^{LW}}}{\gamma_{lv}} + \frac{2\sqrt{\gamma_{sv}^+\gamma_{lv}^-}}{\gamma_{lv}} + \frac{2\sqrt{\gamma_{sv}^-\gamma_{lv}^+}}{\gamma_{lv}} \quad (2)$$

which, in the absence of polar interactions, reduces to:

$$\cos\theta = -1 + \frac{2\sqrt{\gamma_{sv}^{LW}\gamma_{lv}^{LW}}}{\gamma_{lv}} \quad (3)$$

Contact angles on bacterial lawns and substratum surfaces with the apolar 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 (γ_{bv}^{LW}) and bacterial surface (γ_{lv}^{LW}), after which contact angles with water and formamide were used in

equation (2) to give the acid–base component (γ_{sv}^{AB}) and its parameters (γ_{sv}^+) and (γ_{sv}^-), according to:

$$\gamma_s^{AB} = 2\sqrt{\gamma_{sv}^+\gamma_{sv}^-} \quad (4)$$

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

$$\Delta G_{slb}^{LW} = -2 \left(\sqrt{\gamma_{bv}^{LW}} - \sqrt{\gamma_{lv}^{LW}} \right) \left(\sqrt{\gamma_{sv}^{LW}} - \sqrt{\gamma_{lv}^{LW}} \right) \quad (5)$$

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

$$\Delta G_{slb}^{AB} = 2 \left[\left(\sqrt{\gamma_{bv}^+} - \sqrt{\gamma_{sv}^+} \right) \left(\sqrt{\gamma_{bv}^-} - \sqrt{\gamma_{sv}^-} \right) - \left(\sqrt{\gamma_{bv}^+} - \sqrt{\gamma_{lv}^+} \right) \left(\sqrt{\gamma_{bv}^-} - \sqrt{\gamma_{lv}^-} \right) - \left(\sqrt{\gamma_{sv}^+} - \sqrt{\gamma_{lv}^+} \right) \left(\sqrt{\gamma_{sv}^-} - \sqrt{\gamma_{lv}^-} \right) \right] \quad (6)$$

The free energy of adhesion at contact (ΔG_{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.

Upon adhesion, a bacterium may undergo a small deformation to give a contact area of πa^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 ; taken as 0.7 μm , which is equivalent to a sphere of equal volume to the rod-shaped bacteria under study), and to obtain an upper limit for free energy of adhesion per bacterium, values derived from equations (5) and (6) were multiplied by πr^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 sphere–plane geometry. Lifshitz–Van der Waals interaction energy as a function of separation distance was calculated using Van Oss (1994):

$$\Delta G^{LW}(l) = -\frac{A}{6} \left[\frac{2r(l+r)}{l(l+2r)} - \ln\left(\frac{l+2r}{l}\right) \right] \quad (7)$$

where l is the separation distance, r is the radius of the bacterium, and A is the Hamaker constant, as determined from Van Oss (1994):

$$\Delta G_{slb}^{LW} = \frac{A}{12\pi l_0^2} \quad (8)$$

where ΔG_{slb}^{LW} is obtained, as described above, from surface thermodynamic analyses, i.e. equation (5), and l_0 is the minimum separation distance between the outermost cell surface and the substratum (0.157 nm; Van Oss, 1994).

Electrostatic interaction energy as a function of separation distance was also calculated for the sphere–plane geometry, using Lyklema (2005):

$$\Delta G^{EL}(l) = \frac{8\pi rcz^2 F^2 \Phi_{bl} \Phi_{sl}}{\kappa^2 RT} \ln[1 + \exp(-\kappa l)] \quad (9)$$

where z is the valency of the ions, c is the ion concentration, F is the Faraday constant, R is the universal gas constant, κ is the reciprocal Debye length, and Φ_{bl} and Φ_{sl} are the surface potentials of the bacterium and the 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 (Van Wagenen & Andrade, 1980), yielding -46 and -15 mV for bare glass and PEO-brush-coated glass, respectively (Kaper *et al.*, 2003).

