In situ characterization of bacterial extracellular polymeric substances by AFM

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Abstract

Although the production of extracellular polymeric substances (EPS) has long been recognized as an important factor in bacterial adhesion processes, little is known about the supramolecular organization of EPS adsorbed on solid surfaces. In this paper, atomic force microscopy (AFM) is used to probe, under aqueous conditions, the nanoscale morphology and molecular interactions of polystyrene substrata after adhesion of the Gram-negative bacterium Azospirillum brasilense under different experimental conditions. After cell adhesion under favourable conditions (24 h contact time at 30 °C), topographic images revealed that the substratum surface was covered by a continuous layer of adsorbed substances, ≈ 2 nm thick, from which supramolecular aggregates were protruding. These adsorbed substances, attributed to proteinaceous EPS, were found to cause a significant change of substratum solvation properties: adhesion force mapping performed over 5 × 5 μm areas with a silicon nitride probe showed adhesion forces of 0.8 ± 0.2 nN (n = 1024) magnitude on bare substrata, while forces of only 0.2 ± 0.2 nN magnitude were found after cell adhesion. Finally, when the cell adhesion test was performed under unfavourable conditions (2 h at 30 °C or 24 h at 4 °C), there was no/little indication of the presence of an adsorbed layer and of a change of substratum solvation properties. The correlation between the AFM data and the cell adhesion behaviour provides evidence for the involvement of proteinaceous EPS in the adhesion of A. brasilense to solid surfaces. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Atomic force microscopy; Bacterial adhesion; Biofilms; Extracellular polymeric substances; Proteins

1. Introduction

Bacterial adhesion and biofilm formation processes are encountered both in natural environments and in industrial processes [1]. Their consequences can be either beneficial, such as in biotechnology (wastewater treatment, bioremediation, immobilized cells in reactors), or detrimental, such as in industrial systems (fouling, contamination) and in medicine (accumulation on teeth, implants and prosthetic devices). Various steps are involved in the formation of bacterial biofilms: (i) conditioning of solid substrata by adsorption of (macro)molecules, (ii) transport of the cells towards the substratum surface (diffusion, sedimentation, convection, active transport),

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(iii) physicochemical interactions between bacteria and the substratum, referred to as the initial adhesion step, (iv) production of extracellular polymeric substances (EPS) which may both strengthen adhesion to the substratum and lead to cell–cell attachment, (v) multiplication of the attached cells leading to the formation of microcolonies and biofilms.

The initial adhesion process can be understood in the light of classical physicochemical theories [2]. Long-range van der Waals and electrostatic interactions, on the one hand, and hydrophobic interactions, on the other hand, can be respectively described by the DLVO (Derjaguin, Landau, Verwey and Overbeek) theory of colloid stability and the balance of interfacial free energies involved in the creation of a new cell–substratum interface and in the destruction of the interface of each partner with water.

However, in most cases the surfaces of microbial cells and substrata are not atomically smooth and rigid, as it is assumed in the above approaches, but are coated with lyophilic macromolecules forming loosely structured layers with loops and tails protruding into the solution. This gives rise to macromolecular interactions [3] that can be either attractive (polymer bridging) or repulsive (steric hindrance) depending on the surface coverage and on the affinity of macromolecules for the solvent. The production of EPS by bacteria has been observed by electron microscopy [4,5] but this technique cannot provide information in the hydrated state and often requires complicated preparation procedures. In contrast, atomic force microscopy (AFM) has the ability to image surface morphology in aqueous conditions, without any chemical fixation or drying step. In particular, AFM has recently proved useful in imaging the morphology of bacterial biofilms on solid surfaces. Bremer et al. [6] examined the topography of hydrated bacterial biofilms on a copper surface. Steele et al. [7] used the AFM to observe bacteria and bacterial exopolymers in their hydrated forms in order to elucidate the process of stainless steel corrosion. Beech [8] reported images of biofilms developed either by Pseudomonas species on copper, or by a marine isolate of sulphate-reducing bacterium on stainless steel. Beech et al. [9] showed that AFM allows estimation of the width and height of bacterial cells, of the thickness and width of exopolymeric capsule and bacterial flagella, as well as characterization of substratum roughness, including measurements of depth and diameter of individual corrosion pits. AFM in liquids was used to investigate the effect of iron coatings on the interactions of Shewanella putrefaciens with silica glass surfaces [10]. Corrosion, biofilm formation and the adhesion of different, corrosion-enhancing microorganisms to different surfaces were studied in aqueous environment by AFM [11].

