

**Review Article**

Microbial Cells Force Spectroscopy by Atomic Force Microscopy: A Review

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Abstract: Bacterial adhesion and biofilm formation are important phenomena which can produce both detrimental and beneficial effects in several fields. Research is thus focused on the modulation of the properties of material surfaces in order to design and develop substrates able to control bacterial adhesion process, which is the first trigger event of biofilm formation. Several theoretical predictions and experimental procedures have been developed to investigate the physical, chemical and biological mechanisms regulating the attachment of bacteria to solid substrates. Nevertheless, a comprehensive understanding has not been achieved yet, limiting the capability of individuating effective technological strategies to achieve the desired bacterial adhesion behavior. The development of new experimental procedures able to furnish deeper information about bacterial adhesion mechanism is thus needed. Microbial cell force spectroscopy (MCFS) is an atomic force microscopy (AFM) based technique, consisting in the detection of force-distance curves using particular probes obtained immobilizing bacterial cells at the free end of a flexible microcantilever, which allows the detection of the different kinds of cell-surface interaction forces. In this work, we review the state of the art in the development of MCFS, focusing on its working principle and applications. A brief description of the current models and conventional experimental procedures used to evaluate bacterial adhesion to surfaces is reported. Then, the instrumentation and the working principle, the current procedures used to prepare bacterial cells probes and the main applications of the technique are described with the aim of pointing out the advantages of the technique and the limits which still have to be overcome.

Keywords: Atomic Force Microscopy, Force Spectroscopy, Adhesion, Bacteria

1. Introduction

The ability of bacteria to adhere to solid surfaces, proliferate and form biofilms is an important concern in several fields, including health-related and industrial domains. In biomedical field, bacterial adhesion phenomena and the consequent biofilm formation on medical device surfaces are the first trigger event of infections [1, 2], which represent one of the major causes of failure of medical devices and long-term implants, such as dentures, contact lenses, catheters, orthopedic and cardiovascular prosthesis [3-5].

Indeed, biofilm formation is often associated to the formation of an extracellular polymeric substance (EPS), which synergistically contributes with its sessile microbial community, making it more resistant to immune defences and antibiotic treatment, frequently leading to the necessity of surgical removal of implants with obvious economic consequences. Furthermore, microorganisms organized in sessile communities, once biofilm is formed, are often able to survive also in dry environmental conditions, hugely increasing the risk of infections spreading, especially in predisposed environments, such as hospitals and healthcare

units. Surgical instruments, such as scalpels, drips and catheters, as well as common surfaces for near-patient clinical areas, represent a source of bacterial contamination [6] and are thought to produce relevant transmission pathways of hospital-acquired infections (HAIs), which are a primary worldwide health-related concern [7]. Bacterial adhesion can also have detrimental effects in several industrial fields. Biofouling of industrial and bioprocess engineering equipments, such as water distribution pipes, water treatments facilities, cooling towers, reactor surfaces, heat exchangers, ion-exchangers, etc. can cause loss of process efficiency as well as contamination or difficulties in maintaining sterility [8-10]. Furthermore, when bacteria are in close contact with a metal surface, corrosion reactions can be facilitated by microbial activity (i.e., biocorrosion), leading to the failure of metal substrates [11]. Because of all these detrimental effects and the emergence of restrictive legislation regarding the negative effects of cleaning agents [12], there is a great interest, in different fields, in the design and development of surfaces able to prevent bacterial attachment and the consequent bacterial infections. Otherwise, in some cases, the adhesion of microorganisms to surfaces can produce positive effects. For example, the intestinal microbiota plays a key role in human health [13]. In the industrial domain, the most significant example of benefic effects of bacterial adhesion is represented by bioleaching hydrometallurgical implants. Bioleaching is a process used to recover metals from sulphide ores through the use of living microorganisms activity, in a much more cost-effective and environmentally friendly manner than the traditional heap leaching [14]. The enhancement of bacterial cells attachment to mineral surfaces is, in this case, important to improve the process effectiveness. Therefore, in parallel with the increasing efforts to eradicate bacterial adhesion, attempts are being made with the opposite aim of increasing the attachment of microorganism onto both biotic and abiotic surfaces. In both cases, the lack of a comprehensive understanding of the microbial adhesion process limits the technological progresses. Several efforts are thus focused on understanding the physical, chemical and biological mechanisms regulating the bacterial attachment in order to individuate the most influencing materials surfaces properties and develop effective technological strategies to control and modulate bacterial adhesion to surfaces. Various theoretical predictions, describing adhesion process of bacteria from the point of view of the involved physical interactions (i.e., van der Waals, electrostatic, hydrophobic, and steric interactions), and experimental approaches, generally consisting in the qualitative or statistical evaluation of bacteria attached on a surface after a certain period of time, have been developed to predict and measure the adhesion of bacteria to surfaces. Nevertheless, discrepancies between theoretical models and experimental data have been observed demonstrating the still not exhaustive comprehension of the involved mechanisms.