Acid–base interaction energy, as a function of separation distance, was calculated for the sphere–plane geometry using (Van Oss, 1994):

$$\Delta G^{\text{AB}}(l) = 2\Delta G_{\text{slb}}^{\text{AB}} \pi r \lambda \exp \frac{l_0 - l}{\lambda} \quad (10)$$

where $\Delta G_{\text{slb}}^{\text{AB}}$ was determined using equation (6), and λ is the correlation length of molecules in the liquid medium (estimated to be 0.6 nm for hydrophilic bacteria, and 13 nm for hydrophobic bacteria; Van Oss, 1994).

The total interaction energy [$\Delta G(l)$] was obtained by summation of Lifshitz–Van der Waals, electrostatic and acid–base interaction energies.

AFM. V-shaped silicon nitride tipless cantilevers from Park Scientific Instruments, with a spring constant of 0.06 N m $^{-1}$, as provided by the manufacturer, were pre-coated by placing the extremity of the cantilever in 0.01 % poly-L-lysine solution for 10 s, followed by drying in air for at least 15 min. Next, the cantilevers were coated with bacteria by placing the extremity of the cantilever in a concentrated suspension of a *P. aeruginosa* strain for 60 s, followed by drying in air for at least 30 min. As a control, three cantilevers were prepared with a poly-L-lysine coating only. 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. Spring constants were also experimentally determined by measuring the resonance frequency of each tip used, from which the spring constant could be calculated according to:

$$k = af^3 \quad (11)$$

where k is the spring constant, f is the true resonance frequency, and a is a proportionality constant provided by Veeco. Spring constants measured were similar to those provided by the manufacturer (0.06 N m $^{-1}$).

A Nanoscope III AFM (Digital Instruments), operating in the contact mode, was used to measure interaction forces. Measurements were done at room temperature in a PBS solution. Force–distance curves were taken with a z -displacement of up to 2000 nm, and found to be independent of the scan rate over the frequency range 0.5 – 18.9 Hz, which demonstrates the absence of hydrodynamic artifacts. A scan rate of 1.99 Hz was preferred, however, since this yielded the smallest standard deviation in the parameters measured. 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 were in contact were used to convert the voltage into cantilever deflection. Conversion of deflection into force was carried out as described by Dufrene *et al.* (2001). The approach part of a force–distance curve was fitted to a negative exponential:

$$F(l) = F_0 e^{-\frac{l}{A}} \quad (12)$$

in which F is the measured force at separation distance l , F_0 is the force at zero separation, and A 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 ($E_{\text{repulsion}}$) to be overcome to establish close contact. The retract curve regularly indicated an adhesive force, where its integration yields an adhesive energy (E_{adhesion}). Furthermore, the percentage occurrence of adhesive interactions (% $_{\text{adhesion}}$) was registered.

For each bacterial strain, six cantilevers were prepared, equally divided over three separate bacterial cultures. Fifteen 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 measurements for the six *P. aeruginosa* strains are listed in Table 1. Initial deposition

Table 1. Initial deposition rate, adhesion after 4 h, and percentage detachment of *P. aeruginosa* strains interacting with glass, and the percentage reductions in these values obtained when the strains interacted with PEO-brush-coated glass

Initial deposition rate (j_0) and adhesion after 4 h (n_{4h}) were measured on glass, and the reduction in these values in the presence a PEO-brush coating are given. The percentage detachment in the number of adhering *P. aeruginosa* stimulated by a passing air bubble, as a measure for their retention on glass and PEO-brush-coated glass, is also shown. Bacterial adhesion and retention were determined in a parallel plate flow chamber. Values are means \pm SD for three measurements.

