Review

Title: Atomic Force Microscopy Studies of Bioprocess Engineering Surfaces – Imaging, Interactions and Mechanical Properties Mediating Bacterial Adhesion.

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Abbreviations: **DVLO,** Derjaguin, Landau, Verwey, Overbeek theory; **XDVLO,** Extended Derjaguin, Landau, Verwey, Overbeek theory; **EPS,** Extracellular Polymeric Substance; **LB-EPS,** Loosly Bound Extracellular Polymeric Substance; **TB-EPS,** Tightly Bound Extracellular Polymeric Substance; **SMFS,** Single Molecule Force Spectroscopy; **SCFS,** Single Cell Force Spectroscopy.

Abstract

The detrimental effect of bacterial biofilms on process engineering surfaces is well documented. Thus, interest in the early stages of bacterial biofilm formation; in particular bacterial adhesion and the production of anti-fouling coatings has grown exponentially as a field. During this time, Atomic force microscopy (AFM) has emerged as a critical tool on the evaluation of bacterial adhesion. Due to its versatility AFM offers not only insight into the topographical landscape and mechanical properties of the engineering surfaces, but elucidates, through direct quantification the topographical and biomechnical properties of the foulants The aim of this review is to collate the current research on bacterial adhesion, both theoretical and practical, and outline how AFM as a technique is uniquely equipped to provide further insight into the nanoscale world at the bioprocess engineering surface. .

1 Introduction

Bacterial contamination of water based industrial infrastructure is unavoidable, consequently, the formation of bacterial biofilm on process engineering surfaces is a substantial issue for many industrial processes. A bacterial biofilm consists of a heterogeneous consortium of sessile organisms embedded within a gel like support matrix formed from exopolysaccharides, proteins, and extracellular DNA, typically adhered to a solid support structure. The formation of a biofilm offers bacteria considerable advantages over the planktonic state, including a higher concentration of nutrients and aqueous gasses, increased proximity to other cells, and protection from biocidal, chemical and biological attack. Additionally, biofilm formation is known to result in numerous detrimental effects on process engineering infrastructure, including reduction in the efficacy of cooling towers, heat exchangers, ion exchangers and, filtration membranes, as well as causing a substantial level of end product spoilage. The combined fiscal impact of bacterial bioburden is likely to amount to many millions, if not billions of pounds of lost income due to reductions in process efficiency and repairs. Biofilms are of particular concern as the innate resistance offered by the structure often result in removal being almost impossible, while simultaneously facilitating the re-contamination of any downstream infrastructure that may have been successfully cleaned.

Several control strategies are often employed in an effort to control bacterial biofouling, typically this will involve the use of toxic chemical such as sodium hypochlorite, glutaraldehyde, isothiozoline, and chlorine dosing. However, these strategies are non-specific, damaging to infrastructure and often ineffective in the removal of fully established biofilms. Furthermore, tightening of legislation on the use of antimicrobials has further limited their efficacy, therefore highlighting the need for the implementation of new strategies. As bacterial adhesion marks the initiation of biofilm formation the creation novel anti-adhesion compounds and coatings offers promising solutions to the biofouling problem. Typically, these compounds and coatings alter surface chemistry therefore modifying the strength of binding forces facilitating bacterial adhesion. Hence furthering our understanding of the fundamental bacterial substrate interactions that promote primary adhesion is essential, as the application of such knowledge is paramount in the formation and design of these strategies (1–3). Despite this, development of such technologies is limited primarily due to the lack of understanding of the forces governing theses interactions. Theoretical models predicting the interactions between bacteria and substrates are well established, with comprehensive modeling of the van der Waals, electrostatic and Lewis acid-base interactions described in the extended DVLO (XDVLO) theory (4). Despite the comprehensive nature of this model, discrepancies in the predictions when compared to experimentally derived data exposes a number of substantial flaws (5–7), particularly in the description of the interaction between biological moieties.

Atomic force microscopy (AFM) offer an answer to this issue allowing unique insights into the interaction processes of biological systems. Application of traditional AFM techniques allow for the unparalleled visualization and, characterization of the substrate topography at both the micro and nanometer scale. This has been applied to the characterization of bioprocess engineering surfaces such as stainless steel to measure surface roughness of different finishes pore size determination of separation membranes and study of cellular surfaces (8–10) AFM is not just an imaging device it also has a force measurement capability that has provided novel insight into the interactions of bioprocess engineering surfaces this includes the direct quantification of forces at the microbial surface and nano-mechanics allowing elucidation of the interplay of all of these factors under *in situ* conditions (11,12).

Through the course of this review the authors hopes to highlight the versatility of AFM. Summarize the landmark research that guided the application of the technology in the characterization of substrates, bacterial cells, and the adhesion forces and how these studies, build upon current microbial adhesion models that impact the characterization and optimization of bioprocess engineering surfaces.

