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Topical Review

Single-bacterium nanomechanics in biomedicine: unravelling the dynamics of bacterial cells

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Abstract

The use of the atomic force microscope (AFM) in microbiology has progressed significantly throughout the years since its first application as a high-resolution imaging instrument. Modern AFM setups are capable of characterizing the nanomechanical behaviour of bacterial cells at both the cellular and molecular levels, where elastic properties and adhesion forces of single bacterium cells can be examined under different experimental conditions. Considering that bacterial and biofilm-mediated infections continue to challenge the biomedical field, it is important to understand the biophysical events leading towards bacterial adhesion and colonization on both biological and non-biological substrates. The purpose of this review is to present the latest findings concerning the field of single-bacterium nanomechanics, and discuss future trends and applications of nanoindentation and single-cell force spectroscopy techniques in biomedicine.

Keywords: AFM, bacterial adhesion, microbiology, nanomechanics, force-spectroscopy

(Some figures may appear in colour only in the online journal)

1. Introduction

Bacterial adhesion remains the focus of numerous research groups as it is considered one of the most important factors for bacterial colonization, pathogenesis and biofilm formation [1]. Bacteria have the ability of binding to both natural and artificial substrates such as industrial equipment, tubing, medical devices, prosthetic elements, mucosa and teeth [2, 3]. They can also adhere to other bacteria from the same or different species, developing intricate *biofilms* that possess strong antibacterial resistance and can be very difficult to eliminate [4]. Biofilm accumulation is a process which is not only confined to the medical environment, and several beneficial applications of biofilm have been reported in water remediation, microbial fuel cells, as protective layers against pathogenic organisms and in microbial leaching [5–8]. These applications would require bacterial adhesion to be enhanced rather than prevented. In biomedicine however, biofilms are the initiating point for several human pathologies ranging from *conjunctivitis* to *urinary tract infections*. In oral health, tooth decay and periodontal disease are initiated and maintained by the formation of a complex biofilm on the surface of teeth [9]. The long term stability and success of titanium dental implants can be severely compromised if colonized by biofilms [10, 11]. Therefore, characterizing bacterial adhesion

to inert or biological surfaces is a significant step in the search for new therapeutic approaches against biofilm-mediated pathologies.

The development of the atomic force microscope (AFM) in 1986 was a major breakthrough in modern microscopy [12]. During this year, Binnig *et al* introduced a new microscope capable of exploring surfaces by scanning the sample at a distance of only a few nanometres and recording the interaction between the sample surface and a specialized probe [13]. The capacity of the AFM to interact directly with a sample provides it with unprecedented capabilities and applications in many fields of science ranging from nanotechnology to biology [14, 15]. This allows modern AFM to (a) obtain high resolution images of biological samples, (b) acquire information on the nanomechanical properties of the sample (i.e. elasticity), and (c) measure adhesive forces between cells and surfaces [16, 17]. Furthermore, since little to no sample-preparation is necessary, biological samples can be imaged and probed in liquid environments in their live and physiological states without disrupting native morphology and properties [18].

As stated above, the many advantages of atomic force microscopy make it a reliable tool for biological research with diverse applications in biomaterials, medicine, food sciences and microbiology [19]. This review will focus on the latest findings concerning the field of single-bacterium nanomechanics, and discuss future trends and applications of nanoindentation and single-cell force spectroscopy techniques in biomedicine.

2. Importance of understanding the biomechanical properties of bacteria and their interaction with surfaces

One of the most important characteristics of microbial virulence is the ability of bacteria to interact and adhere to diverse molecules, surfaces and cells via membranes. Bacteria have been reported to interrelate with hard surfaces, host cells, and other bacteria from the same or different species [20]. The initial attachment of bacteria to surfaces occurs in a two-step process [21]. The first stage, known as 'docking stage', describes the non-specific reversible attachment between microbial cells and substrate mediated by long range interactions (i.e. Van der Waals, electrostatic forces). Once bacteria are immediately adjacent to the surface, specific and close-range interactions are produced as a result of ligand-receptor coupling and diverse other chemical connections [22]. In this 'locking stage', molecules on the bacterial surface and appendages such as fimbriae, pili and capsules interact directly with the host surface generating strong and irreversible binding [23]. This adhesion process between a microbial cell and surface depends on many factors including structure and properties of bacteria and substrate, the bacteria species involved and the surrounding environment [24].

The adhesion of bacteria to artificial surfaces and other microbial cells is the crucial initial factor in the formation and maturation of a *biofilm* [25]. A biofilm can be described as a highly organized population of adhered bacteria embedded in an exopolysaccharide and protein matrix [26, 27]. Biofilm formation comprises attachment of initial colonizers to a given substrate and consecutive co-aggregation of late colonizing species which change their bacterial composition, phenotype and pathogenic properties to adapt to the established biofilm setting [28, 29]. Biofilms allow bacteria to grow and survive in hostile environments, act as a reservoir for the dispersion of bacterial cells into the surroundings and present an increased resistance to antibiotic treatment and mechanical removal [30, 31].

