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REVIEWS

Characterizing Bacteria Adhesion to Substrate and Early Biofilm Formation Using Atomic Force Microscopy: A Review

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Abstract | Bacterial adherence has received continued interest, as it is the most important and crucial step in the development of a biofilm. Bacterial interactions with various surfaces are characterized by examining their morphology and physico-chemical parameters. Atomic force microscopy (AFM) is an attractive tool that not only provides high-resolution images at nanometric range but also provides information on the interaction forces. AFM with functionalized probes can be used to measure interaction forces as small as a few picoNewtons. This review describes the aspects of initial biofilm formation i.e., bacterial adhesion and how AFM can be used to study the interaction forces.

1 Introduction

Microorganisms are ubiquitously present as biofilms in nature. A biofilm is a community of microorganisms irreversibly attached to a surface and enclosed in self-produced extracellular polymeric substances (EPS). The resident bacteria in a biofilm exhibits an altered phenotype compared to corresponding planktonic cells.[1] The polymeric matrix of biofilms serves as a barrier as well as a storage facility for nutrients and minerals.[2] Quorum sensing helps the bacteria within the biofilms to synchronize target gene expression and function in a co-coordinated manner with certain biological activity; in some ways—mimicking multicellular organisms.[3] The first step in the formation of a biofilm is the adhesion of bacteria to conditioned surfaces either live or inert. The molecular and physical interactions that govern the bacterial adhesion to biomaterials depend on the surface properties of bacteria and biomaterial, as well as the associated fluid flow conditions. Generally, the bacterial adhesion to a substrate is said to be a two-phase process, (1) initial and reversible physical phase followed by (2) a time-dependent and irreversible molecular phase.[4] In phase one, bacteria move to the substratum surface by Brownian motion, van der Waals attraction forces, gravitational forces and the effect of surface electrostatic charge and hydrophobic interactions.[5] The attachment of a bacterial cell on to a substratum is the most important and crucial step in the development of a biofilm. Thus many studies have focused on these two specific phases of attachment of bacterial cells to various surfaces.

Bacterial interactions with various surfaces are characterized by examining their morphology and physico-chemical parameters. Electron microscopic methods require extensive sample preparation that could result in alteration to the surface morphology. These methods are used to determine cell surface physico-chemical properties such as zeta potential, contact angle, etc. Though these properties are useful to model the bacteria substrate interaction, it is important to realize that they yield averaged data from many cells. With the invention of atomic force microscopy (AFM) and its significant improvements over the last two decades, it is possible to image as well as determine the forces of interaction between bacteria and substrate at nano-levels without significant sample preparation.[6] AFM allows biological samples to be imaged in three-dimensional topographic views in their native environment (Figures 1 and 2). In addition, AFM could detect the smallest practical force range from ≈5 pN.[7,8] This review will provide the basic information on the phases of bacterial adhesion, bacterial surface interaction