2 Basic Principles

AFM is a form of high resolution microscopy from the family of scanning probe microscopies (SPM) developed in the mid-1980s the components of which are detailed in figure 1 (13). AFMs defining features are its resolution, demonstrated to be in the order of fractions of a nanometer, and versatility as a force sensor. AFM operates two major modes; imaging and force spectroscopy, the former can be further divided into two major categories of imaging; contact and intermittent-contact, the basic principles employed during operation remain the same regardless of application. Other AFM imaging techniques have been developed that map properties such as conductance and friction across a surface, however it is contact and intermittent contact mode that have mainly been applied to the study of process engineering surfaces.

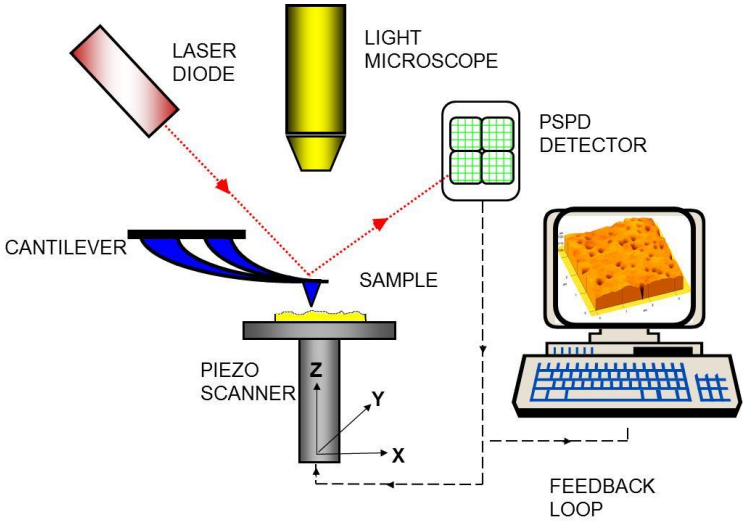


Figure 1 Schematic representation of the atomic force microscope

During measurement AFM records the nanoscale attractive and repulsive forces of the sample material by the deflection of a sharp tipped cantilever, which is systematically scanned across the surface. These forces are measured via the displacement of a laser reflected off the upper gold-coated side of the cantilever onto a quadratic photosensitive photodiode (PSPD). In contact mode, the AFM cantilever is brought into intimate contact with the sample material, the material is then rastered beneath the cantilever and axial and longitudinal deflections in the cantilever recorded via the photodiode. Intimate contact with the sample material is essential, and maintained through the implementation of a DC feedback amplifier controlling a piezoelectric motor to maintain a set level of deflection in the cantilever. This ensures that the force applied to the sample is constant and controlled to prevent sample damage. The forces in the bent cantilever maintain the imaging tip in direct contact with the surface. The destructive forces applied to the sample as a result of imaging can be mitigated through imaging in a liquid media and/or changing to intermittent-contact imaging. Additionally imaging in liquid allows for the removal of the strong attractive forces (capillary forces) on the AFM tip and cantilever caused by the adsorbed water layers at the surface of the sample further reducing the forces applied to the sample due to the tip. Imaging in a liquid media has the additional advantage of allowing for the characterization of sample materials such as membranes and biological contaminant under process- relevant conditions. However, due to the mobility of some bacterial species and the generally weak forces binding the cells to the surface, immobilization of the cells is often required to prevent destruction of the sample (14–16). Comprehensive review of the methods employed in the immobilization of bacterial cells can be found here (17). However, for the purposes of this review a brief summery shall be supplied. Typically immobilization takes one of two form; mechanical, or chemical. Mechanical immobilization traps the cells within an inert matrix for example, gelatin, agar or membranes (14,18–20). This methodology has been

refined to include the use of functionalized surfaces such as lithographically patterned silica and polydimethylsiloxane (PDMS) (15,21–24). Chemical immobilization typically make use of surface chemistry to bind the cell to substrate. The target for immobilization varies and includes surface charge in the case of poly-L-lysine to the crosslinking of carboxyl groups (25–30). While chemical techniques typically result in a high level of immobilization and specificity in orientation care should be taken in selecting an appropriate technique. Chemical fixation, through its very nature alters the surface chemistry of the cell and therefore alters not only the surface properties but also the viability of the cell. Hence, it is critical that an appropriate methodology is selected that takes into consideration the goals of the experimentation.