In this study, AFM is used to characterize, under aqueous conditions, the supramolecular organization of bacterial EPS adsorbed onto solid substrata. To this end, we investigate the changes in morphology and molecular interactions of polystyrene substrata after adhesion of the Gram-negative bacterium Azospirillum brasilense, a nitrogen-fixing bacterium living in close association with plant roots. In previous studies, correlations were established between the density of A. brasilense cells adhering onto polystyrene substrata and the protein concentration at the substratum surface detected by X-ray photoelectron spectroscopy (XPS) after cell detachment [12,13]. This lead to the conclusion that extracellular proteins play a key role in the adhesion of A. brasilense to model substrata. However, little is known about the supramolecular organization of these proteins.

2. Materials and methods

The strain A. brasilense Sp7 (ATCC 29,145), kindly supplied by Professor J. Vanderleyden (F.A. Janssens Laboratory of Genetics, Katholieke Universiteit Leuven, Belgium), was kept and restreaked every 3 weeks on fresh plates of Luria-Bertani complex medium [14] supplemented with 2.5 mM CaCl$_2$ and 2.5 mM MgSO$_4$ (LB*). Bacteria were cultivated at 30 °C in standard Erlenmeyer flasks placed on a rotary shaker (100 rpm). Precultures were carried out during 10–12 h in 50-ml flasks containing 10 ml LB*, by inoculating with material collected on several colonies from a fresh stock plate. The cultures
were made in 1-l flasks broth, by inoculating 2 ml of preculture in 200 ml LB**. During the culture, the cell concentration increased from $6 \times 10^8$ to $2 \times 10^9$ cells ml$^{-1}$. Cells were collected 12 h following inoculation, corresponding to the exponential growth phase. They were harvested by centrifugation at 13,400 × g for 10 min, washed three times by resuspension in demineralized water and centrifugation and finally resuspended in water to a cellular concentration of $6 \times 10^8$ cells ml$^{-1}$.

Polystyrene squares of about $1 \times 1$ cm were cut from the bottom part of polystyrene Petri dishes (Merck, Belgium) and attached to steel sample pucks (Digital Instruments, Santa Barbara, CA) using an epoxy glue (Araldite, Sodiema, France). Cell adhesion tests were performed in static conditions as described elsewhere [12]. Briefly, substrata were deposited on the bottom of polystyrene Petri dishes and incubated for 2 or 24 h, at 30 or 4 °C, with 30 ml of a cell suspension. Substrata were then rinsed by gentle agitation (15 s) in three baths of MilliQ water and the wet samples were carefully mounted in the AFM liquid cell while avoiding dewetting. The bottom parts of the polystyrene Petri dishes were observed under the optical microscope to confirm that the cell adhesion densities were similar to those reported earlier [12].

AFM imaging and force–distance measurements were made at room temperature ($\approx 20$ °C) using a commercial optical lever microscope equipped with a liquid cell (Nanoscope III, Digital Instruments, Santa Barbara, CA, USA). Contact mode topographic images were recorded in both height and deflection modes. While height images provided quantitative topographic information, deflection images often revealed finer surface details. The imaging force was kept below 1 nN and the scan rate in the range of 2–4 Hz. Oxide-sharpened microfabricated Si$_3$N$_4$ cantilevers with spring constants ranging from 0.03 to 0.5 N m$^{-1}$ and typical radii of curvature of the probes $\approx 20$ nm were obtained from Park Scientific Instruments (Mountain View, CA, USA). The slope of the retraction force curves in the region where probe and sample are in contact was used to convert the position sensitive detector voltage into cantilever deflection. Adhesion maps were obtained by recording $32 \times 32$ force–distance curves over well-defined areas, calculating the adhesion force for each force curve and displaying adhesion force values as grey levels.