Strain	Glass			PEO-brush-coated glass		
	j_0 (10^2 cm $^{-2}$ s $^{-1}$)	n_{4h} (10^6 cm $^{-2}$)	Detachment (%)	Reduction in j_0 (%)	Reduction in n_{4h} (%)	Detachment (%)
Non-adhesive strains						
6487	18 \pm 4	7.9 \pm 3.2	2 \pm 7	94 \pm 4	95 \pm 4	83 \pm 14
ATCC 19582	10 \pm 3	4.8 \pm 0.5	32 \pm 2	95 \pm 2	98 \pm 2	86 \pm 20
KEI 1025	18 \pm 5	9.6 \pm 4.4	16 \pm 43	97 \pm 2	95 \pm 3	99 \pm 1
Mean	15 \pm 4	7.4 \pm 2.7	17 \pm 17	95 \pm 3	96 \pm 3	89 \pm 12
Adhesive strains						
D1	13 \pm 6	3.6 \pm 1.7	2 \pm 6	55 \pm 4	65 \pm 18	97 \pm 4
6354	6 \pm 2	2.5 \pm 0.3	16 \pm 27	33 \pm 18	72 \pm 9	87 \pm 12
# 3	5 \pm 3	2.5 \pm 1.5	51 \pm 33	63 \pm 9	55 \pm 15	73 \pm 10
Mean	8 \pm 4	2.9 \pm 1.2	23 \pm 22	50 \pm 10	64 \pm 14	86 \pm 9

rates of the bacteria on glass were between 5×10^2 and $18 \times 10^2 \text{ cm}^{-2} \text{ s}^{-1}$, and adhesion after 4 h was between 2.5×10^6 and $9.6 \times 10^6 \text{ cm}^{-2}$. Three bacterial strains showed mean reductions by the brush coating, as compared with glass, of 95 and 96 % in j_0 and n_{4h} , respectively; these strains are denoted as 'non-adhesive' hereafter. The three other strains showed mean reductions of 50 and 64 %, and are denoted as 'adhesive'. Both non-adhesive and adhesive strains showed relatively low mean removal percentages from glass by an air bubble (17 and 23 %), and relatively high mean removal percentages from brush-coated glass (89 and 86 %).

Bacterial characteristics

Table 2 summarizes surface characteristics of all *P. aeruginosa* strains used. For both non-adhesive and adhesive strains, a mean of 14 % of the population were motile, with a mean speed of around $11 \mu\text{m s}^{-1}$. 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 (mean θ_w , 22°), while the adhesive strains were very hydrophobic (mean θ_w , 132°). Furthermore, adhesive strains reduced the surface tension of an aqueous suspension by a mean value of 19 mJ m^{-2} , indicating release of surface-active extracellular substances, while the non-adhesive strains released a minimal amount of these substances, which did not affect the surface tension of their suspension liquid.

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 given in Table 3. All strains were attracted towards glass, with a mean free energy of adhesion (ΔG_{slb}) of $-47 \times 10^{-16} \text{ J}$ for the non-adhesive strains, and $-767 \times 10^{-16} \text{ J}$ 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 were comparable for all strains.

Extended-DLVO theory

In contrast to the thermodynamic approach, the extended-DLVO theory provides the opportunity to incorporate the 3D 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 to stretch into the aqueous surroundings to a thickness of 23.7 nm. Therefore, it was assumed that the Lifshitz–Van der Waals interaction energy between the brush surface and the bacterium was negligibly small, and arose solely from the underlying glass surface. Thus, the Lifshitz–Van der Waals interaction energy at closest approach to the brush was calculated as acting at a separation distance of 23.7 nm from the glass surface. Since it has been suggested that water is strongly immobilized inside a brush, and that zeta potentials reflect the potential at the outside of the brush (Kaper *et al.*, 2003), electrostatic interactions were calculated with respect to the outer brush surface. Similarly, the short-range (Van Oss, 1994) acid–base interactions were also calculated with respect to the outer brush surface. However, the density of acid–base interacting groups is lower in a swollen state than during contact-angle measurements, and this 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 ethylene oxide monomers in the swollen brush (118 nm^3 ; Roosjen *et al.*, 2004), and the dry volume of a PEO chain (16 nm^3 ; Van Krevelen, 1976), 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 mean distance dependence of the interaction energies between non-adhesive or adhesive strains and glass or

Table 2. Percentage of motile bacteria, motility, zeta potential (ζ), water contact angle (θ_w), and change in surface tension ($\Delta\gamma_{lv}$) of suspensions of *P. aeruginosa*

Values are means \pm SD for three measurements.