Currently in biomedicine, biofilm-mediated diseases continue to be highly problematic for health care systems around the world [32, 33]. Despite great progressions in biocompatibility, *Staphylococcus*-related colonization and infection continue to be a major problem in orthopaedic implants [34]. *Pseudomonas aeruginosa* biofilms are a major threat in cystic fibrosis patients [35], and peritoneal dialyses catheters are prone to colonization and infection with *Staphylococcus epidermidis*, which can be nearly impossible to eradicate [36]. It has been clearly demonstrated that both tooth decay and periodontitis, the most frequent oral diseases, are caused by a complex bacterial biofilm build-up on tooth surfaces [37] and that presence of specific pathogenic bacteria such as *Porphyromonas gingivalis*, *Treponema denticola and Tanerella forsythia* in dental biofilm is strongly related to periodontal disease [38, 39].

Additionally, many worries currently exist regarding the increase of antibiotic resistant strains of bacteria such as MRSA (methicillin-resistant *Staphylococcus aureus*) and VRE (vancomycin-resistant *Enterococcus*) [40]. More alarmingly, reports of extensively drug-resistant and totally resistant strains of *Mycobacterium tuberculosis* are a threat for healthcare systems around the world [41, 42]. Bacteria have shown resistance to an elevated number of antibiotics available in the market, and the availability of new drugs for therapies against non-susceptible strains is very limited for years to come [43]. These worrying facts have emphasized the importance of characterizing and understanding the biophysical properties of bacteria related to (a) adhesion and colonization of biological and non-biological substrates and (b) bacterial nanomechanical properties such as cell wall elasticity and adhesive behaviour, for the development and evaluation of new effective antibacterial therapies. Furthermore, describing the fundamental forces at play during the initial attachment of bacterial cells to surfaces is essential for understanding the process of biofilm establishment and subsistence.

3. Bacterial nanomechanics

The basic setup on which the AFM is configured is 'simple' yet very effective. The *tip* or *probe* of the AFM is scanned over the sample either in direct contact or in the close vicinity of the surface [44]. The tip is attached to the end of a silicon-nitride *cantilever*; hence variations on the surface height and topography will cause some degree of vertical deflection on

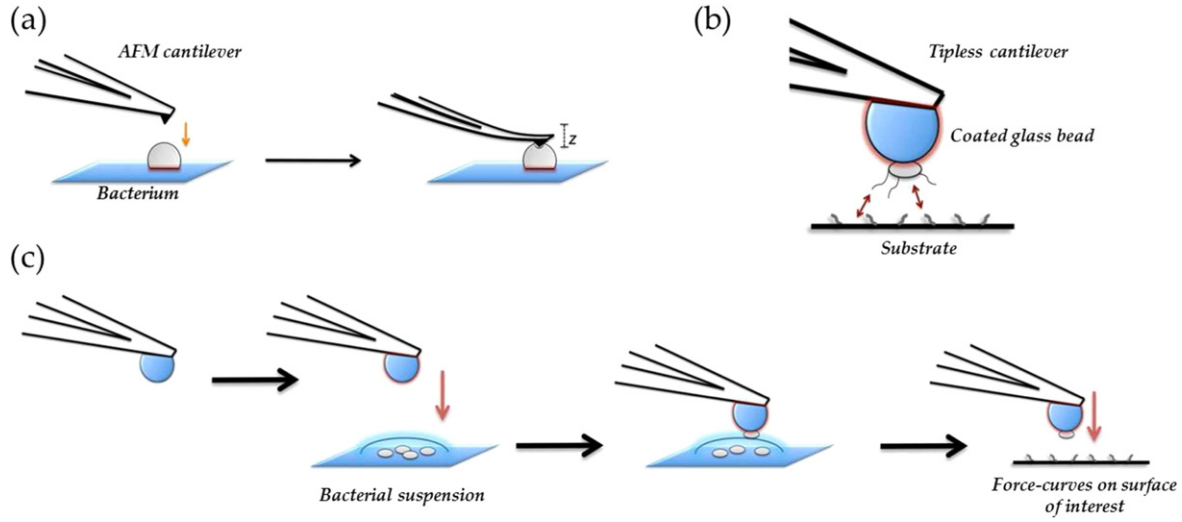

Figure 1. Schematic representation of bacterial nanomechanics experiments for (a) nanoindentation and (b), (c) single-cell force spectroscopy (SCFS). In nanoindentation techniques, a bacterial cell is immobilized on a substrate and an AFM probe is approached until a certain degree of indentation occurs on the cell surface. Plotting the cantilever deflection versus movement (z-distance) can give information on bacterial elasticity through Young's modulus (a). SCFS requires functionalization of a cantilever with a single bacterium to measure adhesive interactions between the sample and the bacterial surface (wall and appendages) (b). This can be obtained by attaching glass microspheres to tipless cantilevers and coating them with an immobilizing biological substance (i.e. poly-L-lysine, poly-DOPA). Modern AFM can effectively measure adhesive forces between the probe and the sample by transducing vertical deflection into force, and generating a correspondent force curve for analysis.