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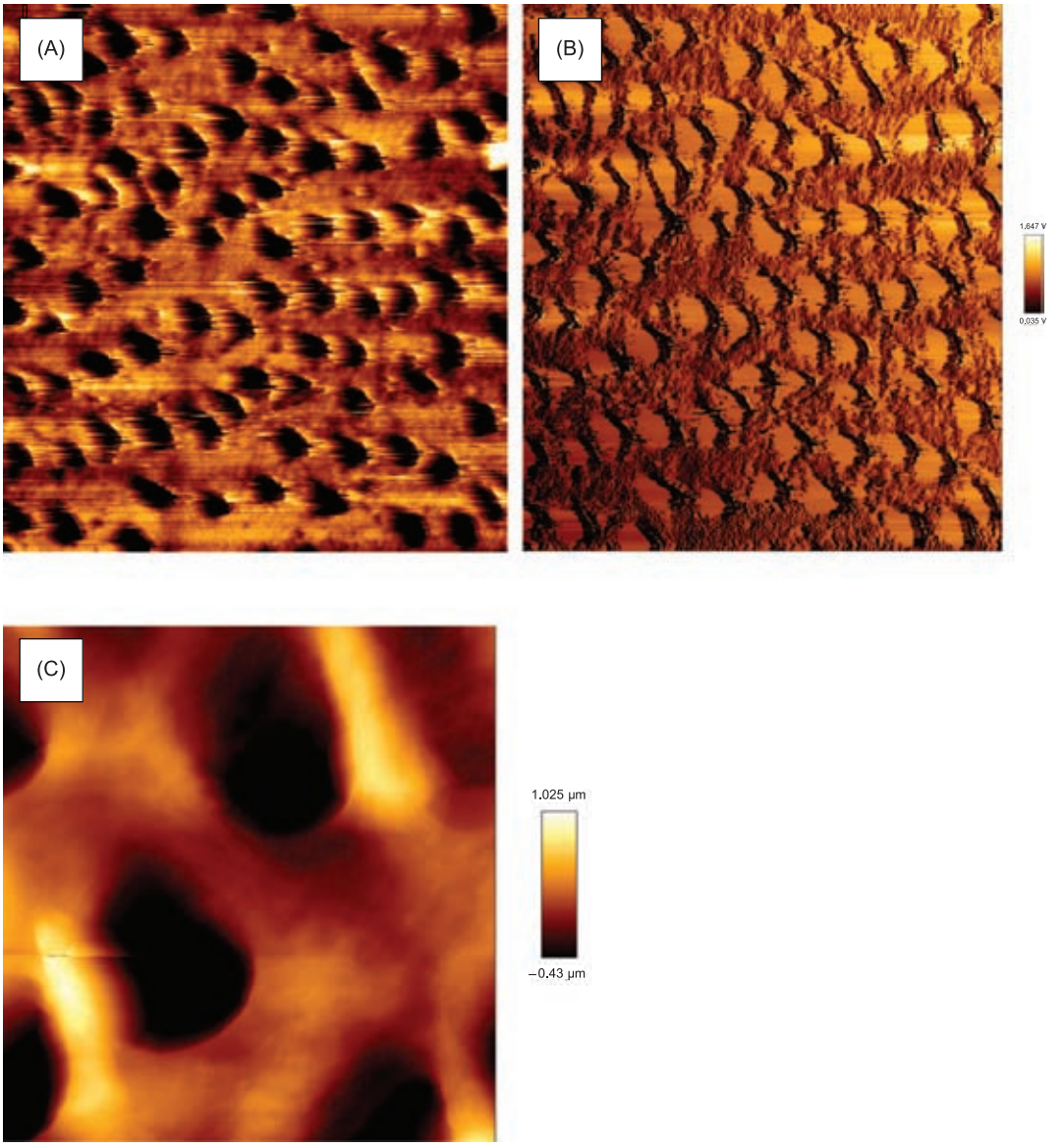

Figure 1: AFM images of the dentin surface (adjacent to the root canal). (A) Shows a three dimensional surface topography (height) image from a 50 µ × 50 µ window (B) shows a three dimensional surface topography (amplitude) image from a 50 µ × 50 µ window and (C) shows a three dimensional surface topography (height) image from a 10 µ × 10 µ window.

and how AFM can be used to measure these interaction forces.

2 Bacterial-Surface Interaction

Microbial cell surface properties regulate the interfacial phenomena such as bacterial adhesion, biomineralization, and biofilm formation. Surface charges are inherently important for bacteria adhesion to a substrate. The net surface charge on bacterial cells has been reported to be negative.[9] The value of the surface charge is dependent on the species, as surface chemical groups in differing species of bacteria are different. In addition, as phenotypic expression in bacteria may be affected by culture media, nutrients and age, the surface charge would also be dependent on these variables.[10] Electric double layers arise because the net charge of a particle affects the distribution of ions in the media in the immediate interfacial region. If a particle (example: a bacterium) is negatively charged, it would induce positively charged ions to move on to its surface. The immediate layer of induced charges are tightly bound to the particle forming the Stern layer, whereas those slightly further away are more diffused and less firmly attached. Within this there is an imaginary boundary called the slipping plane,