Strain	Motile bacteria (%)	Motility ($\mu\text{m s}^{-1}$)	ζ (mV)	θ_w ($^\circ$)	$\Delta\gamma_{lv}$ (mJ m^{-2})
Non-adhesive strains					
6487	18 ± 9	13 ± 4	-14 ± 1	24 ± 1	0 ± 2
ATCC 19582	15 ± 8	12 ± 2	-15 ± 3	19 ± 3	-3 ± 3
KEI 1025	10 ± 5	8 ± 3	-12 ± 3	22 ± 3	-3 ± 2
Mean	14 ± 7	11 ± 3	-14 ± 2	22 ± 2	-2 ± 2
Adhesive strains					
D1	19 ± 4	12 ± 2	-12 ± 2	127 ± 16	-18 ± 3
6354	13 ± 3	11 ± 3	-18 ± 3	131 ± 4	-16 ± 4
# 3	9 ± 6	10 ± 4	-11 ± 2	137 ± 9	-22 ± 4
Mean	14 ± 4	11 ± 3	-14 ± 2	132 ± 9	-19 ± 4

Table 3. Upper limits for the free energy of adhesion (10^{-16} J), according to a thermodynamic approach, between glass or PEO-brush-coated glass and the *P. aeruginosa* strains

Lifshitz–Van der Waals (LW) and acid–base (AB) components of free energy were measured at contact (slb interface).

Strain	Glass			PEO-brush-coated glass		
	ΔG_{slb}^{LW}	ΔG_{slb}^{AB}	ΔG_{slb}	ΔG_{slb}^{LW}	ΔG_{slb}^{AB}	ΔG_{slb}
Non-adhesive strains						
6487	-32	-15	-48	-41	352	311
ATCC 19582	-30	9	-21	-38	352	314
KEI 1025	-46	-26	-72	-59	370	311
Mean	-36	-11	-47	-46	358	312
Adhesive strains						
D1	-39	-640	-680	-50	-161	-211
6354	-39	-763	-802	-49	-228	-277
# 3	-30	-790	-820	-38	-257	-295
Mean	-36	-731	-767	-46	-215	-261

PEO-brush-coated glass, calculated as described above, is shown in Fig. 1. Interaction energies at the minimal separation distance are given 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 led to a relatively strong attraction to glass. The non-adhesive strains had a negligible Lifshitz–Van der

Waals interaction energy with the PEO-brush-coated glass, leaving repulsive acid–base and electrostatic contributions, yielding a total repulsive interaction. In contrast, the interaction of the adhesive strains with the PEO-brush-coated glass was dominated by an attractive acid–base interaction, yielding a mean attractive total interaction of -1.1×10^{-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 given 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) was significantly lower than that between the bacteria-coated cantilevers and glass ($P < 0.001$, two-sided Student's *t* test). Furthermore, the adhesive energy upon retract ($-30 \pm 28 \times 10^{-16}$ J) was significantly higher ($P < 0.001$) for a poly-L-lysine-coated cantilever in the absence of bacteria. Thus, it was concluded that the cantilevers were 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 between approach towards glass or brush-coated glass. However, upon retract of non-adhesive strains from a brush-coated