When imaging of soft samples such as biological samples or filtration membranes is required application of contact mode imaging may not be suitable due to the intimate contact of the sharp imaging tip with the surface, in this case intermittent contact or tapping mode is implemented. During tapping mode, the cantilever is driven into oscillation at a frequency close to that of the cantilevers resonance. The oscillation of the cantilever is then monitored as the sample material is scanned with any alterations in the cantilevers oscillation corrected through the implementation of a direct feedback loop, causing the movement of a piezo-scanner, which is used to produce the image. The change in the oscillation as a function of the movement of the piezo-scanner is then recorded as a topography image. Simultaneously, alteration in the phase angle of the oscillations at the tip of the cantilever and the free oscillation of the cantilever can be recorded. This allows for the characterization of the phase angle across the surface; termed the phase contrast. Phase contrast mapping allows for a qualitative analyses of the differences in the mechanical properties across a surface of heterogeneous material. Tapping mode offers a number of advantages in the imaging of softer materials, most predominantly tapping mode minimizes the destructive lateral forces of the technique on the sample material.

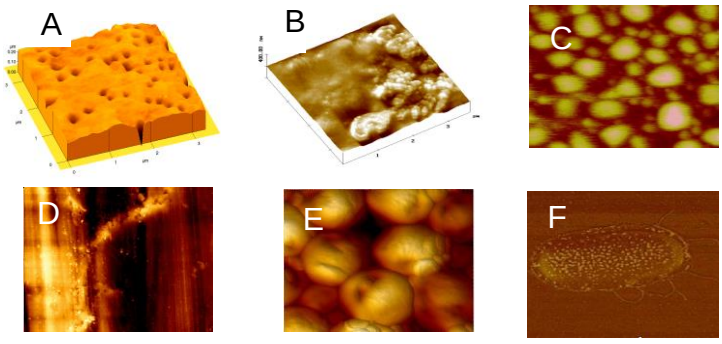


Figure 2. AFM images of bioprocess engineering surfaces (A) Cyclopore microfiltration membrane (3.2µm^2) (B) Humic acid layer fouling an ultrafiltration membrane (3.5µm^2) (C) Air bubbles at a membrane surface (0.75µm^2) (D) Stainless steel process plant surface. (10µm^2).(E) Lawn of *Saccharomyces cerevisiae* cells (brewing yeast NCYC 1681) showing budding scars (15 µm^2) (F) *Shewanella oneidensis* cell showing protein clusters at surface formed during anaerobic respiration and microbial fuel cell operation (1.2µm^2)

Figure 2 shows a number of example AFM images captured in our laboratory outlining the application of AFM in bioprocess engineering. The first three images are at membrane surfaces showing features important to process performance such as pore size and distribution (Figure 2 (A)) Figure 2 (B) shows an ultrafiltration membrane purposely fouled with humic acids; a common foulant of process engineering surfaces consisting of the biproducts of organic matter biodegradation, Figure 2 (C) details bubbles at a membrane surface, which formed under certain operating conditions and are reported to participate in biofouling reduction (31). Finally figure 2 (D) details surface defect in a stainless steel surface as a result of surface polishing. These first four images highlight how AFM is capable of characterisaing a number of surface artifacts associated with fouling. Figure 2 also details AFM images that suggest molecular features of the microbial cell wall, in this particular case protein clusters involved in electron transport under anaerobic conditions (Figure 2 (E)); and yeast budding scars (Figure 2 (F)).

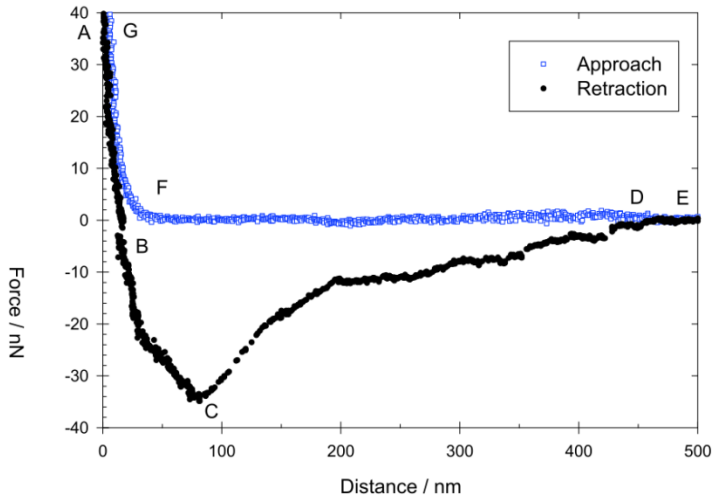


Figure 3. AFM Force spectroscopy of a single stationary phase *S. cerevisiae* cell probe against freshly cleaved mica in 0.01M NaCl pH5