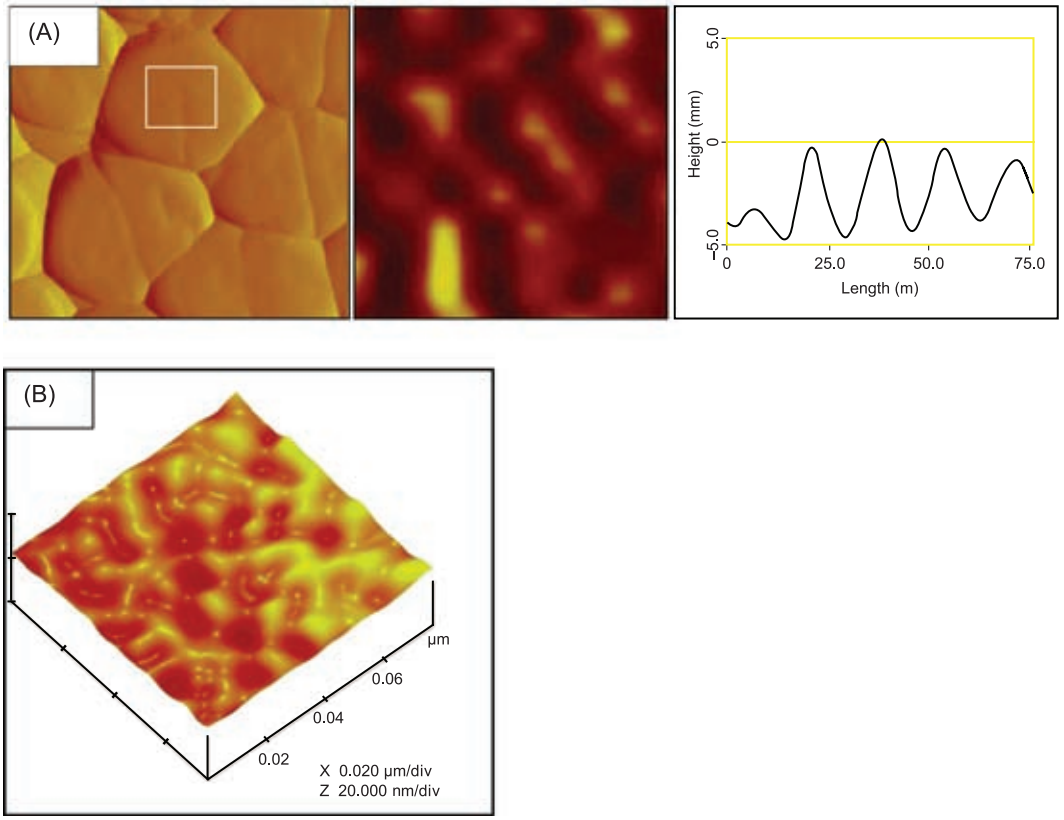

Figure 2: (A) The AFM surface profile of *Enterococcus faecalis* cells. The image of the entire bacterial cell is given in left figure ($2 \times 2\ \mu$m) and the boxed area shows the region zoomed to analyze the profile (middle figure). The quantitative measurement of the surface roughness, taken by 'cursor profile' is shown in the right figure. (B) The three dimensional topographic image (height) of *E. faecalis* cells.

where the ZETA potential is determined.[11] The surface charge of bacteria is a complex problem, as there are gel-like structures on the outer surface of the cell with ionogenic groups. As the ionic strength in the suspending medium is reduced, the ionic atmosphere of the bacterium increases. Ionic groups that lie further from the shear plane will not possess counterions.[12] Whilst it is logical to believe that long-range electrostatic forces can influence the initial phase of bacterial adhesion, there are studies that show that adhesion is unaffected by these long-range electrostatic forces. Harkes *et al.* found that there was no correlation between the zeta potentials and contact angles of the bacterium with the adhesion values found.[13]

There are few studies relating to ZETA potential and adhesion of bacteria to mineralized structures and dental tissues. Weerkamp *et al.*[14] studied the effect of ZETA potential on bacterial adhesion to saliva coated/uncoated enamel. They found that there was great variability in adhesion of various bacteria to the same surface. Olsson *et al.*[15] studied the interference of bacteria adhesion on hydroxyapatite treated with alkyl phosphates and nonionic surfactants. It was reported that hydroxyapatite treated by alkyl phosphates reduced the ZETA potential to highly negative. On the other hand, when the mixture of alkyl phosphate and nonionic surfactant was used, it reduced the ZETA potential to near zero. This scenario may reduce the propensity of bacteria adhesion to hydroxyapatite. Another study examined the relationship between ZETA potential and cariogenic potential of seven species of acidogenic streptococcal bacteria. The authors reported that few species of bacteria that were more cariogenic, besides being more acidogenic, were all having smaller negative ZETA potential, making them highly adherent on dental tissues, which were naturally negatively charged.[16] Hence, ZETA potential has a direct bearing on the adhesion of bacteria to mineralized structures and dental tissues.