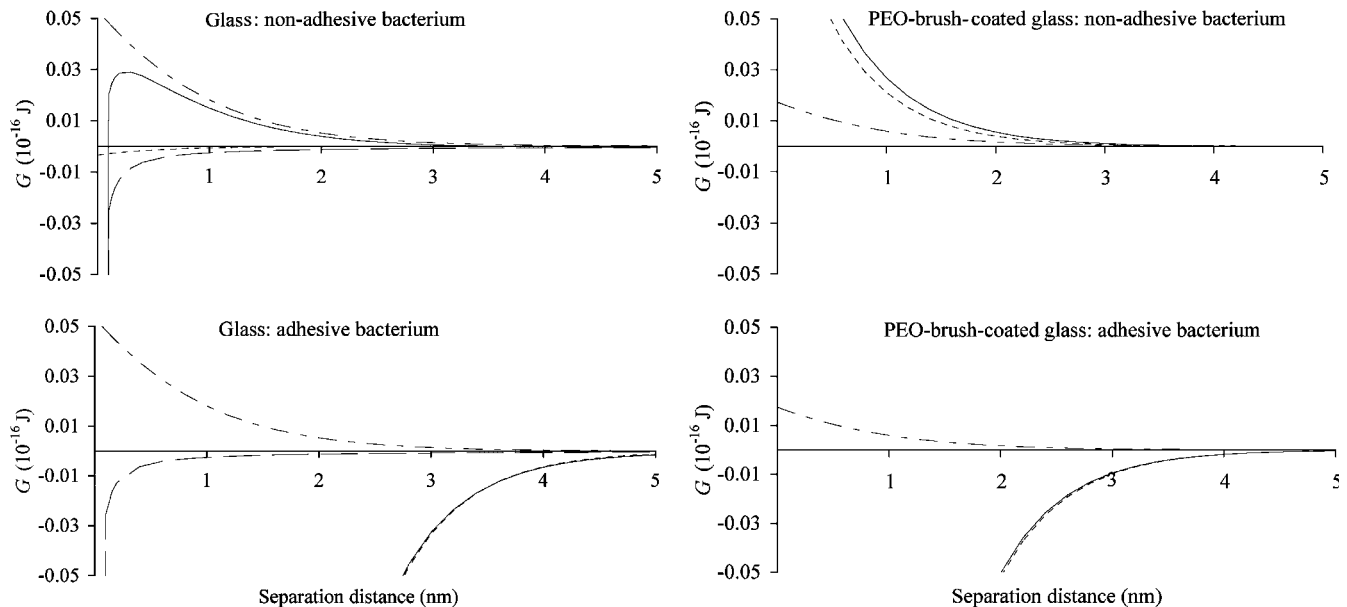


Fig. 1. Interaction energies, according to the extended-DLVO theory, between a representative non-adhesive or adhesive *P. aeruginosa* strain and glass or PEO-brush-coated glass, as a function of separation distance. The $\Delta G^{LW}(l)$ for PEO-brush-coated glass was assumed to originate from the glass and to operate over a distance of 23.7 nm. The interaction energy is represented by the continuous line, while the electrostatic, Lifshitz–Van der Waals and acid–base interaction energies are represented by the dashed–dotted, dashed and dotted lines, respectively.

Table 4. Interaction energies (10^{-16} J) between glass or PEO-brush-coated glass and *P. aeruginosa* strains, according to the extended-DLVO theory, at the minimal separation distance (l_0) of 0.157 nm

Mean values are shown. 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.

Strain	Glass				PEO-brush-coated glass			
	ΔG^{EL}	ΔG^{LW}	ΔG^{AB}	ΔG	ΔG^{EL}	ΔG^{LW}	ΔG^{AB}	ΔG
Non-adhesive strains								
6487	0.047	-0.014	-0.0037	0.029	0.015	-0.00011	0.084	0.099
ATCC 19582	0.050	-0.013	0.0021	0.035	0.016	-0.00010	0.084	0.10
KEI 1025	0.040	-0.021	-0.0062	0.013	0.013	-0.00015	0.089	0.10
Mean	0.046	-0.016	-0.0026	0.027	0.015	-0.00012	0.086	0.10
Adhesive strains								
D1	0.040	-0.018	-3.3	-3.3	0.013	-0.00013	-0.84	-0.83
6354	0.060	-0.017	-4.0	-4.0	0.020	-0.00013	-1.2	-1.2
# 3	0.037	-0.013	-4.1	-4.1	0.012	-0.00010	-1.3	-1.3
Mean	0.046	-0.016	-3.8	-3.8	0.015	-0.00012	-1.1	-1.1

surface, a proportionally lower percentage of force–distance curves demonstrated adhesion than those 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 a mean adhesion energy of -1.5×10^{-16} J, which is significantly more negative than that of the non-adhesive strains ($P < 0.001$). Note that, based on repulsion energy, *P. aeruginosa* D1 seems to fit better in the non-adhesive group, but based on decay length and percentage adhesion, it fits in the adhesive group. Since

the AFM measurements were done on 90 force–distance curves, the mean differences between the adhesive and non-adhesive group are significant, despite the large standard deviation values.