Table 1. Summary of immobilization techniques used to attach bacterial cells to surfaces (for nanoindentation) or modified cantilevers (for single-cell force spectroscopy, SCFS).

Immobilization	Reference	Considerations
Physical entrapment	• Van der Mei *et al* (2000)	• *Streptococcus salivarius* captured in polycarbonate filtering membranes
Gel immobilization	• Beckmann *et al* (2006)	• Immobilization of *Escherichia coli* on gelatin-coated mica surfaces
Poly-L-lysine	• Da Silva and Teschke (2003)	• Immobilization of *E. coli* on poly-L-lysine covered mica
	• Ovchinnikova *et al* (2013)	• *Staphylococcus aureus* immobilized to poly-L-lysine treated tipless cantilevers
Poly-DOPA	• Beaussart *et al* (2013)	• *Lactobacillus plantarum* attached to poly-DOPA coated colloidal probes

the cantilever [45]. To perceive this deflection the AFM reflects a *laser beam* from the backside of the cantilever directly towards a *position-sensitive photodiode*; therefore, any bending in the cantilever will change the position of the laser beam on the detector [46].

The introduction of the AFM in microbiology has opened exciting new approaches to study the nanomechanic behaviour of bacterial cells (figure 1). The capacity of probing living bacteria under physiological conditions grants researchers with information on the elastic behaviour, membrane-molecule properties and turgor pressure of individual cells [47, 48]. Additionally, upon knowing the *spring constant* of a given cantilever based on its *sensitivity* and *resonant frequency*, calibration can be performed so that the vertical deflection can be transduced into force in accordance to *Hooke's law* ($F = kd$, where F is force, k is the cantilever spring constant and d is vertical deflection) [49]. Therefore, the AFM can precisely measure the adhesive force between the probe and the sample [50] and generate a *force curve* as a function of the distance between the both. Dwelling time (time the cell probe is in contact with the substrate) can be modified to obtain precise data on bond-strengthening of specific membrane receptors. The most important information yielded by force curves are (a) maximum adhesion strength, (b) adhesion force and (c) nature of binding interactions. The construction and interpretation of force curves will not be discussed in this review, since this subject has been considered extensively in recent publications [51, 52].

Finally, to assure reliability of data obtained by bacterial nanomechanic experiments, it is important to note that firm attachment of bacterial cells to probe or sample surfaces must be achieved [53]. Before attachment of a bacterium, cantilevers and substrates must be coated with an adhesive substance to promote firm adhesion throughout the force measurements and avoid detachment as a result of vertical and lateral shear forces [54]. Some substances previously employed for cell immobilization in AFM nanomechanic experiments include glutaraldehyde, poly-L-lysine, and polyethyleneimine [55–59] (table 1).

3.1. Nanoindentation of single bacterium cells

Atomic force microscopy allows accurate measurement of cell elasticity and hardness. After immobilizing a single cell to a glass surface, an AFM probe is approached towards the cell surface with a determined loading force until a certain degree of indentation occurs [60]. By plotting the deflection of the cantilever versus the *z*-direction movement, a force curve is obtained by which the Young's modulus can be calculated for each cell. As a result, bacterial cell elasticity can be studied under different conditions and give insights on cell properties and behaviour. Several studies have successfully determined the elastic and hardness properties of living bacterial cells. Mechanical properties of *Escherichia coli* membranes were characterized by probing the cell surface with an AFM cantilever by Longo *et al* [61], where researchers found that the membrane of *E. coli* was not mechanically uniform but presented stiffer areas possibly associated with intracellular structures. Furthermore, AFM nanoindentation was employed to study the mechanical properties of seven different bacterial strains in efforts to understand their aggregation behaviour [62]. Authors demonstrated a strong correlation between the studied nanomechanical parameters and the macroscopic aggregation of these strains, providing further evidence on the effectiveness of AFM nanomechanics for predicting bacterial behaviour.