3. Results

Fig. 1A and B show AFM height and deflection images recorded under water for the surface of polystyrene. The root-mean-square surface roughness (Rr.m.s.) determined over 25 μm$^2$ areas was $1.7 \pm 0.4$ nm (SD), indicating that the surface was fairly smooth. Height and deflection images of polystyrene after adhesion of *A. brasilense* under favourable conditions, i.e. 24 h contact time at 30 °C, are shown in Fig. 1C and D. While the Rr.m.s. did not change significantly ($1.9 \pm 0.3$ nm) compared to bare substrata, the images clearly showed dotlike features of $8 \pm 2$ nm height, uniformly distributed across the surface as well as streaks oriented in the scanning direction.

It is worth noting that while optical microscopy observations confirmed that the density of adhering cells was significant and similar to that reported previously (Table 1; for details, see [12]), adhering cells were never observed on the AFM images. Two interpretations may account for this observation. First, we previously showed that after 24 h contact time at 30 °C, the adhesion pattern was heterogeneous: cells occupied only $\approx 10\%$ of the surface in the form of aggregates and the size of uncovered zones was much larger than the cell dimension [13]. Therefore, it is likely that some of the small $5 \times 5$ μm areas examined by AFM were indeed devoid of cells. Second, due to the micrometer size of the cells and to the fairly small cell/substratum contact area, one may expect that any adhering cell will easily be detached from the substratum by the scanning probe.

To evaluate the thickness of the layer of the adsorbed material, a $1 \times 1$ μm image was first recorded at large forces ($\approx 10$ nN) for short period of times, followed by imaging a $2 \times 2$ μm image of the same area under normal load. Fig. 2 shows that imaging at high forces resulted in pushing loosely bound material along the scan-
ning direction, thereby revealing the underlying substratum. The thickness of the layer removed from the $1 \times 1 \, \mu m$ area was measured on vertical cross-sections (Fig. 2A, bottom) and found to be $2.3 \pm 0.3 \, nm$. As a first approximation, it may be considered that the material accumulated on the two sides of this $1 \times 1 \, \mu m$ zone occupies a volume of about $2 \times (5 \, nm \, height \times 200 \, nm \, width \times 1 \, \mu m \, length) = 0.002 \, \mu m^3$. This corresponds roughly to the volume of the removed adsorbed layer, i.e. $2 \, nm \, height \times 1 \times 1 \, \mu m = 0.002 \, \mu m^3$, indicating that dispersion or dissolution of the adsorbed material in the aqueous phase was minimal.

Lowering the contact time (2 h) or the temperature ($4 \, ^\circ C$) was previously shown to cause a dramatic reduction of the adhesion density (Table 1; for details, see [12]). As shown in Fig. 3A and B, no features could be detected at the substratum surface when the contact time was 2 h instead of 24 h. When the test was performed at $4 \, ^\circ C$, instead of $30 \, ^\circ C$, dot-like features were observed at the surface but with a much smaller coverage compared to $30 \, ^\circ C$ (Fig. 3C and D).

In addition to topographic imaging, AFM force–distance curves were used to determine interaction forces acting between the silicon nitride probe and the substratum surface. Fig. 4A presents a typical force–distance curve recorded under water on bare polystyrene. It showed significant adhesion forces, of $0.8 \pm 0.2 \, nN (n = 1024)$ magnitude. In contrast, Fig. 4B shows that the curves obtained for polystyrene after adhesion

![AFM images](image_url)