For this reason the development of new experimental procedures able to furnish deeper information about bacterial adhesion mechanism appears as a fundamental need.

Microbial cell force spectroscopy (MCFS) is a recently developed atomic force microscopy (AFM) based technique, in which force-distance curves are acquire through the use of an AFM probe at the free end of which one or more bacteria are immobilized [15, 16]. The capability of the technique of manipulating a single or few bacterial cells and measuring the distance-dependent cells-surfaces interactions with relation to the distance, the characteristics of the liquid environment, and the cell-surface contact time potentially allows one to obtain further information in respect to conventional statistic experimental methods and to quantitative evaluate the interaction forces involved in each phase of the adhesion process, which is necessary to establish a comprehensive understanding of the adhesion phenomena.

In this work, the state of the art and the potentialities of MCFS are reviewed. A brief description of the current models and conventional experimental procedures used to evaluate bacterial adhesion to surfaces is reported in order to highlight the potentiality of MCFS in providing still missing information about the implicated phenomena. Therefore, the instrumentation and the working principle, the current procedures used to prepare bacterial cells probes and the main applications of the technique are described with the aim of pointing out the advantages and the limits of the technique which still have to be overcome.

2. Bacterial Adhesion Mechanisms and Theoretical Models

From a qualitative point of view, bacterial adhesion to a surface can be described as a two-phases process, consisting in a first reversible 'physical adhesion' and a second irreversible attachment dominated by molecular and biological mechanisms [17]. 'Physical adhesion' is the first crucial step of bacteria attachment and consists in a reversible adsorption process, mainly governed by van der Waals, electrostatic and hydrophobic interactions as well as hydrogen binding. This phase is thought to be mainly influenced by some bacteria and surface physical characteristics, like the surface charge and hydrophobicity. The environmental conditions can also affect the process, inducing chemotaxis and hapoptaxis, or simply influencing the bacterial Brownian motion and the gravitational forces. In studying an antiadherent material, we must also take account of other molecules eventually present in the environment in which our material will be used, in fact, in this case, these molecules can act as bridge of adhesion between bacteria and our surface. After the first physical adhesion phase, a firmer (irreversible) bacterial attachment occurs, thanks to the presence, on bacteria surfaces, of polymeric structures, such as pili, capsules or fimbriae, characterized by functional groups (adhesins) which mediate the adhesion of bacteria onto surfaces. Once bacterial adhesion occurs, biofilm formation mechanism starts: the bacteria begin to multiply forming microcolonies, then reached a certain size of the population, they will begin to produce EPS and then to form

biofilms.

Several efforts have been done to find a mathematical model to describe and predict bacterial adhesion onto surfaces. Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory [18, 19] was used to explain bacterial adhesion for the first time in 1971 by Marshall *et al.* [20]. The net force acting on a single bacterium because of the presence of a flat surface is described as the sum of van der Waals and Coulomb interactions. The application of DLVO theory to bacterial adhesion process was the first step to mathematically describe the adhesion of living microorganisms to surfaces. Nevertheless, some important factors are not taken into account and a certain discrepancy between DLVO model and experimental data has been observed. Among these factors, hydrophobic interactions have been proven to have a crucial effect in bacterial adhesion process, especially in the case of hydrophobic surfaces. For example, some studies experimentally showed that bacterial adhesion on hydrophobic Teflon surfaces is higher than on glass, although predictions based on DLVO theory express van der Waals interactions stronger in correspondence of glass than of Teflon [21]. In order to take into account hydrophobic interactions, the thermodynamic approach was introduced [22]. This model is based on the experimental measurement of the contact angle (of bacteria and substrates) and the application of Dupré equation, in which the free energy per unit surface area (ΔG^{adh}) is given by

$$\Delta G^{adh} = \gamma_{BS} - \gamma_{BL} - \gamma_{SL} \quad (1)$$

where γ_{BS} , γ_{BL} and γ_{SL} represent the interfacial energy of the bacterium-substratum, bacterium-liquid and substratum-liquid interfaces, respectively. According to this model, adhesion is favored if ΔG^{adh} is negative, i.e., if γ_{BS} is smaller than the sum of γ_{BL} and γ_{SL} . Several bacterial adhesion experimental data seem to be explained by this model [23, 24], but some cases are still not explicable by thermodynamic theory [25, 26]. This incongruence has been ascribed to the fact that the thermodynamic approach, using Dupré equation, assumes that the process is reversible, which is often not the case. Furthermore, it is an equilibrium and not distance-dependent model that does not allow a kinetic interpretation. Also, the thermodynamic approach assumes the formation of a new bacterium-substrate interface, at the expense of the substratum-medium interface (the strength of the interaction is calculated when the contact between the bacterial cell and the surface is achieved). If a new cell-substratum interface is not formed the theory is not applicable. Because of the limits of DLVO theory and thermodynamic approach, van Oss introduced an extension of the DLVO theory in which also the hydrophobic interactions are taken into account [27]. In this model, known as the extended DLVO (XDLVO) theory, the total adhesion energy is expressed as

$$\Delta G^{adh} = \Delta G^{vdW} + \Delta G^{el} + \Delta G^{AB} \quad (2)$$

where ΔG^{vdW} is the Lifshitz-van der Waals interaction term, ΔG^{el} is the electrostatic interaction term and ΔG^{AB} is the Lewis Acid-Base interaction term, which takes into account the hydrophobic attraction, the hydrophilic repulsion, and the structural forces, respectively.