DISCUSSION

PEO brush coatings are supposed to create a barrier between the surface and any approaching bacterium, thus providing a generic anti-adhesive effect (Harris, 1992). In this research, it has been shown that several ‘non-adhesive’ *P. aeruginosa* strains comply with this general rule, as do many other strains and species (Roosjen *et al.*, 2003). However, adhesion of other, ‘adhesive’, *P. aeruginosa* strains was much less suppressed by the brush, although the interaction was

Table 5. Characteristics of the force–distance curves between the *P. aeruginosa* strains on the cantilever and bare glass or PEO-brush-coated glass, as determined by AFM

Repulsive ($E_{\text{repulsion}}$) and adhesive (E_{adhesion}) energies, characteristic decay length (A), and percentage occurrence of adhesive interactions ($\%_{\text{adhesion}}$) were measured. Values are means (\pm SD) for 90 force–distance curves.

Strain	Glass approach curve		Glass retract curve		Brush-coated-glass approach curve		Brush-coated-glass retract curve	
	$E_{\text{repulsion}}$ (10^{-16} J)	A (nm)	E_{adhesion} (10^{-16} J)	$\%_{\text{adhesion}}$	$E_{\text{repulsion}}$ (10^{-16} J)	A (nm)	E_{adhesion} (10^{-16} J)	$\%_{\text{adhesion}}$
Non-adhesive strains								
6487	2.7 \pm 3.2	40 \pm 35	-1.4 \pm 1.4	100	1.2 \pm 1.1	21 \pm 19	-0.2 \pm 0.3	17
ATCC 19582	7.5 \pm 12	56 \pm 73	-9.7 \pm 9.7	71	8.4 \pm 12	74 \pm 72	-0.8 \pm 0.7	29
KEI 1025	1.4 \pm 0.8	40 \pm 45	-0.9 \pm 1.1	100	1.4 \pm 0.8	39 \pm 46	-0.2 \pm 0.3	20
Mean	3.9 \pm 5.3	45 \pm 51	-4.0 \pm 4.1	90	3.7 \pm 4.6	45 \pm 46	-0.4 \pm 0.4	22
Adhesive strains								
D1	5.2 \pm 7.0	146 \pm 211	-3.9 \pm 5.4	75	3.7 \pm 5.1	91 \pm 135	-1.9 \pm 2.5	38
6354	19 \pm 29	301 \pm 375	-1.1 \pm 1.3	33	12 \pm 21	266 \pm 423	-1.8 \pm 2.5	33
# 3	69 \pm 65	565 \pm 260	-0.5 \pm 1.0	17	57 \pm 70	565 \pm 313	-0.8 \pm 1.7	17
Mean	31 \pm 32	337 \pm 282	-1.8 \pm 2.6	42	24 \pm 32	307 \pm 290	-1.5 \pm 2.2	29

relatively weak. Whether or not these strains are able to form a biofilm when allowed to grow remains to be determined, but it is likely that environmentally occurring shear conditions will stimulate their detachment. Another aspect of these weakly interacting organisms is whether, due to the weak interaction and the hydrated nature of the brush, the organisms adhering to the brush will 'realize' that they are indeed on a surface, and commence their battery of phenotypic, and possibly genotypic, changes involved in the transition from adhesion to biofilm growth (Sauer *et al.*, 2002).