In a solution, a bacterium is in constant Brownian motion. It was noted by Zobell in 1943 that bacteria prefer to grow on available surfaces, rather than remain in the aqueous phase.[17] The adhesion

of a bacterium to a surface depends first on its transport to the vicinity of the substrate, followed by the attachment to the substrate and then followed by the development of molecular interactions with the substrate to desist dislodgement.[18] Hence, these events can be thought of as occurring in two phases, an initial instantaneous reversible phase or the physicochemical phase also called phase 1, and a time dependent irreversible molecular and cellular interaction phase also called phase 2.[4,19]

2.1 *Reversible adhesion of bacterium to a substrate*

The forces involved in the initial reversible bacteria-substrate interaction can be classified into long and short-range forces. At a separation distance of >50 nm, only macroscopic cell surface properties play the dominant role in adhesion. Long range van der Waals forces are important in this case. At distances ranging 10–20 nm, both van der Waals and electrostatic charges occur, while at distances <15 nm, specific molecular force in addition to electrostatic and van der Waals forces participate to adhere the bacteria to the substrate. Specific interactions are those that occur between stereochemically complementary surface components, allowing ionic, hydrogen and other chemical bonds. On the other hand, non-specific interactions are regulated by the overall surface properties such as charge and surface free energy. In addition, specific interaction is also influenced by specific metabolic processes and subsequent secretion of substances on cell surface.[20] Cell surface appendages will also contribute to the process of adhesion. When the cell body is very close (10–20 nm range), many appendages are longer than the range of interactive forces and will be able to literally bridge the gap. Interestingly, even when the bacteria appear to be in contact, there is likely to be a thin vicinal layer of water between the cell and the substratum. If bacteria come very close to the substratum so as to squeeze out the thin film of water, the interaction between the cell and the substratum are likely to be hydrophobic in nature. In this case, the area in direct contact is likely to be very small. However, both types of contact may be present at the same time. It must also be realized that a decrease in bacterial concentration in suspension by a factor of ten can desorb adhering bacteria from a solid surface.[21]

2.2 *Irreversible adhesion of bacterium to a substrate*

In this phase, molecular specific interactions between the bacterium and the substratum surface become predominant. The attachment is likely to be firmer and many of the adhesion molecules of the bacteria are located on or near to appendages.[22,23] These appendages include fimbriae, pilli, capsule amongst others, are likely to participate in the interaction. To this end, most molecules responsible for the irreversible adhesion are found on the cell wall. Examples of which are most microbial-surface-component-recognition-adhesive macromolecules (MSCRAMM) on Gram-positive pathogens. These MSCRAMMs contain an N-terminal signal peptide followed by a non-repetitive region called the A region, which in most cases, is responsible for ligand binding complex.[24] Once the bacteria is adherent on the surface, it goes about the business of forming a biofilm. Thus bacterial adhesion is suggested to be the first step in pathogenesis.[25]

3 Methods of Measuring Bacterial Adhesion

Adhesion of a microbial cell is unambiguously defined in terms of the energy required to remove the cell from a surface. Bacterial adhesion to surfaces can be measured by biochemical methods wherein molecular structures involved adhesion are determined,[26,27] or by physicochemical methods. The macroscopic methods such as contact angle measurements and zeta potential could provide surface thermodynamic analysis between interacting surfaces. However, a microscopic method such as AFM has been used to understand the bacterial adhesion forces at a single cell level. There are, in general, three different methods to study adhesion of bacteria to a substratum. These are:

1. Optical microscopy, laser scanning confocal or scanning electron microscopy, can be used to measure the association of microbial populations on a surface. These methods may be combined with dyes, fluorescence and antibodies, and use or not-use of laminar flow techniques. These methods measure the overall results of adhesion events between bacteria and the substratum surface but do not provide direct evidence of the increase or decrease of the adhesion force resulting from treatment of the substratum.[28]
2. Direct measurement of adhesion force by methods involving separation of cell and substratum shows direct evidence of increase or decrease in adhesion force. It fails to take into account the overall effect on the substratum, but rather takes into account the interaction of specific points of the substratum.[29]
3. Macroscopic techniques involving measurement of contact angles. This method measures the surface energies and gives an understanding of the potential of an adhesion event.[30,31]

4 Atomic Force Microscope

The improvement in the sensitivity and resolution of AFM has provided an important complement to the standard optical and electron microscopic techniques. In addition to the high-resolution imaging, AFM has proven to be a useful tool to measure interaction forces, a mode known as force spectroscopy. In this review, we will focus on the AFM force measurements rather than the imaging aspect.