Indentation nanomechanics were also used to demonstrate the effect of two antibacterial agents, ticarcillin and tobramycin, on the cell wall of *P. aeruginosa* [63]. Subsequently, the same authors determined the mechanism of action of a novel antibacterial agent on the cell wall of resistant strains of *P. aeruginosa* [64]. Bacteria treated with ticarcillin and tobramycin had lower Young's modulus than untreated cells, and moreover, novel-drug CX1 treatment dramatically reduced cell elasticity. In another *in vitro* study, researchers investigated the effect of a low-molecular weight alginate oligosaccharide (*OligoG*) on the mechanics of *Acinetobacter baumannii* and *P. aeruginosa* biofilms [65]. Similarly, Wu and Zhou employed nanoindentation essays to evaluate the Young's modulus of *Mycobacterium sp*. before and after treatment with antimycobacterial agents ethambutol and isoniazid [66], providing new understandings on the biomechanical interactions between antimycobacterial drugs and *Mycobacterium* cell wall components.

Some considerations must be made when studying bacterial cells with nanoindentation techniques. Bacteria are not homogeneous in nature and therefore, traditional colloid-science deformation models should be modified to take into account the heterogeneous structure and mechanic behaviour of microbial surfaces [67]. To solve this issue, Chen *et al* proposed a new model to evaluate elastic deformation of a bacterial cell under an applied AFM load [68]. Researchers employed strains of *Streptococcus salivarius*, *S. aureus* and *S. epidermidis* attached to tip-less cantilevers and recorded the *z*-displacement against loading force, until a maximum force of 3 nN was reached. By comparing strains with different surface thickness and characteristics they suggested gram-positive bacterial cells show two distinct areas of elastic behaviour being (a) a deformable 'cylinder-like' contact area on the cell surface and (b) the rigid cell core. Bacteria stiffness was found to be strongly related to the mechanics of the contact area, suggesting a strong correlation between bacterial surface characteristics and elastic behaviour.

3.2. Force spectroscopy of bacterial cells

3.2.1. Functionalization of AFM cantilevers for adhesion probing. For a force curve to have significance in microbiology, an AFM tip must be functionalized with a bacterial cell or molecule for probing against a surface of interest [69]. Ideally, to characterize bacterial adhesion on the cellular and sub-cellular levels, a single bacterial cell should be employed to obtain force curves on a substrate of interest. Multiple bacterial cells attached to a probe can give inconsistent readings, and an unknown number of cells during probing make it nearly impossible to replicate the same measurements and compare different studies. Nowadays, *single-cell force spectroscopy* of bacteria is an interesting approach to evaluate adhesive interactions between cell-surface and cell-cell components.

As above mentioned, single-cell force spectroscopy requires the functionalization of a cantilever with a single bacterium cell [70]. This process is not simple and therefore previous treatment of the cantilever is necessary to ensure integrity of the modified probe throughout the readings. As previously mentioned, glutaraldehyde, poly-L-lysine, and polyethyleneimine have been utilized for immobilization, however, there have been some concerns on the effectiveness of some of these substances to maintain cell viability and effective attachment throughout measurements [71, 72]. An interesting and promising alternative for cantilever functionalization is the use of probes coated with the bio-inspired adhesive polymer *poly-dopamine* [73, 74] (table 2). Kang and Elimelech described *poly-DOPA* for functionalization of AFM cantilevers with single bacterial cells for force spectroscopy. The authors immobilized *E. coli* on poly-DOPA coated cantilevers and compared their adhesive behaviour to that of glutaraldehyde-fixed cell probes. They reported glutaraldehyde not only inactivated the cell probes but also affected the adhesive properties of the bacteria when measured on quartz [75], whereas poly-DOPA did not affect cell viability before and during measurements. In later studies, *Massilia timonae*, *P. aeruginosa* and *Bacillus subtilis* biofilm formation on steel was studied with DOPA-coated functionalized cantilevers [76], where adhesion forces were successfully recorded for all strains.

Recently, researchers raised some concerns about viability of bacteria attached directly to silicon-nitride cantilevers, possibly due to overheating by laser reflection after repetitive readings [71]. Furthermore, these techniques do not always allow uniform contact between the microbial cell and studied surface as a result of random placement of the single bacterium on the cantilever tip. To overcome this issue, Beaussart *et al* developed a protocol by attaching a DOPA-coated glass micro bead to a tip-less cantilever, under which a single *Lactobacillus plantarum* cell was immobilized to

Table 2. Recent experiments employing poly-DOPA modified cantilevers for measuring adhesion forces in live bacterial cells.