**Fig. 1.** AFM height (A, C) and deflection (B, D) images ($5 \times 5 \, \mu m$) recorded under water for polystyrene substrata: (A, B) bare polystyrene, (C, D) polystyrene after adhesion of *A. brasilense* cells under favourable conditions (24 h contact time at $30 \, ^\circ C$); $z$-range = 25 nm (A, C). Similar results were obtained in different regions of several independent samples.
Table 1
Summary of data obtained for polystyrene substrata (PS) after adhesion of *A. brasilense* cells under various conditions

<table>
<thead>
<tr>
<th>Substratum</th>
<th>Adhesion density (10^4 cells cm(^{-2})) ± SD</th>
<th>N/C(^a) AFM data</th>
<th>Adsorbed layer</th>
<th>Adhesion force (nN)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare PS</td>
<td>–</td>
<td>0.00</td>
<td>–</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>PS after cell adhesion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h; 30 °C</td>
<td>587 ± 61</td>
<td>0.08</td>
<td>+++</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>2 h; 30 °C</td>
<td>&lt;1</td>
<td>0.01</td>
<td>–</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>24 h; 4 °C</td>
<td>&lt;1</td>
<td>0.03</td>
<td>+</td>
<td>0.8 ± 0.6</td>
</tr>
</tbody>
</table>

Previously published data [12]: adhesion density after rinsing; N/C atomic concentration ratio determined by XPS after detachment of the adhering cells. AFM data (this paper): occurrence of an adsorbed layer, as visualized by topographic imaging; adhesion force, as determined from force-distance curves.

\(^a\) Mean of two independent determinations, the difference between duplicates being smaller than 15%; the N/C ratio was shown to reflect the protein surface concentration [12].

\(^b\) Mean of 1024 measurements, ± SD.

of *A. brasilense* under favourable (24 h contact time at 30 °C) showed either no adhesion or very small adhesion forces (0.2 ± 0.2 nN; \(n = 1024\)). This difference was further illustrated in the adhesion force maps, Fig. 4C and D, characterized by bright and dark levels, respectively. It was also reflected in the resulting adhesion force histograms, Fig. 4E and F. The lack of a significant contrast in the maps as well as the fairly narrow distributions of the histograms indicate that the 1024 adhesion forces probed in different \(x, y\) locations were fairly reproducible, suggesting that the surface properties of the two substrata were homogeneous. Finally, adhesion forces of 0.5 ± 0.3 (\(n = 1024\)) and 0.8 ± 0.6 (\(n = 1024\)) nN magnitude were observed when the contact time was 2 h and when the cell adhesion test was performed at 4 °C, respectively.

4. Discussion

Characterizing the organization of adsorbed macromolecules in situ and at high spatial resolution is an important step towards a molecular understanding of bacterial adhesion phenomena. Using AFM topographic imaging and force–distance curves, we have investigated the morphology and molecular interactions of polystyrene substrata after adhesion of *A. brasilense* under different experimental conditions.

4.1. Nature of adsorbed EPS

AFM topographic images obtained after cell adhesion under favourable conditions (24 h contact time at 30 °C) reveal that substantial amounts of adsorbed material has accumulated at the substratum surface during the course of the adhesion process (Fig. 1). Several pieces of evidence indicate that this material consists of extracellular proteins. First, we previously showed that during the adhesion process, extracellular proteins are released into the solution and adsorb at the substratum surface [12,13]. The high surface protein concentration is reflected by the high N/C ratios detected by XPS (Table 1). Second, the \(\approx 8\) nm height of the dotlike structures observed in the AFM images is roughly consistent with the size of globular proteins; by way of example, the sizes of hydrated lysozyme and bovin serum albumin are 4.5 \(\times\) 3.0 \(\times\) 3.0 and 11.6 \(\times\) 2.7 \(\times\) 2.7 nm, respectively [15]. Third, the streaks oriented in the scanning direction reveal that loosely bound material is being displaced by the scanning probe; the presence of loosely bound material is further demonstrated by imaging at high loads which apparently caused complete displacement of the adsorbed material (Fig. 2). Similar probe-induced
displacements have been reported when imaging various adsorbed proteins by contact mode AFM [16–19]. Taken together, the above observations lead us to believe that the material observed at the substratum surface consists essentially of proteinaceous EPS produced by *A. brasilense* during the course of the adhesion process.