Application of AFM is not limited to the characterization of topographical landscapes, in fact AFMs greatest asset lies in its ability to directly quantify the nanomechanical properties of a material. This is achieved through the generation of force-displacement curves; a graphical representation of the deflection of the cantilever as a function of the tip-sample separation distance as the tip is brought into contact with the sample. Figure 3 shows a typical force-displacement curve, in this case a single *S.cerevisiae* cell probe against(stationary phase) freshly cleaved mica. The force-displacement curve is treated as two curves; the approach

curve (E,D,F,G) and the retraction curve (A-D). At position E the sample and probe are in complete isolation this continues until position F wherein long range electrostatic forces begin to repel the probe. The probe is then pressed further towards the surface by the extension of the piezo scanner, through the electrostatic repulsion experienced at F until contact with the sample surface is achieved. At this point a number of nano-mechanial properties can be defined, such as elastic and plastic deformation regions and the yield point. The bending of the cantilever continues at the constant, defined approach speed until a predefined loading force is experienced (G). Point A defines the start of the retraction curve, the probe is retracted from the surface and any compression in the probe or surface is present to point B. Point C represents the separation of the probe from the sample material, in this case the probe exhibits some adhesion with the substrate as seen through the deflection of the cantilever in the opposite direction culminating in the snapping of the attractive forces at the apex wherein the cantilever returns to a state of zero deflection (E). This characteristic behavior allows for the generation of a number of key variables, for example, at point C the maximum adhesive force can be defined as the maximum deflection of the cantilever. Similarly, the energy of adhesion can also be defined as the area between the approach and retract curves. With regards to biological samples, this area is often large and not as “clean” as non-biological samples. This is for a variety of reasons, including multiple fracture points caused by the fracture of different macromolecules; discussed in depth later in this review, and the tendency for elastic deformation of biological samples. The nanomechanical properties of the substrate can be derived from the contact regions (F-G and A-B) and the adhesive properties from region B-D, however it is worth noting that these variables are dependent on the loading rate of the measurement.

Comparison of force-deflection curves collected from soft biological samples to those taken at samples with significantly higher mechanical properties such as silica can allow for the characterization of nanomechanical properties, such as Young’s modulus and turgor pressure. This is achieved by processing of the curves through a Herztian model, which describes the elastic deformation of two homogenous bodies touching under load, assuming a parabolic indenter and that the cantilever is of significantly greater thickness than that of the indentation depth, the force can be is defined by the equation;

$$F(\delta)_{parabolic} = \frac{4E\sqrt{R}}{3(1-\nu^2)}\delta^{3/2}$$

where R is the radius of the indenter, δ is the indentation depth, E the Young’s modulus and v the poisson ratio. Assuming the prerequisites are met the definition of the indentation depth as a function of the applied force is then possible. However, in reality the first assumption; that the both bodies are homogenous, is often incorrect and in fact the substrate is heterogeneous in nature. Hence, multiple force curves at multiple surface locations this should be considered

when analyzing a homogenous substrate.. Furthermore, the act of nanoindentation is by its very definition a destructive process and as a result consecutive indentation of the same geographical location may result in variation.

It is worth noting that with the interpretation of force curves determination of the contact point is often necessary. This is particularly challenging for biological samples for several reasons. Firstly, there is the definition of contact, wherein contact of any description defines the zero point. This would be easily discernable if both objects were essentially smooth on the given scale of measurement; in this case the nanometer scale. However, this is far from the case and in fact both topographies, especially the biological one are likely to respectively rough due to the presence of surface macromolecules to name one obstruction. Methods for the calculation of the precise contact point have been developed, wherein scattered laser light shone between the colloid and the sample is detected using a near-field scanning optical microscope or inverted light microscope however they are far from simply employed. Secondly, on approaching the sample if the attractive forces exceed the spring constant of the cantilever then the cantilever will snap into contact with the sample, and then further drawn into the sample by the attractive forces. This results in two issues, firstly the contact point is almost impossible to define and secondly it is then necessary to assume that no indentation of the sample has occurred. While this is easily mitigated through selection of a cantilever with an appropriate spring constant it does limit resolution.

3 Bacterial Adhesion and Biofouling

3.1 Mechanisms and Theoretical Models

Surface adhesion is a multistage event, consisting of initial or reversible adhesion followed by permanent or irreversible adhesion. Reversible adhesion defines the most crucial step in adsorption of the cell to the substrate, involving the simple, non-specific interaction of a number of fundamental physical effects including; van der Waals forces, Brownian motion, electrostatic, hydrophobic and acid-base interactions. While irreversible adhesion is more complex as it is the product of the characteristics of the cell, and predominately mediated by polymeric structures on the bacterial surface such as pili, flagella, adhesins and capsule components (slime layer, glycocalyx).