Authors	Probe preparation	Cantilever spring constant (k)	Additional remarks
Kang and Elimelech (2009)	• Poly-DOPA coated cantilevers • Bacterial cells attached directly to cantilevers	• 0.058 ± 0.005 N m^{-1}	• Cell viability maintained during experiments
Harimawan *et al* (2011)	• Poly-DOPA coated cantilevers • Bacterial cells attached directly to cantilevers	• 0.12 ± 0.01 N m^{-1}	• Bacterial binding confirmed by SEM measurements
Beaussart *et al* (2013a)	• Poly-DOPA coated microsphere glued to a tipless cantilever • Bacterial cells attached to microsphere	• Not reported	• Poly-DOPA did not affect adhesion measurements • Colloidal probes allowed control of contact area between bacterium-substrate
Herman *et al* (2013)	• Poly-DOPA coated microsphere glued to a tipless cantilever • Bacterial cells attached to microsphere	• Not reported	• Cell viability maintained during experiments
Sullan *et al* (2014)	• Poly-DOPA coated microsphere glued to a tipless cantilever • Bacterial cells attached to microsphere	• 0.04–0.08 N m^{-1}	• Cell viability maintained during experiments

assess viability and adhesion to a quartz surface. Reported advantages of utilizing a colloidal probe for bacterial force spectroscopy include (a) better control of the bacteria-substrate contact area, and (b) cell viability maintained throughout experimentation. More importantly, the use of poly-DOPA does not interfere with adhesion measurements during force probing of single cells.

Further work carried out by Herman *et al* evaluated the adhesion of *S. epidermidis* to fibrinogen-coated surfaces [77]. Interestingly, adhesion frequency and mean adhesion forces increased with longer dwelling times although some cells did not show this phenomenon, probably due to the expected heterogeneity of bacterial samples. In yet another study using functionalized colloid probes, researchers investigated pili-mediated adhesion of *Lactobacillus rhamnosus* GG to mucin, epithelial cells and hydrophobic surfaces [78]. The study reported adhesion forces governing *L. rhamnosus* GG are dependent on the nature of substrate and presence of pili on the bacterial surface, and confirmed previous findings that DOPA-coated colloidal probes allow consistent force measurements without affecting cell viability.

In summary, the use of coated colloid functionalized probes appears to be a reliable approach for the *in vitro* study of microbial adhesion. This technique is simple, allows effective positioning of a single cell, preserves cell integrity and viability, and yields consistent results when used for measuring adhesion between bacteria and both biological and non-biological surfaces.

3.2.2. Probing bacteria–surface interactions. Understanding the initial adhesion process between bacteria and hard surfaces is of vital importance for many fields of biomedicine and materials science. The AFM has proven to be a dependable tool for the characterization of forces in the *nano*- and *pico*-newton scales [79], and therefore, reliable information regarding adhesion forces between microbial cells and non-biological substrates can be obtained (table 3).

Many studies have employed AFM force spectroscopy with microbial probes to evaluate interactions between bacteria and different substrates. Loskill *et al* investigated the adhesive behaviour of *Staphylococcus carnosus* when placed into contact with hydrophobic and hydrophilic modified silicon wafers [80]. By employing native and thickened silicon oxide layers, they concluded that surface composition of the substrate directly influences bacterial adhesion mainly by differences in the strength of van der Walls forces. In another study focusing on bacterial adhesion to hard surfaces, Zhang *et al* evaluated the binding of *E. coli* to corundum and hematite nanoparticles [81]. Authors observed variations in adhesion force depending on the size of the bacterial cell, attributing this to differences in the effective contact area between cell and surface. Although this investigation was mainly focused towards environmental research, authors suggest these findings can also be applied to future biomedical studies.

Single-cell force spectroscopy is also a useful technique to determine the adhesive interaction between bacteria and

Table 3. Summary of latest findings in the field of AFM bacterial force-spectroscopy.

Authors	Bacteria species	Immobilization agent	Main findings
	Gram positive (+)		
Younes *et al* (2012) [82]	*Staphylococcus aureus*	• Poly-L-lysine	• *S. aureus-lactobacilli* adhesion force was stronger (2.2–6.4 nN) compared to *S. aureus* co-aggregation (2.2–3.4 nN)
Mei *et al* (2009) [83]	*Streptococcus mitis, sanguinis, sobrinus and mutans*	• Poly-L-lysine	• Early tooth-colonizing bacteria showed stronger adhesion to enamel compared to late strains • Increasing contact time from 0 to 120 s leads to increased adhesion forces (0.7 nN compared to 10.3 nN respectively)
Le *et al* (2013) [89]	*Lactococcus lactis*	• Polyethylenimine	• Characterization of adhesion of *L. lactis* to pig gastric mucin (PGM) coated surfaces
Beaussart *et al* (2013) [90]	*Staphylococcus epidermidis*	• Poly-DOPA	• Adhesion of *S. epidermidis* to *Candida albicans* is mediated by Als proteins and O-mannosylations expressed by *C. albicans* in different stages of development
	Gram negative (–)		
Zhang *et al* (2011) [81]	*Escherichia coli*	• Gelatin	• Adhesion of *E. coli* to nanoparticle-coated surfaces is influenced by particle size
El-Kirat-Chatel *et al* (2014) [93]	*Pseudomonas fluorescens*	• Poly-DOPA	• LapA mediated adhesion for *P. fluorescens* was characterized by employing wild-type and LapA+ mutant bacterial cells
	Mixed species		
Qu *et al* (2013) [85]	*Staphylococcus aureus, Pseudomonas aeruginosa, Serratia marcescens*	• Not reported	• Bacterial adhesion to Ag-coated lens cases is lower than to poly-propylene surfaces (0.6 ± 0.6 nN versus 11.9 ± 8.8 nN for *P. aeruginosa* at 90 s dwelling time)