4.2. Supramolecular organization of the adsorbed EPS layer

The question as to whether the dots observed after cell adhesion under favourable conditions reflect individual proteins may be discussed. Contact mode imaging of individual proteins adsorbed under water is known to be difficult due to probe-induced displacement resulting from high lateral and loading forces [16,19]. On the other hand, supramolecular aggregates form more stable structures that can be easily imaged by AFM [16,20]; in particular, collagen adsorbed onto polycarbonate substrata showed similar dotlike features which were attributed to aggregated ends of collagen molecules pointing perpendicular from the surface [20]. We therefore suggest that dotlike structures observed here are supramolecular assemblages of proteins, rather than individual protein molecules. Validation of this interpretation would require further experiments using for instance near-field scanning optical microscopy with fluorescent-probe-labelled proteins.

Several observations indicate that the regions which are not covered with dotlike structures are coated by a continuous layer of adsorbed proteins. First, images recorded after scanning at large forces (Fig. 2) provide direct evidence for the presence of a continuous layer: the measured step height reveals a layer thickness of \( \approx 2 \) nm while the volume of the material accumulated on the sides of the \( 1 \times 1 \) \( \mu \)m area (\( \approx 0.002 \) \( \mu \)m\(^3\)) corresponds to the volume occupied by a \( \approx 2 \) nm thick continuous layer. The ability to perform scratching experiments by applying high forces is thus a valuable approach to evaluate the thickness of adsorbed EPS layers as well as their mechanical stability. Second, adhesion force maps recorded on \( 5 \times 5 \) \( \mu \)m areas together with their corresponding histograms (Fig. 4; for more details, see dis-

![Fig. 2. Scratching experiment. To assess the thickness of the adsorbed layer, a \( 1 \times 1 \) \( \mu \)m image was first recorded at large forces and high rates, followed by imaging \( 2 \times 2 \) \( \mu \)m height (A) and deflection (B) images of the same area under small forces. A cross-section taken along the line indicated by the arrow is shown beneath the height image and reveals a film thickness of \( \approx 2 \) nm.](image-url)
Fig. 3. AFM height (A, C) and deflection (B, D) images (5 × 5 μm) recorded under water for polystyrene substrata after cell adhesion under unfavourable conditions: during 2 h at 30 °C (A, B) and during 24 h at 4 °C (C, D); z-range = 25 nm (A, C).

cussion below) show a decrease of adhesion forces in most locations of the map, suggesting that the protein coverage is continuous on the scale of the probe (≈ 50 nm). It may therefore be concluded that proteinaceous EPS accumulate at the sub-stratum surface in the form of a thin, continuous layer from which supramolecular assemblages are protruding.

The results may be compared with images obtained by scanning electron microscopy (SEM). SEM examination of fixed samples, Fig. 5, showed that cells left in contact with a support for 24 h at 30 °C are surrounded by small particles attributable to EPS [12]. This observation is in contrast with the AFM data indicating a continuous coverage by a ≈ 2 nm thick EPS layer and suggests that reorganization of the adsorbed molecules occurred during the SEM preparation procedure (glutaraldehyde fixation, dehydration). This emphasizes the distinct advantages of AFM over SEM: first, the possibility to probe adsorbed layers directly in the hydrated state; second, the ability to measure critical dimensions on the nanoscale, such as the height of supramolecular structures or the thickness of adsorbed layers.

4.3. Changes of interfacial interactions upon EPS adsorption

AFM force–distance curves recorded between the silicon nitride probe and bare polystyrene reveal adhesion forces of 0.8 ± 0.2 nN magnitude (Fig. 4A). Assuming that the probe and the polystyrene surfaces are respectively hydrophilic
and hydrophobic, these results are consistent with previous measurements between model hydrophilic/hydrophobic surfaces. For instance, adhesion forces of $0.9 \pm 0.4$ nN magnitude were measured under water between probes and model substrata functionalized with alkanethiol self-assembled monolayers terminated respectively with OH and CH$_3$ groups [21].