While potentially constituting a useful tool for assessing the first physicochemical stage of bacterial adhesion to surfaces, these models do not take into account molecular interactions and biological phenomena. This limits the possibility of finding a complete validation through the use of conventional experimental methods, which evaluate the bacterial adhesion as the overall result of physical, chemical and biological effects. In addition to the various characteristics of the substrates, for well understanding the phenomena of adhesion, it is necessary to consider also the fundamental cellular differences among the various bacterial species.

3. Conventional Methods to Quantify Bacterial Adhesion onto Surfaces

The most common experimental way to evaluate the adhesion of bacteria to different surfaces is represented by static assays methods, consisting in overloading the surface with a cells suspension for a determined period of time and counting the adhered cells by microscopy techniques, e.g., scanning electron microscopy (SEM), optical microscopy, scanning confocal laser microscopy and AFM, or by viable bacteria counting methods, such as colony forming units (CFU) plate counting, radio labeling, 5-cyano-2,3-ditolyltetrazolium chloride (CTC) staining [28], and BioTimer Assay [29, 30]. Flow systems, such as parallel-plate flow chambers [31], radial flow chambers [32] and rotating discs [33], have also been used to simulate the in vivo dynamic mechanical stress state and to obtain global probabilistic measurements of the bacterial adhesion strength, by evaluating the adhered bacteria before and after the application of a known shear stress. Furthermore, on-line microscopic detection methods allow the quantitative calculation of deposition and desorption rates.

In all the conventional experimental methods, the evaluation of the bacterial adhesion strength to substrates is qualitative (in the case of static assays) or probabilistic (in the case of fluid shear assays), being obtained by measurements carried out on numerous populations of bacteria. Moreover, the bacteria adhere to the surface for a relatively long period of time (from few minutes to several days). This means that the adhesion is produced by the overall interactions between the bacteria and the surface, i.e., physicochemical interactions, molecular interactions and bacterium-bacterium interactions that occur when certain bacteria have already adhered to the surfaces, furnishing information about the overall adhesion mechanism, but impeding the investigation of the bacterium-surface interactions regulating each single step.

To obtain quantitative information about thermodynamic

and kinetic mechanisms regulating the single phases of bacterial adhesion to a surface different techniques are required. In this context, MCFS represents an interesting possibility to overcome the limits of conventional experimental methods, thanks to its capability of quantitatively evaluating the adhesion forces between bacteria and surfaces in each adhesion phase and in response to different environmental conditions.

4. Bacterial Cell Force Spectroscopy

4.1. Force Spectroscopy by Atomic Force Microscopy

AFM, introduced for the first time in 1986 [34], is nowadays a well established technique for the characterization of surfaces morphology at the nanometer scale. In AFM imaging, the sample surface is scanned by a probe consisting in a flexible microcantilever with a sharp tip positioned at its free end. The interaction forces between the tip and the analyzed sample produce a deflection, in the case of 'static-mode' AFM, or a variation in the dynamic behavior of the cantilever (oscillation amplitude, resonance frequency and phase shift) in the case of 'dynamic mode' AFM. The response of the cantilever to the interaction force with the sample is monitored through an optical lever system, which produces a signal in each point of the scanned area, from the processing of which the morphology of the sample can be reconstructed.

The capability of AFM to measure interaction forces between the probe and the sample allowed the extension of the technique to the detection, beyond the topography, of a wide number of physical parameters at the nanometer scale, such as mechanical [35, 36], electric [37-39], and magnetic [40]. Furthermore, the possibility of performing AFM measurements in liquid environment, have provided the opportunity of its application in biology and microbiology field [41, 42]. For example, AFM has been widely used to image the morphology of single bacterial cells and biofilms on solid substrates, both in dried and hydrated states [43] and in different environmental conditions [44] as well as to investigate the nanomechanical properties of cells [45-49].

The recording of force-distance curves consists in the detection of the cantilever deflection, proportional to the tip-sample interaction force, as a function of the piezoelectric displacement, i.e., the tip-sample distance, when the tip is made approach the sample surface and retract. It is one of the most versatile tools provided by AFM instrumentation and is the basis of MCFS.