Initially, attempts to model bacterial adhesion focused on the use simple colloidal models such as Derjaguin-Landau-Verwey-Overbeek (DVLO) (16,32,33). However experimental results have typically been shown to correlate poorly to predictions made by this method, as the DVLO theory only considers electrostatic and van der Waals forces (5). This may be a result of the inherent assumptions of DVLO theory, which suggests that pH and ionic strength are the defining features of bacterial adhesion in solution; while ignoring the effect of hydrophobicity, or, the incorrect estimation of key variables (6,7).The result of these discrepancies resulted in the combination of the DLVO theory and surface thermodynamics. While this remediated some

of the discrepancies of the DVLO theory a number of other phenomenon were still unaccounted for, predominantly as a result of the assumptions of thermodynamic theory in which the reaction is assumed to be reversible, and that a new intimate bacterium substrate interface is formed (34–37). van Oss further extended the DVLO theory to include hydrophobic interactions (4). Extended DVLO theory (XDVLO) as it became known, expressed the total adhesion energy of a spherical object; the cell, against a semi-infinite plane; the substrate at a discrete distance to be a result of the sum of the van der Waals, electrostatic and lewis acid-base interactions and is defined by the equation:

$$\Delta G^{TOT}(d) = \Delta G^{LW}(d) + \Delta G^{EL}(d) + \Delta G^{AB}(d)$$

wherein $\Delta G^{LW}(d), \Delta G^{EL}(d)$ and $\Delta G^{AB}(d)$ are the free energies of the Lischitz-van der Waals, electrostatic and Lewis acid-base interactions respectively at a given distance. At a discrete distance d, $\Delta G^{LW}(d), \Delta G^{EL}(d)$ and $\Delta G^{AB}(d)$ can be defined as:

$$\Delta G^{LW}(d) = -\frac{A}{6}\left[\frac{a}{d} + \frac{a}{d+2a} + ln\left(\frac{d}{d+2a}\right)\right]$$

$$\Delta G^{EL}(d) = \pi\varepsilon a(\zeta_1^2 + \zeta_2^2)\left[\frac{2\zeta_1\zeta_2}{\zeta_1^2+\zeta_2^2} ln\frac{1+exp(-\kappa d)}{1+exp(-\kappa d)} + In\{1 - exp\ (-2\kappa d)\}\right]$$

$$\Delta G^{AB}(d) = 2\pi a\lambda\Delta G_{adh}^{AB}\exp\left[\frac{(d_0 - d)}{\lambda}\right]$$

wherein a is the radius of the sphere, A is the Haymaker constant and defined as:

$$A = -12\pi d_0^2 \Delta G_{adh}^{LW}$$

ζ is the zeta potential, ε is the permittivity of the medium K is the electric double layer thickness, λ is the correlation length if molecules in a liquid medium and d_0 is the closest separation distance between the sphere and plane. Finally, ΔG_{adh}^{LW} and ΔG_{adh}^{AB} are the free energies of adhesion for the Lischitz-van der Waals, and Lewis acid-base interactions as defined by the LW-AB approach. Through the implementation of this model a series of separation distance against free energy graphs can be created theorizing the interactions between the cell and the substrate. Through interpretation of these graphs maximum and minimum energy requirements can be deduced for a given separation distance and hence favorability of the adhesion event. However, while the theory proves to be a powerful tool in the modeling of the initial stages of bacterial adhesion the model still fails to take into consideration the biological aspect of the interaction such as the effects due to pilli, flagella and fails to account for non-spherical cell shapes.. AFM offers a unique opportunity to remedy these issues. Firstly, through the application of force spectroscopy; to be discussed in the section 4, AFM allows for the direct quantification and comparison of experimentally derived approach curves and the theoretical separation distance against free energy curves predicted by these models. Secondly, through the characterization of the folding/unfolding pathways of surface macromolecules and the use of non-spherical cell probes further refinement of the model through the implementation of a fourth biological factor in the XDVLO theory may be possible. Consequently, the implementation of a

more rigorous theoretical framework will be possible for predicting and preventing biofouling at bioprocess engineering surfaces

3.2 Substratum Characteristics

3.2.1 Surface Roughness

The effect of substratum characteristics on the adhesion of bacteria has been a point of interest ever since the discovery of the detrimental effects of biofilms, with both surface roughness and nanomechanical properties being implicated with increasing retention of bacteria [31]. However, due to its complex nature elucidating the complete mechanism of bacterial attachment and biofouling of bioprocess surfaces remains a challenge. As shown by the XDVLO theory the physiochemical interactions are understood to a degree although not comprehensively; the impact of surface topography on the adhesive characteristics remains less well defined. To begin to elucidate the mechanism by which bacterial adhesion is influenced by surface roughness a method for quantifying surface roughness is required. A number of techniques are available to researchers to accomplish this such as stylus and optical type profilometers and dynamic contact angle. These techniques offer a number of advances such as being relatively simple to implement and not particularly resource intensive, but their resolution is limited to the microscale and offer no further insight into the substrate characteristics. As a result, more researchers are applying AFM to the characterization of such surfaces. AFM offers a number of key features not achievable through other means, most notably its ability to resolve the surface roughness at the nanometer scale: a scale that has grown in interest over the last few years (38,39).