The AFM has following major components: an AFM tip, piezoelectric scanner, laser diode, photodetector and an electrical feedback system. In an AFM, the AFM tip serves as a surface profiler, which is used to measure the surface features in the vertical direction. The AFM tip probes over the surface of interest while sensing the interaction between the tip and the sample. A laser is pointed at the reflective surface, on the back of an AFM tip and a sensor picks up the laser light reflected from there. The reflected laser beam strikes a position-sensitive photo-detector consisting of four-segment photodetector. The differences between the segments of photo-detector of signals indicate the position of the laser spot on the detector and thus the angular deflections of the cantilever. The magnitude of the deflected beams is received as a signal and is digitally processed either as a topographic image or interaction force profile of the sample.

The AFM has been used to measure the interaction force between bacteria and substrate. In general this is achieved by (1) attaching bacteria to an AFM tip and testing it against the substratum to measure the interaction force[32] or (2) by allowing the bacterium to settle onto the substratum and applying a force through the AFM tip to remove the cell.[29] Both these methods would measure the force of removal of the bacterium from the substrate, and give a direct measure of the effect of chemical treatment on adhesion.

4.1 *Measuring interaction forces by using a cell probe method*

AFM tips can be used as a cell probe by attaching the cell of interest to generate quantitative data of interaction-forces.[33,34] Cell probe technique quantifies the initial interaction forces of reversible adhesion phase between bacteria and a material surface. The forces are measured in the perpendicular direction. Immobilization of a bacterial cell to flat surfaces without hampering the ultrastructural properties and viability is a huge challenge to apply AFM in microbial research. The microbial samples are soft and flexible in aqueous media, thus requiring substrate surface modification to ensure firm attachment. The immobilized cells should be able to resist the lateral friction forces exerted by the AFM tip during scanning.[35] The earlier AFM studies were conducted using dried microbial cells due to the ease of sample preparation. However, this has undergone tremendous development to deal with imaging of live cells in aqueous environments and study the interactions between microbes and their surroundings.[36]

Cells could be immobilized on a surface by electrostatic interactions,[37] entrapment and adsorption techniques.[38] Glutaraldehyde treatment to create covalent crosslinking between bacterial surface and the polyethyleneimine coated tips[39] have been discarded in the recent studies due to the effect on cell structure and properties. Electrostatic interactions of microbes to surfaces that have been coated with positively charged substances such as poly-L-lysine,[40] polyethyleneimine[37] or gelatine[41] are commonly used for immobilization. However, cell detachment and effect of these substances on cell viability are few of the limiting factors. Adsorption technique for immobilization is based on highly adhesive polyphenolic proteins.[38] This is known to facilitate firm immobilization of viable cells.

The microbes can also be immobilized onto a tip-less cantilever or AFM tip to study the force of interaction with a substrate. Cell probes with a single bacterium could be prepared using a polydopamine adhesive.[42] Another commonly used approach is the use of polyethylene glycol (PEG) crosslinker, which provides free movement as well as prevents sample denaturation.[43] The AFM tip is initially derivatized with amino groups and then reacted with PEG crosslinker that directly attaches to proteins through lysine residues.[44] Adsorption of bacteria onto poly-L-lysine coated glass beads and subsequently attaching cell-coated bead to a cantilever by using epoxy resin have also been used.[33] Though, the cell probe method has undergone tremendous improvements this approach still presents certain limitations. The need to use a physicochemical treatment to stabilize the cells on to the AFM tips may affect the cell surface properties. Detachment forces between bacteria and surface cannot be quantified, as the lateral forces of interaction can not be measured using this technique.

4.2 *Measuring interaction forces by using a cell peeling method*

Cell peeling method has been introduced to quantify the lateral adhesion forces at cell-substrate interface in aqueous medium using AFM equipped with unmodified cantilever tips.[29,45] In this method, a bacterium is allowed to interact with the substrate and subsequently peeled off vertically from

the surface. A microcantilever was used for the first time to measure the detachment force of an adhered cell.[46] The cell adheres to a material surface kept on a microscope stage that moves at a constant velocity in X-Y direction. Lateral forces are applied when the tip of a microcantilever touches the cell surface. This leads to the cantilever deflection corresponding to the cell deformation and shear force required for cell detachment. The maximum force that appears in the force displacement curves was the shear force required to detach the cell and the area under the curve is the total energy necessary for cell detachment.