Indeed, force-distance curves measurements are generally used for the mechanical characterization of samples for a wide range of applications (from ultrathin coatings to living cells), but can also furnish information about interaction and adhesion forces between the probe and the sample.

A typical cantilever deflection versus tip-sample distance curve is sketched in Fig. 1.

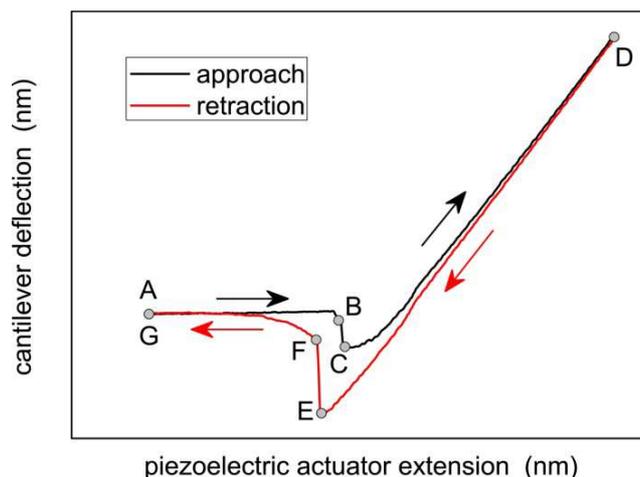


Figure 1. Example of a cantilever deflection versus piezoelectric actuator extension curve. At the beginning of the experiment, the tip is far from the sample surface (point A). Then, the tip approaches the surface experiencing long-range interactions (region A-B) until it abruptly jumps into contact with the surface (point C). Further increasing the extension of the piezo-actuator (i.e., decreasing the cantilever-sample separation) results in the upward deflection of the cantilever (region C-D). Retracting the tip from the surface, the cantilever deflection is decreased (region D-E) until the tip abruptly jumps off the contact with the surface when the cantilever restoring force overcomes the tip-sample adhesive and capillary forces (point F). Then, only long-range forces are experienced by the tip (region F-G) until the initial extension of the piezoelectric actuator is reached (point G).

At the beginning of the experiment (point A) the piezo-element is completely retracted, a large distance separates the tip from the surface and no tip-sample interaction forces exist. The tip is then moved towards the sample surface by acting on the piezo-element, which extends thus reducing the tip-sample distance. During the first phase of approach (region A-B), the probe is subjected to long-range attractive (van der Waals or electrostatic) or repulsive (electrostatic) interaction forces, which produce the cantilever bending toward the sample or upward and are balanced by the cantilever elastic force. When attractive interactions become dominant in respect of the cantilever elastic restoring, the tip 'jumps into contact' with the surface (point C). The further extension of piezo-element produces the cantilever bending upward under the effect of repulsive forces acting on the tip because of the overlapping of the electron orbitals of the probe and the sample (region C-D). After reaching the maximum preset value of the piezo-element extension, the tip is retracted from the sample surface (region D-E). When the cantilever elastic restoring overcomes the adhesive forces created during the contact and capillary forces, the latter being present if the experiment is performed in air, the detaching of the tip from the surface occurs (point E - 'Jump-off-contact') [50]. When the cantilever is further retracted from the surface, only long-range tip-sample forces are experienced by the cantilever (region F-G) until the initial separation is reached (point G).

These curves can be processed and transformed into curves representing the normal force applied to the sample by the probe due to the bending of the cantilever as a

function of the penetration depth, which are analogous to those retrieved with standard micro- and nano-indentation and can be used to evaluate the sample indentation modulus and hardness [51-56]. In addition, AFM force-distance curves can furnish quantitative information about the adhesion force between the probe and the surface, which corresponds to the force acting on the cantilever immediately before the tip-sample detachment ('pull-off force').

This capability of the technique has been widely applied for the study of the adhesion mechanisms between surfaces using both standard AFM Si (or SiN) tips [57] and the so called 'colloidal probes', consisting in standard tipless cantilever at the free end of which a microparticle is immobilized [58]. Moreover, beside the measurement of these 'not specific' forces, the so-called chemical force microscopy (CFM) has been developed [59,60] which takes advantage of probes functionalized with specific molecules to measure the receptor-ligand binding forces as well as to perform the molecular recognition and mapping on the sample surface [61-63].