Several studies have been completed detailing the effect of surface roughness on bacterial adhesion (10-18). The consensus of the aforementioned studies being that on the micro-scale, bacterial adhesion is at its peak when the arithmetic roughness (R_a) of the surface nears the diameter of the cell. This correlation between surface adhesion and surface roughness has been attributed to a number of factors, including the maximization of surface contact area, the accumulation of organic contaminants and protection from sheer stresses (40–42). Consequently, modification of substrate R_a has become a primary focus in the formation of antifouling strategies.

Interest in minimizing the surface roughness of substrate materials has resulted in a growth in the characterization and application of nanoscale topographies (43–47). However, nanoscale interactions have proved to be more complex with a number of fundamental contradictions arising (48–54). For example, a significant increase in the adhesion of *Escherichia coli* and *Pseudomonas aeruginosa* on nanorough thin film titanium substrates was observed in comparison to a flat reference samples(46). The authors attribute this to the physical stimulus of the nanotextured surface. However, the authors proceed to describe the absence of flagella on *P. aeruginosa* cells bound to same surface, suggesting changes in cell surface characteristics. Conversely, in direct opposition to the previously mentioned study Ivanova *et al.* reported that

on comparable titanium surfaces adhesion of *P aeruginosa* and *S. aereus* were inversely proportional to the roughness of the surface (43,47). However it is worth noting that a number of the studies performed on titanium contain quantities of TiO_2, a known antibacterial. It is therefore difficult to distinguish alterations in adhesion caused by the nanoscale roughness of the surface as opposed to those caused by the antimicrobial activity.

In a more recent study the effect of nanotapography and grain size, on the adhesion of *E. coli, P. aeruginosa, S. epidermidus* and *S. aureus* was observed on shot peened SS316L stainless steel (55). During the study the authors suggest that increases in the nanoscale roughness of material notably increased the adhesion rate of both *S. aureus* and *S. epidermidus* while having no significant effect on the adhesion rate of the two Gram negative species. The research group were additionally able to conclude that the refinement of the grain size, as a result of shot peening did not significantly alter adhesion rates.

3.2.3 Conditioning Layers

Adsorption of organic matter to surfaces in aqueous environments and the corresponding impact on bacterial adhesion rates has been well documented (56–62). A major contributing factor to the formation of an organic conditioning layer is the deposition of extracellular polymeric substance (EPS). EPS is not a defined mixture but rather the collective name given to a number of soluble macromolecules produced by bacteria. EPS has been demonstrated to consist of a temporally dynamic mix of polysaccharides, DNA, lipids, proteins and humic substances (63–66). EPS can be loosely grouped into two forms when viewed with regards to planktonic cells, loosely bound (LB-EPS) and tightly bound (TB-EPS), both varying in composition and the former being the primary form of EPS conditioning layers and composed primarily of bacterial proteins and the latter bacterial capsids (67,68). While the formation of organic conditioning layers effects all surfaces within a process engineering environment, by far the most researched area is in the organic fouling of membranes (69–71).

Application of membrane separation technologies has become a substantial part of a number of industrial processes including membrane bioreactors, desalination plants and food processing. However, with all sizes of membrane; microfiltration, ultrafiltration, nanofiltration and reverse osmosis (RO), biofouling is of particular concern as colonization of the membrane will typically result in reductions in membrane separation efficiency and eventually biodeteriation of the separation membrane surface and surrounding infrastructure (72).

In a recent study, Suwarno *et al.,* characterized the effects of a number of conditioning layer contaminants on bacterial adhesion of RO membranes (73). During the study, the researchers observed no trend in organic fouling and surface roughness with an increase in surface roughness of 19nm when the membrane was fouled with alginic acid, this was compared to a 21nm and 15nm decrease in surface roughness for a bovine serum albumin fouled membrane and membrane bioreactor permeate fouled membrane, respectively. Suwarno *et al.,* continued to use AFM to characterize the adhesive properties of the membranes, during the force

measurement study it was found that the adhesive force exhibited at the membrane surface was significantly increased from the baseline when exposed to the model foulants; alginic acid and bovine serum albumin [55]. However, the change in adhesion was less pronounced with contamination of membrane bioreactor permeate; an increase of 110nN was observed. The group attributed this to be a result of non-uniform distribution of the foulant material. The research identified an increase in bacterial adhesion as a result of the biopolymer fouling. Bacterial adhesion to the membrane bioreactor permeate fouled membranes was found to be in the order of four times higher than that of the virgin membranes indicating that these changes in surface roughness and adhesion correlate to an increase in bacterial retention.