Kogure *et al.* (1998) have shown that motility increases adhesion to a bare glass substratum. This has been attributed to the increase in collisions with the substratum surface (Morisaki *et al.*, 1999). Also, a decrease in zeta potential is known to increase adhesion to a bare substratum, and this is caused by reduced electrostatic repulsion (Van Loosdrecht *et al.*, 1987). In this study, motilities and zeta potentials were not distinctive for adhesive and non-adhesive strains, and could therefore not be the reason for the difference in adhesion behaviour. The adhesive strains used in this study released surfactive substances. *P. aeruginosa* strains are known to produce a biosurfactant called rhamnolipid (Lang, 2002), which has a potential role in their adhesion to substratum surfaces. Also, in another study (Wei *et al.*, 2003), adherence of a *Pseudomonas* sp. to a PEO coating has been found, and also attributed to the release of surfactive substances. Furthermore, AFM showed relatively long mean decay lengths (337 nm) with glass for the adhesive strains. Decay lengths of interaction of 10–33 nm between a bacterium and a bare surface are known (Vadillo-Rodriguez *et al.*, 2003); these are in the same range as the non-adhesive strains in the present study (45 nm), and are attributable 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, since the substances of which this layer consists can penetrate the PEO chains.

Results from both the thermodynamic approach and the extended-DLVO theory indicate attractive interaction of the adhesive bacteria with the brush-coated glass, but the values differ by two orders of magnitude, i.e. -211×10^{-16} to -295×10^{-16} J, and -0.8×10^{-16} to -1.3×10^{-16} J, respectively, as can be seen by comparison of Tables 3 and 4. The thermodynamic approach assumes direct contact between the bacterium and the substratum, with the formation of a new bacterium–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 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 to be the most reliable. Indeed, the

values for the interaction energy per bacterium, calculated using the extended-DLVO theory, were in the same range as the adhesive energies derived from direct measurements using AFM, although it may not be evident a priori that the contact between the substratum surface and the bacterial AFM probe involves a single bacterial cell. However, the decay length of the interaction (337 nm for the adhesive strains) was relatively short compared with the bacterial cell dimensions, which made it unlikely that more than one bacterium was involved in force–distance measurements. It is difficult to estimate whether one may expect a perfect match between the extended-DLVO interaction energies and the adhesive energies derived from AFM. Since, in AFM, it is probable that detachment is forced upon the system, elastic stretch energies between polymer chains on the cell surfaces were included in the measurements, and these were not accounted for in the extended-DLVO calculations.

Both the thermodynamic approach and the extended-DLVO theory 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 (Norde, 2003). 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 favourable release of water, and hence to a total attractive interaction free energy. Indeed, the adhesive bacteria used in this research had 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 (Furness *et al.*, 1998), and attraction of PEO to non-polar surfaces has also been demonstrated (Sheth *et al.*, 2000).

The thermodynamic approach and the extended-DLVO theory have been applied here to explain the adhesion of adhesive *P. aeruginosa* strains to PEO brushes in comparison with glass, but these theories do not explain the mechanisms involved in the adhesion of the different strains to glass. This reflects our earlier statement that physicochemistry is less able to explain adhesion of different bacterial strains to the same substratum than it is to explain adhesion of one bacterial strain to different substrata, because not all bacterial variability is included in physicochemical properties (Pratt-Terpstra *et al.*, 1988).

For future practical applications of PEO brushes, it should be mentioned that most bacteria are hydrophilic in nature ($\theta_w < 60^\circ$; Van der Mei *et al.*, 1998), and not all bacteria produce substantial amounts of extracellular substances (Kiers *et al.*, 2001). Furthermore, AFM indicated that, for non-adhesive strains, adhesive interaction was reduced by

the application of a PEO-brush coating, as was also found in a study by Razatos *et al.* (2000). For the adhesive bacteria, adhesion was reduced by 33–63 %, which is comparable to other surface-modification techniques (Nomura *et al.*, 1997; Hogt *et al.*, 1986). Moreover, the energies of adhesion, as calculated using the thermodynamic and extended-DLVO analyses of all strains interacting with a brush coating, were small when compared with their energies of adhesion to a glass surface. Accordingly, both the adhesive and the non-adhesive strains could be readily removed by passing an air bubble. In conclusion, cell surface hydrophobicity and the release of surfactants seem to be the main factors involved in adhesion of *P. aeruginosa* strains to PEO-brush coatings.

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