4.2. Bacterial Cell Force Spectroscopy by Atomic Force Microscopy

As a natural extension of the colloidal probes approach, MCFS has been proposed for investigating the phenomena underlying the bacterial adhesion to surfaces by immobilizing cells on an AFM probe and recording force-distance curves [15, 16]. The interest in the development of MCFS is due to the unique capability of separately and quantitatively detecting all the interaction forces acting on the cells in each phase of the approaching and anchoring process, which cannot be evaluated with any other technique and which can be summarized as follows [64]:

- *Attractive long-range van der Waals and electrostatic forces* can be detected through the measurement of the approaching curve, which is, in this case, characterized by the negative deflection of the cantilever, i.e., its bending towards the surface and/or by the 'jump to contact peak';

- *Repulsive 'double-layer' electrostatic forces*, due to the cells and substrate surface charge, can be also detected through the measurement of the approaching curve. The cantilever deflection is, in this case, positive (it bends upward) and the 'jump-to-contact peak' does not occur;

- At high salt concentration, due to the repulsive interactions between hydrated ions bound to the cell and the surface, also *repulsive hydration forces* can occur and be visible on the approaching curve, which will be characterized by a positive cantilever deflection and the lack of the 'jump to contact peak';

- *Solvation forces*, caused by ordering of non-polar liquid molecules between the two liquid-solid interfaces (cell-liquid and surface-liquid), can be visible, creating serial peaks on the approaching curve, due to the oscillations of attractive and repulsive forces;

- As already mentioned, the *adhesion force* between the

cells and the surface can be evaluated through the detection of the retracting curve and the calculation of the pull-off force;

- *Hydrophobic interactions* can also be observable on the retracting curve, producing a gradual pull-off instead of an instantaneous jump off contact;

- *Bonds stretching*, due for example to multi-domain proteins stretching, can be also observed on retracting curves, giving rise to multiple detachment peaks;

- Furthermore, information about *bond strengthening*, which could be due for example to the formation of hydrogen bonds, can be obtained by measuring retraction curves after different time of contact between the cells and the substrate.

Nevertheless, all the potentialities of MCFS have still not been completely exploited, mainly due to the lack of standardized measurement procedures and the difficulties of data interpretation, which result to be strongly dependent on the measurement conditions and, in particular, on the characteristics of the used bacterial cell probes.

4.3. Cell Probes

Cell probes are generally obtained by the immobilization of cells on commercial cantilevers (Si or SiN), with spring constant in the range 0.01-0.5 N/m.

Immobilization of bacteria on the AFM probe is the most critical phase of MCFS technique. Indeed the characteristics of the probe can strongly influence the operation mode of the technique and the data interpretation. Due to the numerous factors involved, a standard procedure to produce bacterial cell probes has not been defined yet and their characteristics as well as the preparation method have to be chosen considering the specific experiment.

The first requirement to be achieved is the high adhesion of cells on the probe surface, which, in order to avoid cells detachment, must be stronger than the adhesive interactions with the analyzed surfaces experienced during the measurements. At the same time, the characteristics of immobilized cells should be minimally modified in respect to their physiological conditions, in order to obtain biologically meaningful information. Furthermore, if a quantitative comparison between measurements carried out with different probes is necessary, the probe preparation process must be reproducible and the number of attached bacteria interacting with the surface should be known and controllable.

Different kinds of bacterial cell probes have been proposed, which can be classified on the basis of the shape of the modified cantilever ('with tip' or 'tipless' probes), of the number of attached bacteria ('multiple bacterial cells' and 'single bacterial cell' probes, which are sketched in Fig. 2a and b, respectively) or of the method used to make bacteria adhere on the cantilever surface, i.e., (i) the surface chemical modification of the probes; (ii) the attachment of a microsphere, covered with bacteria, to the cantilever surface (Colloidal probes) or (iii) the physical entrapment of cells, through the use of the recently introduced technique of FluidFM.

In the following paragraphs the proposed methods to

prepare bacterial probes are described and discussed, with particular focus on their advantages and disadvantages.

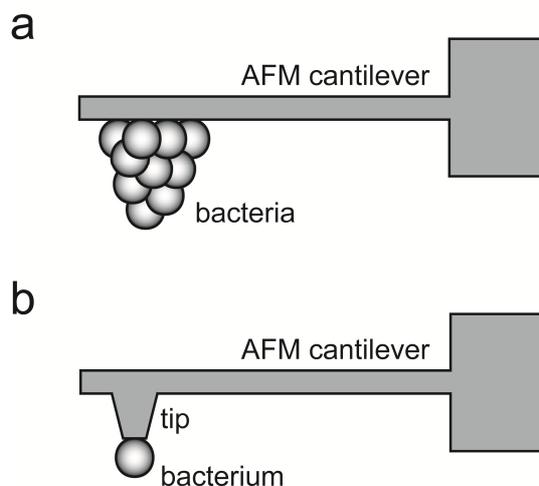


Figure 2. Sketch of (a) 'multiple bacterial cell' and (b) 'single bacterial cell' probes.

4.3.1. Chemical Modification of the Cantilever Surface

The chemical modification of the cantilever surface is the easiest and most common way to prepare bacterial cell probes, which has been proposed and used since the first introduction of MCFS [15, 16]. Chemical methods are based on a two-steps procedure: (i) the modification of the cantilever surface through substances able to improve cells adhesion; (ii) the transfer of bacterial cells on the cantilever surfaces. The different procedures proposed to perform the two phases are described in the following paragraphs.