different cell types. The capacity of *S. aureus* to adhere to mucosa and co-aggregate with each other is a critical step in the pathogenesis of female urogenital infections, being a determining factor for epithelial-biofilm formation in patients with bacterial vaginosis. However, certain probiotic lactobacilli strains have been shown to prevent or resolve these established infections. In an effort to explain the biofilm-disrupting effect of lactobacilli, Younes *et al* compared the adhesion forces between lactobacilli and *S. aureus* to the forces mediating staphylococcal co-aggregation [82]. Poly-L-lysine was used for immobilization and all measurements were carried out in physiological solution. Authors reported both maximum adhesion force and adhesion energy between *S. aureus* and Lactobacilli were significantly increased compared to *S. aureus* co-attachment in a time-dependant manner. It is important to note that in the above studies, although authors used the same cantilevers and consistent results were obtained, cantilevers were covered with an undetermined number of bacteria and therefore effective single-cell measurements is debatable.

Recently, AFM bacterial probes have also been utilized to evaluate interactions between oral bacteria and hard tooth surfaces. To study the nature of the forces responsible for bond-strengthening of four oral *Streptococci* strains to saliva-coated enamel surfaces, Mei *et al* employed Poisson analyses of adhesion-force distribution obtained by AFM nanomechanic probing [83]. Researchers observed that both maximum-adhesion force and number of minor adhesion peaks increased with longer surface delay times. Also, initial adhesion was significantly weaker than after bond-strengthening for all studied strains. Increased hydrogen-bonding forces found for initial colonizing streptococci could help explain the higher affinity of these strains to saliva-coated enamel, providing interesting new insights on the biophysics of early-phase dental biofilm formation.

A later study by Wessel *et al* employed AFM bacterial probes to measure the interaction of microbial cells to saliva-coated enamel surfaces [84]. Using bovine tooth surfaces coated in human saliva, they evaluated the attachment forces of seven strains of oral bacteria. Adhering microbiome bacteria were found to present higher overall adhesion forces than planktonic bacteria, demonstrating that attachment forces towards tooth surfaces determine the adhesive behaviour of each strain. These findings reflect the importance of the initial bacteria–surface interactions for bacterial adhesion and colonization of hard surfaces leading towards biofilm formation in the oral cavity.

An interesting approach to measure bacterial–hard surface interactions with AFM force spectroscopy has been employed by Qu *et al* for the study of ophthalmological devices [85]. In this paper, the adhesion strength of *P. aeruginosa*, *S. aureus*, and *Serratia marcescens* to contact lens (CL), polypropylene and Ag-impregnated cases was evaluated. For all three strains, higher surface delays increased adhesion forces and bond-strengthening occurred at dwelling times of 10–30 s. Ag-impregnated surfaces decreased the adhesion force of bacteria compared to CL and standard polypropylene. Based on their findings, researchers suggested combining Ag-impregnated lens-cases and an antimicrobial lens solution for increased antibacterial efficiency in the maintenance of contact lens.

In a similar study, authors studied the adhesion of nine bacterial strains involved in microbial keratitis to contact lenses and two polypropylene lens-cases [86]. Polypropylene cases modified with a silica nanoparticles-based brush-coating showed up to a 10-fold reduction in bacterial adhesion. AFM measurements were paired with biofilm removal essays to demonstrate that the decreased adhesive force on brush-coated polypropylene was related to a more effective cleaning process, and authors consequently concluded that the surface of lens-cases plays an important role in the adhesion of all studied strains.

3.2.3. Identification of important cell-surface binding receptors for bacterial adhesion. The specific adhesion of bacteria to surfaces, cells and molecules is mediated by both membrane receptors (i.e. adhesins) and characteristics of the substrate and therefore, it is important to understand the dynamics of the interaction between individual ligand molecules and their respective receptors. Highly precise AFM techniques have been developed to characterize the morphology and function of these molecules and complement existing data from traditional microscopy methods [87]. Therefore, precise information can be obtained on the ligand-receptor complexes that modulate bacterial adhesion to hard biotic and abiotic surfaces.