3.3 Force Spectroscopy

Implementation of AFM for the characterization of bacterial nano-mechanics has been widely accepted as an essential tool in the microbiological community. Eager adoption of AFM by the community is the result of the ability of AFM to resolve the nano-mechanical properties of cells on all levels, from single molecules to consortia of multiple cells such as biofilms in the nano and picoNewton range (74). Understanding the nano-mechanics of bacterial cells with regards to the cellular capsid, elastic modulus and turgor pressure is of particular importance in the further refinement of the understanding of reversible attachment. While, characterization of membrane bound polyproteins, adhesins and cellular organelles will further understanding of the mechanisms of irreversible attachment.

3.3.1 Single Molecule Force Spectroscopy (SMFS)

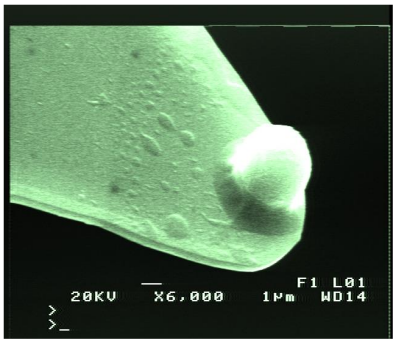
Surface macromolecules are essential for the ubiquitous successe of microbes within the environment, mediating a number of physiological processes including adhesion. Chemical functionalization of the cantilever has allowed AFM to be applied in the characterisation of a number of surface bound receptors important in adhesion under *in situ* conditions including lipopolysaccharides, pilli and adhesins (75–82). The study of surface macromolecules has facilitated the rapid development of SMFS techniques, and ensured a comprehensive understanding of the fundamental mechanics governing tip-molecules interactions (83–85).

Microbial surface proteins can be generally broken down into two groups: functional surface proteins such as adhesins and surface bound long chain macromolecules such as pili. AFM characterisation of long chain surface macromolecules is complex. However, through the use of established models such as the Worm Like Chain (WLC) and Freely Jointed Chain (FJC) models a wealth of information on adhesion can be ascertained. Use of the WLC and FJC models allow for the definition of the entropic elasticity of the molecules and as the result the contour length (L_o) (86,87). If the structure of the target macromolecule is unknown, definition of L_o allows for the confirmation of the experimentally derived data through the use of a normal (Gaussian) distribution, while simultaneously partially elucidating the unbinding pathway (88,89). This method for the definition of L_o was further expanded through the work of Farrance *et al.,* wherein an idealized theoretical tethered surfaces and the probability of two such

surfaces contacting is used to predict the distribution of experimentally derived data (90). In a recent example, SMFS was applied to demonstrate the role of *P. aeruginosa* type IV pili (91). During the study the group untilised a *pilT* mutant to demonstrate low-affinity, high-avidity model for type IV pili adhesion through the application of the WLC model. The group concluded that each subunit of PilA contained a adhesintope capable of binding the surface allowing adhesion along the full length of the molecule.

3.3.2 Single Cell Force Spectroscopy

Pioneered through the construction of a *S. cerevisiae* cell probes by Bowen *et al.,* single-cell force spectroscopy (SCFM) has become a fundamental technique in adhesive force characterization (92). This technique is of particular importance in the field of bacterial adhesion as it allows for the simultaneous quantification of all factors governing adhesion under *in situ* conditions, therefore reducing error as the result of the interpretation of discrete elements. Selection of an appropriate immobilization technique is imperative in the construction of an AFM cell probe (Figure 4) . As a result a number of techniques have been utilized to functionalize cantilevers including chemical fixation, electrostatic and wet adhesives (93–100). With the optimum method allowing for the immobilization of the target cell with minimal effect of the technique of the viability, and physiochemical properties of the immobilized cell. Further consideration needs to be taken in to the size of the target cell, with larger cells; such as *S cerivisae* (~10µm) being easily immobilized directly to the cantilever surface. While immobilization of smaller cells and spores; such as bacteria (~1µm) require further steps. In one such method to immobilize a single bacterial cell, a colloidal sphere was immobilized onto the tip a AFM cantilever. This colloid was then further functionalized with polydopamine to facilitate the immobilization of a single bacterium through AFM micromanipulation (101). This technique offered a number of significant advantages over comparable techniques for the immobilization of single cells. Firstly, through the use of confocal microscopy the technique was shown to immobilize bacterial cells with minimal effect of the target cells membrane integrity, and as a result the viability of that cell. Additionally, the technique allows for the immobilization of the target cell in a highly organized manner further increasing the repeatability of the measurements. Finally the technique was found to be applicable to a number of target species (99).