Cantilever surface modification. Several substances have been used in order to promote bacterial adhesion through different mechanisms, including physisorption, covalent binding or immobilization through adhesive proteins. Bacteria are characterized by negatively charged surfaces. Therefore the physisorption on the probe surfaces can be promoted by coating the cantilevers with positively charged polymeric films, such as poly-L-lysine [15, 65-69] and poly(ethylene imine) (PEI) [16, 70-74]. Several authors successfully used only physisorption methods, using poly-L-lysine, to immobilize bacteria on AFM cantilevers, but in this case bacteria-surface binding is weak and cells detachments can occur, limiting the capability of performing numerous serial measurements.

A stronger immobilisation can be achieved by glutaraldehyde covalent crosslinking [16]. A pellet of bacterial cells, previously suspended in glutaraldehyde, is transferred onto a PEI-coated cantilever and further treated with an additional drop of 2:5% vol/vol glutaraldehyde. Cantilevers are then incubated for some hours at 4°C, then rinsed in water and dried. The physical characteristics of cell surfaces, such as the hydrophobicity and surface charge, which are supposed to be the most influencing cells surface properties during the first phase of the adhesion process, have been demonstrated to be not significantly affected by the glutaraldehyde treatment [75], which stimulate the use of this

method in several works [65-69]. Nevertheless, some authors observed that cross-linking of proteins induced on the entire cells surface can produce significant changes in the chemical structure of bacteria surfaces, affecting the mechanical properties and the viability of cells, leading to possible variations in their adhesion behavior [76, 77]. Therefore, the approach has been modified activating bovine serum albumin (BSA) coated probes with glutaraldehyde (coated cantilevers have been incubated in 2% glutaraldehyde for 10 minutes at room temperature). In this way, chemical groups were activated on the cantilever surface instead of bacteria, affecting the cells surface chemistry only on the side which is in contact with the probe surface [76].

Despite the wide use of chemical methods to prepare bacterial cell probes, it has been observed that approaches which require exposure of cells to chemicals, distilled water or drying, could affect cells viability and surface properties, for example rearranging the surface charge during the adsorption onto positively charged cantilever surfaces or, as already mentioned, cross linking proteins by glutaraldehyde treatments [78, 79]. For this reason, the use of adhesive polyphenolic proteins, which does not require the use of chemicals or drying and does not trigger any undesirable biological response, has been proposed as an alternative immobilization method. For example, Kang et al. [79] proposed the use of a polydopamine coated cantilever and Zeng et al. [80] introduced the use of Cell-Tak™, a commercial wet adhesive composed of naturally derived polyphenolic proteins, which were demonstrated to not significantly affect cells viability.

Transfer of bacterial cells on the cantilever surface. After the modification of the surface of the probe, bacteria have to be transferred on the cantilever surface. Different procedures have been proposed with the aim of obtaining multiple bacterial cells probes or single cell probes. The first and most common way to prepare multiple cells probes is the method proposed by Razatos et al. [16], which consists in manually transferring a pellet of bacterial cells onto the cantilever. Cells can then then fixed by glutaraldehyde treatment [16, 70, 71, 73, 74] or just rinsed with the same medium used for AFM force measurement, generally deionized water or phosphatebuffered saline (PBS) buffer [81]. An alternative method to prepare multiple cells probes with reduced denaturation of cells consists in dipping or incubating the functionalized cantilever in a bacterial suspension allowing bacteria to adsorb on the probe surface [15, 66, 68, 69, 76, 82]. Probes are then rinsed to remove unbound bacteria with the same medium which is used to carry out AFM force measurements. The bacterial transfer procedure to prepare multiple bacterial cell probes is easy and for this reason this kind of probes are widely used. Nevertheless, they present the disadvantage of a low reproducibility and an uncertainty in the number of bacteria immobilized on the cantilever surface and interacting with the analyzed surface. This restricting issue impedes the comparison of measurements carried out with different probes. However, measurements

performed with the same probe can be considered comparable as long as bacteria do not detach from the probe surface [68]. The control of the bacterial probe integrity is thus necessary during serial measurements and can be carried out by optical microscopy control or by monitoring the cantilever resonance frequency.

Single cell probes guarantee the contact of one single cell with the analyzed surface, allowing, under certain conditions, the comparison between measurements carried out with different probes. A single cell can be isolated and picked up by the functionalized cantilever through the use of a micromanipulator [77, 83]. Alternatively, bacteria can be spread on a flat surface (generally glass) and a single cell can be picked up by the functionalized cantilever using the manual stage and the step motor of the AFM [15, 65, 67, 80, 84, 85].