Tedjo *et al.*[29] measured the peeling force of interaction (lateral or shear force) after one hour of bacterial adhesion on conditioned polymeric films. The shear force of detachment measured represented the later events of bacteria adhesion such as molecular interactions with the substratum. The detachment force between *Enterococcus faecalis* and polymers using the deflection set-point-applied force calibration curve via AFM has been evaluated.[45] This provided accurate approach to evaluate the adhesion forces for irreversible phase of bacterial adhesion. Sagvolden *et al.* used an inclined atomic microscope cantilever and the laser beam deflection to measure the force.[47] The modification involved a moving substrate that resulted in cell displacement and after the cantilever tip touches, the force on the cell increased. Eventually the cell gets detached from the surface after overcoming the last bond. The detachment forces were calculated from the typical force displacement curves and were in the range of 20 to 200 nN.

Recent study has developed and optimized a quantitative method using the contact mode of AFM to determine the lateral detachment force of the adhered bacterial cell.[48] The study suggested that the scan size of 40×40 mm^2, scan rate of 40 mm/s, could provide sufficient information and measurement accuracy on the cell identification and detachment force measurement. Choosing the cantilever selection with the lowest spring constant first and then stepping up to a harder cantilever until all cells are detached have also been highlighted. This method showed good repeatability and sensitivity to various bacteria/substrata combinations.

4.3 *Measurement of interaction forces*

To measure the force of interaction, the cantilever deflection of the AFM tip is recorded as a function of the vertical displacement of the piezoelectric scanner. The sample is pressed towards the AFM tip and retracted, wherein the raw curves consist of voltage measured as a function of piezo displacement at a given x, y location. Depending on the set-up, the movement of the tip, which has a sensitive spring constant, can be amplified many times such that even movements over distances as small as a silicon atom can be detected and visualized. Movements of the tip whilst it traverses over a surface is recorded using software and is used to make comparisons.[49] Raw force curves consist of a voltage measured on the photodetector as a function of the vertical piezo displacement (z) at a given x, y location. The slope of the retraction curve is then converted into deflection force using Hooke's law:

$$F = -kD \tag{1}$$

where F is the force, D is the deflection and k is the cantilever spring constant. The zero separation distance corresponds to the contact point between the cantilever tip and the sample, when the tip suddenly snaps into contact as it goes past the repulsive forces. At the pull off from the surface, there is a hysteresis of the curve and the size of the hysteresis is dependent on the area of contact, position of cell in contact, surface energy and time of contact between the tip and the substratum surface.[50]

Poisson analysis[26] of the work of adhesion[51] obtained from the measured forces could provide information on the bacterium substrate interaction. Considering the difficulties of Poisson analysis, the work of adhesion could be calculated using the free energy required to detach a single bacterium from a surface. The work of adhesion can be calculated from the area under the entire retraction force-distance curve.[51] Previous study monitored the interaction of *Shewanellaoneidensis* and goethite substrate using AFM at aerobic and anaerobic conditions.[33] When compared to the aerobic conditions, at anaerobic condition, the *S. oneidensis* showed stronger adhesion energy, determined from the measurement forces at the interface with goethite. Andre *et al.* revealed using AFM force curves that peptidoglycan is hidden by other cell wall constituents in *Lactococcus lactis*.[52] The binding forces of the wild type were found to be much higher (250 pN) as compared to the mutants with impaired production of cell wall polysaccharides (71 ± 16 pN).

Theoretically there should be no separation between the approach curve and the retraction curve, at the constant compliant region. However, there can be a separation between the two curves in the constant compliant region, which may lead to errors in the forces measured. There is still controversy regarding how forces should be measured from an AFM force distance curve. It has not been determined what represents the point where the forces acting on the probe tip

is at zero on the curve. Whether the Ducker and Selden method[53] or contact biomechanics method reported by Emerson and Camesano[54] is more appropriate is at best uncertain. Further authors have used various positions of the curve as the contact point between the tip and the substratum, hence the forces of interaction between the tip and the substratum can take on several interpretations.[55] It is imperative to consider that the choice of tip location is determined by the physicochemical conditions such as electrostatic repulsions or ionic strength of the solution. The main difference between these various approaches lies in the spatial definition of the cell surface.[55] This method as described by Razatos *et al.*[32] measures vertical forces of adhesion of a bacterium to the substratum surface within seconds of contact and represents the early events of adhesion.

5 Conclusion

This short review provides an insight into the AFM that is becoming a key technique in microbiology to understand physical properties and interaction forces at higher resolution. The constant improvements in sample preparation techniques, instrumentation, experimental conditions for the living cells and analyses systems are bound to bring improvements in the accuracy and reproducibility of the force measurements. In addition to the elucidation of structure-function relationships of microbial surfaces, these nanoscale observations could be used to understand the molecular interactions associated with soft, complex interfaces.

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