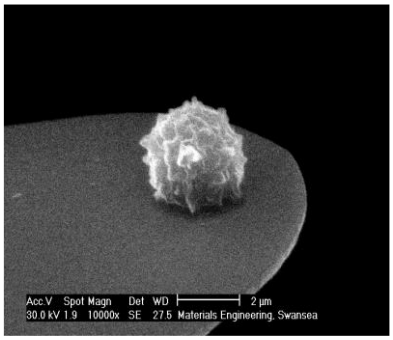


Figure 4. SEM image of a cell probe A) *Saccharomyces cerevisiae* B) *Aspergillus niger*

In a recent study, SCFS was applied to characterize the effect of antifouling polymer brushes on the adhesion of *Yersinia pseudotuberculosis* (102). During the study the group compared the adhesive forces, energy of adhesions and the rupture distance of the brushes against polytetrafluoroethylene (PTFE), polystyrene and glass controls. It was found that the adhesive forces exhibited by the bacteria where sustainably reduced, with a 22% reduction observed for the oligo(ethyleneglycol) methyl ether methacrylate (MeOEGMA) and oligo(ethyleneglycol) methacrylate (HOEGMA) brushes and no less than a 95% reduction for the *N*-(2-hydroxypropyl) methacrylamide (poly(HPMA)), (3-acryloylamino-propyl)- (2-carboxyethyl)-dimethyl-ammonium (CBAA), [2-(methacryloyloxy)ethyl]- dimethyl-(3-sulfopropyl)ammonium hydroxide (SBMA), 2-methacryloyloxyethyl phosphorylcholine (PCMA), and, Poly(2-hydroxyethyl methacrylate) (poly-(HEMA)) brushes. Similarly the energy of adhesion showed a significant decrease from 450, 550 and 7000aJ for the glass, PTFE and PS respectively to 100aJ for the oligo(ethylene glycol) methactylates and 10aJ for the polyzwitterioic and poly(HPMA) brushes. Further analyses of the force-distance curves highlighted a decrease in the number of rupture events with a corresponding increase in the event distance. A further comprehensive study conducted by Aguayo *et al.,* investigated the impact of nanopatterned polycarbonate on *S. aureus* adhesion (103). During the study the group observed an increase in both the adhesive force and energy of adhesion with increasing contact time and nanopatterning. Interestingly, the number and location of rupture events exhibited remained relatively constant, the researchers suggested that the surface and/or capsular receptors were involved in adhesion to both substrates

4 Conclusions

In conclusion, AFM has become an essential tool for the study of bioprocess engineering surfaces. This is exemplified by the comprehensive insight it has provided into the nanoscale forces involved in bacterial adhesion and their mechanical properties. Through the characterization of nanoscale surface topography AFM has allowed for the elucidation of the effect of surface roughness on bacterial adhesion rates adding further experimental evidence to the predictions of adhesion models and in turn informing potential anti-fouling strategies. Studies into microbial conditioning have demonstrated the heterogeneity of the conditioning layer while demonstrating the importance of this layer in bacterial adhesion and alluding to potential methods to prevent, remove or mitigate the effect. Conversely AFM has been used to demonstrate the efficacy of anti-fouling layers. Through the functionalisation of probes attached to AFM cantilevers, SMFS has revolutionised our understanding of the complex interplay between microbial surface macromolecules under *in situ* conditions providing valuable insight

into new strategies of preventing the initiation of irreversible attachment. SCFM has now become a mainstay in microbial applications of AFM, and the study of microbial colonisation of bioprocess engineering surfaces. SCFM studies has demonstrated the efficacy of a number of antifouling strategies while facilitating whole cell studies to further future modelling. AFM is not without its limitations and most applications of the technology still require the immobilisation of the cells prior to measurement, potentially influencing the validity of the measurement. Despite this AFM is still an essential addition to the family of instruments used in the characterization of bioprocess engineering surfaces and bacteria. The technology promises further analytical capabilities for the study of microbial systems with the only limitation being the imagination needed to invent more ingenious functionalised cantilever probes.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure legends

Figure 1. Schematic representation of the Atomic Force Microscope.

Figure 2. AFM images of bioprocess engineering surfaces (A) Cyclopore microfiltration membrane (3.2µm^2) (B) Humic acid layer fouling an ultrafiltration membrane (3.5µm^2) (C) Air bubbles at a membrane surface (0.75µm^2) (D) Stainless steel process plant surface. (10µm^2).(E) Lawn of *Saccharomyces cerevisiae* cells (brewing yeast NCYC 1681) showing

budding scars (15 μm^2) (F) *Shewanella oneidensis* cell showing protein clusters at surface formed during anaerobic respiration and microbial fuel cell operation (1.2μm^2)

Figure 3. AFM Force spectroscopy curve of a single *S.cerevisiae* cell (stationary phase) with freshly cleaved mica in 10-2 M NaCl pH 5.0.

Figure 4. SEM image of a cell probe A) *Saccharomyces cerevisiae* B) *Aspergillus niger*

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