4.3.2. Colloidal Probes

The attachment of bacterial cells directly on the cantilever surface can lead to the lack of a precise control of the probe-surface interacting area and, sometimes, to the contact between the cantilever surface and the analyzed substratum, complicating the interpretation of the results. To obtain a higher control of the interacting area, some authors proposed the use of colloidal probes, consisting in a microsphere attached at the very end of a tipless cantilever, covered with a layer of bacteria or a single bacterial cell. The preparation of bacterial cells colloidal probes is based on a three-steps procedure: (i) the attachment of a micrometric bead on the cantilever free end; (ii) the chemical modification of the bead surface through substances able to improve cells adhesion; (iii) the transfer of bacterial cells on the bead surfaces.

The attachment of the sphere on the AFM cantilever is obtained through the use of a micromanipulator and particular glues, such as epoxy resins [86, 87] or UV-curable glues [88]. The colloidal probe is then functionalized with substances able to increase the bacterial adhesion on the sphere surface, for example polylysine [87, 89], PEI [86], or polydopamine [88]. A single bacterial cell, previously isolated and deposited on a flat substrate, can be picked up by and placed at the apex of the immobilized sphere, using a micromanipulator [86] or the AFM instrumentation [88]. Otherwise, a micromanipulator can be used to cover the sphere with a uniform layer of cells [87, 90].

A different procedure has been proposed by Lower *et al.* [90], who prepared a bacterial cell colloidal probe functionalizing a glass bead with 3-aminopropyltriethoxysilane before the attachment of the bead to the cantilever surface. Bacterial cells were immobilized on the functionalized bead by spinning a cell-bead mixture. The bead has been, then, attached to the cantilever through the use of an epoxy resin.

4.3.3. Physical Entrapment: FluidFM

The bacteria immobilization through chemical modification of the cantilever is the easiest method to prepare bacterial cell probes and does not require any particular AFM instrumentation, being accessible to all users. For this reason it

is the most widespread procedure to produce probes for MCFS measurements.

Nevertheless bacteria immobilization through electrostatic or covalent binding or through bioinspired wet adhesives presents some limitations. Indeed, besides inducing a perturbation of cell characteristics, these methods, often, do not provide sufficient strand to withstand the cell-substrate interaction forces, leading to cells detachment during the experiments. Furthermore bacteria immobilization is irreversible, limiting the biological replicate measurements due to the long time required for the probe preparation.

The recent introduction of FluidFM technology [91] opened the possibility of immobilizing bacteria on AFM cantilevers without using chemicals, but simply by 'physical entrapment' of cells. The FluidFM instrumental apparatus is based on the use of a hollow cantilever which is connected, through micro-sized channels integrated in the AFM chip holder, to a pressure controller.

The immobilization and the release of a single cell on the tip aperture can be obtained by applying underpressure and overpressure, respectively. In this way the modification of cells characteristics is limited and the firm entrapment of the cell guarantees the integrity of the probe also after numerous measurements. Furthermore the cell immobilization is reversible, allowing one to measure the adhesion of many individual cells, and thus to obtain information on cell-to-cell variations [92]. Nevertheless a sophisticated and not commercially available instrumentation as well as a complex fabrication process of the microchanneled cantilever is required, making the technique not accessible to all AFM users.

5. Applications

MCFS was introduced in 1998 with the pioneer works of Bowen *et al.* [15] and Razatos *et al.* [16], which were mainly focused on the definition of the measurement procedure and the investigation of the potentialities of the technique.

Bowen and coworkers demonstrated the possibility of performing bacterial adhesion measurement by gluing a cell on an AFM cantilever [15, 77].

Adhesion force of yeast cells (*Saccharomyces cerevisiae*) on mica and modified mica surfaces were analyzed and correlated to the effects of the time of contact between the cell and the substrates, the life cycle stage of the tested cells and the physical properties of the analyzed substrates, with particular focus on the hydrophobicity characteristics [15, 77]. The adhesive force of cells onto surfaces increased with the increasing of the contact time of the cell onto the surface, showing the response of the cell to the presence of the surface and demonstrating the possibility of monitoring, through MCFS, the bond strengthening between bacteria and surfaces as a function of the contact time. The analysis of the curves shape showed congruent differences in the curves obtained with different cell life cycle stages. Also, the stretching of the cell during the detachment was observed, demonstrating the elastic nature of the cell-surface contact and the possibility of the technique to furnish information

also about cells mechanical properties. The comparison between MCFS data and the qualitative information obtained by counting procedures exhibited a good agreement between the two methods, indicating the complementarity of MCFS with standard procedures. Furthermore, a stronger adhesion force has been observed on hydrophobic surfaces in respect to hydrophilic surfaces, confirming the existence of a strict relationship between surface hydrophobicity and adhesive forces, especially during the first physicochemical phase of bacterial adhesion mechanism.