Strikingly, the curves recorded after the cell adhesion test show a marked reduction in adhesion forces (Fig. 4B). The homogeneous decrease in the brightness of the adhesion map, also reflected in the smaller adhesion force values of the histograms, indicates that this behavior is representative of the whole substratum and therefore that the protein coverage is continuous on the scale of the probe. The reduced adhesion forces may be attributed to a change of solvation interactions at the substratum surface, resulting from the adsorption of the proteinaceous EPS. Dupont-Gillain et al. [22] measured significant adhesion forces between a silicon probe and

Fig. 4. Typical force–distance curves (A, B), adhesion force maps (5 × 5 μm) (C, D) and adhesion force histograms (1024 events over 5 × 5 μm areas) (E, F) recorded under water for bare polystyrene (A, C, E) and for polystyrene after cell adhesion under favourable conditions (B, D, F). Similar results were obtained in different regions of several independent samples.
polystyrene under water while much smaller forces were detected for polystyrene treated by oxygen plasma. This was attributed to a repulsion between the probe and hydrated polymer chains present on the oxidized surface. In this study, similar repulsion may occur between the probe and hydrated proteinaceous moieties. Further work is needed to elucidate to what extent the change of substratum solvation properties affects the cell adhesion behaviour.

4.4. Role of adsorbed EPS in the adhesion process

Table 1 reveals that both the contact time and temperature dramatically affect the cell adhesion density and the protein concentration (N/C ratio) detected at the substratum surface by XPS. These two factors are found to influence the AFM data in the same direction, i.e. (i) lowering the contact time (2 h) or the temperature (4 °C) causes a dramatic reduction of the adhesion density and of the protein surface concentration and (ii) there is no/little evidence for the presence of an adsorbed layer in the AFM topographic images (Fig. 3) and the adhesion forces are not very different from those of bare polystyrene.

The correlation between cell adhesion density, protein concentration and the surface properties of polystyrene substrata after adhesion under different experimental conditions provides a strong indication for the involvement of proteinaceous EPS in the adhesion of *Azospirillum* to solid surfaces. This may be related to previous biochemical or genetic studies. Attachment of *A. brasilense* Cd to sand particles was found to be actively mediated by a network of fibrillar material, presumably proteinaceous [23]. Fibrillar material was also found to anchor the cells to root surfaces [24]. Michiels et al. [25] showed, by the use of protease treatments, that adhesion of *A. brasilense* Sp7 to wheat roots was possibly mediated by proteinaceous compounds. Furthermore, a mutant deficient in both polar and lateral flagella expression failed to adhere to wheat roots. Croes et al. [26] demonstrated that adhesion of *A. brasilense* to wheat roots is mediated by the polar flagellum.

5. Conclusion

This work shows that AFM topographic imaging and force–distance curves can be used to probe the supramolecular organization of bacterial EPS adsorbed on solid substrata and the changes of interfacial interactions associated with the adsorption process. Dramatic changes occur at the surface of polystyrene substrata following the adhesion of *A. brasilense* cells. The surface is covered with adsorbed proteinaceous EPS, in the form a continuous layer from which supramolecular aggregates are protruding. EPS adsorption causes a dramatic change of substratum solvation properties, an effect that may play a role in the cell adhesion process. Changes of experimental conditions (time, temperature) influence the AFM data (occurrence of an adsorbed layer, magnitude of the adhesion force), the adhesion density and the surface protein concentration in the same direction, supporting the idea that proteinaceous EPS play a key role in the adhesion process. An exciting challenge for future research is to measure both the non-specific and specific interactions between single *A. brasilense* cells and AFM
probes modified by proteinaceous EPS, which would provide valuable insight into the mechanisms by which EPS promote bacterial adhesion processes.

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