In a study carried out by Gilbert *et al* [88], AFM tips were coated with the antibiotic drug vancomycin and used to map individual receptor-ligand sites on *Lactococcus lactis.* Vancomycin binds to the bacterial cell wall, more specifically, to the D-Ala-D-Ala terminal of peptidoglycan precursors. Gold AFM cantilevers were covered in a ~1 nm thick layer of vancomycin, and living bacteria were immobilized for probing using porous polymer membranes to ensure cell vitality. Authors reported an increased number of binding sites in the septum region of dividing *L. lactis* cells which are consistent with areas of newly formed peptidoglycan. Moreover, when employing a D-Ala-D-Lac mutant strain of *L. lactis* they reported a significant decrease in adhesion events, suggesting that researchers were able to effectively probe D-Ala-D-Ala terminals with single-molecule functionalized cantilevers. This paper demonstrates that force spectroscopy with modified AFM probes is a valuable method for studying ligand-receptor dynamics in living bacteria.

In another recent paper, Le *et al* measured the interaction of pig gastric mucin (PGM) and *L. lactis* using force spectroscopy [89]. Cantilevers were coated with polyethyleneimine and bacteria were attached directly to the coated tips, and short and long distance interactions (100–200 nm and 600–800 nm respectively) were evaluated. AFM measurements with mucus-binding protein and *pili* defective strains of *L. lactis* demonstrated the influence of each of these receptors in the adhesion to PGM. *Pili* defective bacteria showed a drastic reduction of long-distance interactions, which is consistent with the absence of elongated *pili* appendages on the cell surface, whereas mucus-binding proteins were shown to be responsible for short-range adhesive events.

Candida albicans is a fungal pathogen usually found co-aggregated in infected sites with bacteria such as *S. aureus*. In a paper by Beaussart *et al* authors employed colloidal probes to evaluate the interaction between *S. aureus* and *C. albicans* [90]. A single *S. aureus* cell was attached to a glass bead coated with poly-DOPA and placed over an immobilized *C. albicans* cell. By comparing force curve measurements at different locations researchers were able to determine that *S. aureus* has a higher affinity to yeast tubes compared to yeast cells of *C. albicans*. Additionally, by employing mutant strains of *C. albicans,* they were able to suggest that Als proteins and O-mannosylation present on the fungal surface play a key role in cell adhesion to *S. aureus* by interacting with peptide ligands and lectin receptors on the bacterial membrane. These results are consistent with previous force-spectroscopy studies describing variations in adhesion forces between *C. albicans* and *S. aureus* along the fungal cell surface [91], where authors reported higher adhesion forces in the tip and middle portions of the *C. albicans* germ tube compared to the head region and yeast cells. Differences in polysaccharide and peptide composition on different regions of *C. albicans* may explain differences in adhesion strength to *S. aureus* when probed with AFM nanomechanics.

The probing of *bacterial footprints* with modified AFM probes is an interesting approach described recently by El-Kirat-Chatel and co-workers to identify cell–surface interactions and nanomechanics at the single-cell and single-molecule levels [92]. Bacterial footprints are adhesive biopolymers expressed by bacteria on the cell-substrate interface and are considered important for microbial adhesion. Authors recently analysed remaining bacterial footprints of *Pseudomonas fluorescens* on hydrophobic surfaces using single-molecule force spectroscopy (SMFS). *P. fluorescens* cells express the LapA adhesin, a membrane protein believed to participate actively in cell-adhesion. By using AFM probes functionalized with monoclonal anti-HA antibodies, the accumulation of *LapA* adhesins (containing an HA-tag) at the substrate surface following *P. fluorescens* colonization was described. Researchers demonstrated that accumulation of LapA is involved in the adhesion of *P. fluorescens* to hydrophobic surfaces, and that LapA remains on the surface of the substrate after detachment of bacterial cells has occurred.

In another study, the same authors further characterized the biophysical properties of LapA mediated adhesion in *P.*

fluorescens [93]. Firstly, they demonstrated with single-cell force spectroscopy that the presence of LapA protein on the cell surface increased adhesion of *P. fluorescens* to substrates. Researchers then compared the adhesion of wild type and hyper-adherent LapA+ mutant strains. Results show larger adhesion forces in the LapA+ mutants towards both hydrophobic and hydrophilic surfaces. Furthermore, using SMFS they characterized and mapped single LapA molecules on the surface of *P. fluorescens* cells. Findings indicate *P. fluorescens* express LapA adhesins at a surface density of ~450 sites/μm^2, which are regulated by both biofilm induction and LapA+ mutation. These findings provide new insights into the molecular mechanisms modulating LapA-mediated adhesion in *P. fluorescens*, and demonstrate the utility of the AFM for studying the mechanics of receptors involved in bacterial adhesion.