Razatos et al. [16] studied the adhesion of two different strains of *Escherichia coli* (D21 and D21f2, with truncated lipopolysaccharide (LPS) molecules) on glass and poly(methyl methacrylate) (PMMA) coated glass, with particular focus on electrostatic bacteria-surfaces interactions. The analysis of the measured force-distance curves has been related to the measured zeta-potential of the tested cells. The electrostatic effects in the adhesion mechanism resulted detectable by MCFS, through the analysis of the approaching curves and pull-off forces. The more electronegative specie (D21f2: zeta-potential = -42.3 mV) resulted less attracted by PMMA surface with respect to the less electronegative one (D21: zeta-potential = -28.9 mV).

Hydrophobic and electrostatic interactions were confirmed to play a dominant role in the adhesion of bacteria onto surfaces by Ong et al. [75] and by other subsequent studies [69, 70, 73, 74, 86], which highlighted the congruency between the DLVO and extended DLVO theoretical models and MCFS experimental data.

Several authors investigated the correspondence of MCFS data with theoretical models describing bacterial adhesion mechanisms. Harimawan et al. [84] found a qualitative agreement between MCFS data and classical DLVO and XDLVO models in their study on the adhesion of *Bacillus subtilis* spores and vegetative cells onto stainless steel surfaces. In other works [75, 83] discrepancies between experimental data and DLVO and XDLVO predictions were observed, while a good agreement was found using an 'augmented DLVO model' incorporating not only van der Waals, electrostatic and hydrophobic interactions, but also a steric component, as previously proposed by de Gennes et al. [93].

The increasing of adhesion force between bacteria and substratum surfaces with the increasing of contact time, previously observed by Bowen et al. [77], has been further investigated by Boks et al. [66] with the aim of analyzing the role of substratum hydrophobicity on the bond strengthening between bacteria and surfaces. The analysis of *Staphylococcus epidermidis* versus hydrophilic glass and hydrophobic dimethyldichlorosilane (DDS) coated glass revealed a higher instantaneous adhesion force on hydrophobic surfaces, coherently with previous findings. Also, a higher bond strengthening was observed in correspondence of hydrophilic surfaces, which occurs after a characteristic time of around 30s and is ascribable to the progressive formation of hydrogen bonds.

Loskill et al. [68] extended the study of bacterial adhesion mechanism to the investigation of the role of substrate

subsurface composition, providing the experimental evidence that the bacterial adhesion is tailored not only by the surface properties but also by the substrate composition via long-range van der Waals forces.

MCFS has been also used and demonstrated to be a powerful tool for the investigation of the role, on bacterial adhesion mechanism, of proteins adsorbed onto surfaces. Non-specific binding and ligand/receptor binding were studied by Liu et al. [65] analyzing *S. epidermidis* adhesion on fetal bovine serum (FBS) and fibronectin (FN)-coated self assembled monolayers (SAMs). Proteins modified SAMs were found more favorable for bacterial adhesion than non-coated SAMs, confirming the important role of proteins on bacterial adhesion mechanisms.

After the first fundamental findings about the potentialities of the technique, MCFS found several applications in different fields where the understanding of bacterial adhesion mechanism is fundamental. In medical and biomedical field, MCFS has been used for example to study the interactions between uropathogenic strains of *E. coli* and uroepithelial cells [67]. In dentistry, MCFS has been used to study the effect of heating treatments on the adhesion of *Streptococcus mitis* ATCC49456, *Streptococcus oralis* ATCC 35037, *Streptococcus sanguis* ATCC 10556 to dental enamel [94], to investigate the effect of fluoride treatment of hydroxyapatite on the adhesion of oral bacteria (*S. oralis* and *Staphylococcus carnosus*) [82] and to measure the interactive force between co-aggregating and non co-aggregating oral bacterial pairs (Actinomyces cells immobilized and Streptococci) which are important in the development of dental plaque.

Also bioleaching phenomena have been investigated through the use of MCFS. The adhesion of *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*, which are acidophilic bacteria, essential to the extraction of metals from sulfidic ores, and chalcopyrite surfaces has been investigated by Zhu and coworkers [73, 74].

Biocorrosion phenomena have been studied by Sheng and coworkers, who analyzed the adhesion of anaerobic and aerobic sulfate reducing bacteria (*Desulfovibrio desulfuricans* and a local marine isolate *Pseudomonas spp.* on different metallic surfaces (aluminium, stainless steel 316, mild steel, copper) [70], in different pH conditions [71].

6. Conclusions

The working principle of MCFS, the methods to prepare bacterial cell probes and main applications of the technique have been reviewed and discussed. MCFS presents numerous advantages in respect to conventional experimental methods for the evaluation of bacterial adhesion to surfaces and represents an interesting tool to improve the comprehension of the physical and chemical phenomena regulating bacterial adhesion process. Nevertheless, all the potentialities of the technique have not been completely exploited yet due to some technological limitations, such as the lack of standardized protocols for firmly and reproducibly

immobilizing living bacterial cells on AFM probes.

These represent major challenges which have to be addressed to develop MCFS into a reliable technique for quantitative study of bacterial adhesion on surfaces.

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