4. Where do we go from here?

As discussed throughout this review, AFM force spectroscopy of bacterial cells and molecules has contributed enormously to the understanding of the fundamental forces governing bacterial adhesion to surfaces. Bacteria are complex organisms with many membrane receptors mediating cell adhesion. Force nanoscopy has been employed to understand the dynamics of lipopolysaccharides, peptides and extracellular pili and their contribution in modulating bacterial attachment to surfaces [94, 95]. Although many publications have presented effective approaches to perform single-cell/single-molecule nanomechanics, difficulties such as single-bacterium attachment to cantilevers, effective immobilization of bacteria on substrates and reproducibility of results must be further addressed. Adhesion measurements with cell-spectroscopy are usually non-parametrical and tend to have large standard deviations which make it difficult to extract significant conclusions from the data [96]. Additional efforts should be directed towards developing standardized protocols in the hope of homologating data acquisition and interpretation for different species of bacteria.

Despite significant progress in microbiological research, bacterial colonization and biofilm-related pathologies continue to challenge the fields of food science, orthopaedics, medicine and dentistry [97–99], and therefore the characterization of bacterial virulence factors (i.e. adhesion) and development of antibacterial substrates and agents remain a priority. The improvement of existing force-spectroscopy methods coupled with higher availability of AFM equipment have widened the applicability of these procedures, and there is little doubt that the introduction and development of AFM force-spectroscopy techniques for bacterial nanomechanics offers promising new insights for future biomedical research [100].

Due to the remaining high prevalence of food-related infections, bacterial spectroscopy is being applied as a new method for describing the adhesive behaviour of common food-borne pathogens. Recent force-spectroscopy experiments carried out by Gordesli and Abu-Lail have aimed to characterize the adhesion properties *of Listeria monocytogenes* to silicon nitride surfaces and co-relate them to variations in bacterial temperature [101]. In another report, Goulter-Thorsen *et al* studied the effect of substrate roughness on the attachment of six strains of the food-borne pathogen *E. coli* to different stainless steel surfaces [102] and demonstrated that surface-induced differences in adhesion could be effectively probed by AFM force spectroscopy. These studies yield interesting information on the use of bacterial nanomechanics in the food science field; however, further publications are needed to characterize the nanomechanical behaviour of other food-borne pathogens as well as developing new approaches for disease control and prevention.

Another interesting applicability of bacterial nanomechanics is the development of novel antibacterial therapies against drug-resistant strains of pathogenic bacteria. Conventional methods of antibiotic studies are time consuming and cost ineffective, whereas the potential use of AFM cantilevers as '*nanomechanical biosensors*' can offer real-time results with high sensitivity [40]. The bending of AFM cantilevers due to molecule adsorption has been well documented in the literature [103, 104]. By coating one side of a cantilever with a desired molecule, deflection following ligand–receptor interaction under liquid conditions can be measured and quantified. This method can also be applied in the study of specific interactions between drug molecules and resistant strains of bacteria. Using this technique, one group studied the binding of vancomycin to cantilevers coated in mucopeptide cell-wall precursor analogues, in a multi-cantilever array [105]. By employing both susceptible and drug-resistant analogue precursors (D-Ala-D-Ala and D-Ala-D-Lac, respectively), researchers demonstrated that bending of the coated cantilevers was correlated with the binding ability of vancomycin. Further studies should focus on improving these nanoarray technologies by increasing their sensitivity/specificity and lowering costs, so that these techniques can be employed effectively in both scientific and medical circumstances.

Although certain improvements as user-friendly software and simplified methodologies have been introduced, the AFM continues to be a highly complex tool which non-experts may find difficult to operate. Clinicians and patients could greatly benefit from real-time biochemical and antibiotic resistance AFM essays, therefore, efforts should focus on the development of novel systems capable of being operated in hospitals and medical centres by trained personnel. Improvements in the field of bacterial force spectroscopy should consider real-time video capture during microbial force-spectroscopy measurements, pairing AFM with Raman spectroscopy for mechanical-chemical analysis of samples, and quantitative mechanical imaging.

Finally, it is important to stress that bacterial force-spectroscopy remains an *in vitro* technique and therefore, results may not always be consistent with *in vivo* behaviour of bacterial cells. The true biological environment in which microbial adhesion and colonization takes place is complex and involves many bacterial and host factors, and although

physiological conditions can be currently simulated by the use of liquid chambers, further headway must be made towards successfully translating experimental findings of bacterial force spectroscopy into *in vivo* implications and significances.

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