The biophysics of bacterial infections: Adhesion events in the light of force spectroscopy

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ABSTRACT

Bacterial infections are the most eminent public health challenge of the 21st century. The primary step leading to infection is bacterial adhesion to the surface of host cells or medical devices, which is mediated by a multitude of molecular interactions. At the interface of life sciences and physics, last years advances in atomic force microscopy (AFM)-based force spectroscopy techniques have made possible to measure the forces driving bacteria-cell and bacteria-materials interactions on a single molecule/cell basis (single molecule/cell force spectroscopy).

Among the bacteria-(bio)materials surface interactions, the life-threatening infections associated to medical devices involving *Staphylococcus aureus* and *Escherichia coli* are the most eminent. On the other hand, *Pseudomonas aeruginosa* binding to the pulmonary and urinary tract or the *Helicobacter pylori* binding to the gastric mucosa, are classical examples of bacteria-host cell interactions that end in serious infections.

As we approach the end of the antibiotic era, acquisition of a deeper knowledge of the fundamental forces involved in bacteria – host cells/(bio)materials surface adhesion is crucial for the identification of new ligand-binding events and its assessment as novel targets for alternative anti-infective therapies.

This article aims to highlight the potential of AFM-based force spectroscopy for new targeted therapies development against bacterial infections in which adhesion plays a pivotal role and does not aim to be an extensive overview on the AFM technical capabilities and theory of single molecule force spectroscopy.

1. The force is strong in infection

Antibiotics discovery in the early years of the 20th century has revolutionized medicine, allowing to save millions of lives that would otherwise be lost to infectious diseases (Davies and Davies, 2010). However, antibiotics overuse and misuse led to a dramatic decline in their effectiveness rates, mainly due to emergence of resistant bacterial strains (Lewis, 2013; Ventola, 2015). According to the Center for Diseases Control and Prevention (CDC), at least 2 million people become infected with antibiotic-resistant bacteria and more than 23,000 people die annually as a consequence of these infections (CDC, 2013). In fact, the World Health Organization (WHO) estimates that drug-resistant diseases could kill 10 million each year by 2050 and dramatically damage the economy. More and more common diseases, including respiratory tract and urinary tract infections, once reliably treated with a course of antibiotics, are becoming untreatable due to antimicrobial

resistance (IACG, 2019). As of today, antimicrobial resistance has reached global threat status and countermeasures to tackle superbugs are an urgent need.

The first event in infection and/or biofilm formation (Fig. 1) is bacterial adhesion to host cells and/or (bio)materials surfaces (Stones and Krachler, 2015).

Bacterial cell adhesion is mediated by a multitude of molecular interactions that are either non-specific (hydrogen bonding, hydrophobic, van der Waals, electrostatic and macromolecular forces) or specific (molecular recognition between receptors and ligands) (Busscher et al., 2008; Dufrène, 2015). Therefore, achieving a deeper understanding of the mechanisms of the molecular interactions involved in bacterial adhesion will ultimately allow to design better strategies to control infection, namely by preventing bacterial adhesion and biofilm formation. Downscaling knowledge to these molecular adhesion events has become a tangible reality in 1986, when Binning, Quate and Gerber

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developed the Atomic Force Microscope (AFM) to overcome the drawbacks of its predecessor technique, the scanning tunnelling microscope (STM) (Binnig and Quate, 1986).

In the next sections, a brief overview on AFM-based force spectroscopy technique will be given. Then, its use to untangle adhesion mechanisms of emergent bacterial pathogens that might be of vital importance for development of new infection therapies will be highlighted.

2. Atomic force microscopy (AFM)

In a very simplified approach, we can say that the AFM technique is based on the control of the force acting between the AFM tip and a sample surface (Binnig and Quate, 1986).

2.1. AFM-imaging

In the microbiology field, the AFM high-resolution imaging capacities have largely contributed to this technique growing success as it has made possible to image the structure and ultrastructure of various types of bacterial cells (Beveridge and Graham, 1991; Dufrêne et al., 2017; Maver et al., 2016; Pillet et al., 2014). The main advantages of AFM-imaging over other techniques, like fluorescence microscopy, are that it does not require cell manipulation prior to the experiments (e.g.: staining, fixation,…), can be done in a single cell, is non-destructive and allows to perform experiments in real-time and in physiological conditions (e.g.: in aqueous buffer, controlled temperature and pressure) with nanometer resolution (Dufrêne, 2002; Eghiaian et al., 2014; Mozes et al., 1991). Altogether, these features impact the cells viability and, consequently, the biological strength of the obtained results. More recently, High Speed Atomic Force Microscope (HS-AFM) introduced imaging at nanometer resolution and with a sub-second frame rate (Ando et al., 2001, 2013; 2018).

2.2. AFM-based force spectroscopy: Measure small to think big

Force measurements allow unraveling physical properties and study molecular interactions (Leckband, 2000; Radmacher et al., 1992). Overall, these provide new insights into the structure–function relationships of microbial surfaces, namely receptor-ligand binding, adhesion of microbial to other cells and/or (bio)material surfaces at the single molecule level (Florin et al., 1994; Maver et al., 2016; Moy et al., 1994). Although there are other single molecule force spectroscopy tools, such as optical and magnetic tweezers, here we will only focus on the AFM-based technique.

The sample is mounted on a piezoelectric scanner, which ensures three-dimensional positioning with high resolution. The cantilever deflection is measured using an optical method (laser, photodiode) and the light is then reflected towards a position sensitive photodiode (PSPD). The deflection of the cantilever causes the laser beam to shift on the PSPD. Thus, the PSPD tracks the movement of the cantilever surface by sensing changes in the position of the reflected light. The cantilever deflection is then recorded as a function of the vertical displacement of the piezoelectric scanner, that is, as the sample is pushed towards the tip and retracted from it (bidirectional arrow). The force required for bond breakage is then calculated using the cantilever spring constant times the cantilever deflection (Fig. 2).

In AFM-based force spectroscopy mode, the forces measured are usually in the pN (10^{-12}N) regime and detection of the widest practical force range from 5 pN is possible since the AFM tip is able to probe an extremely small interaction area (tip radius of 5–50 nm) (Müller et al., 2009).

In the next subsections, the main aspects of an AFM-force spectroscopy experiment are briefly presented.

2.2.1. Approach-retract cycle & Force-distance curves

An approach retract cycle is the AFM tip indentation and retraction from the sample surface with controlled force and velocity (Fig. 3). An approach-retract cycle is illustrated in Fig. 3a and can be summarized in: (I) the functionalized AFM probe is approached to the sample with a controllable level of applied force; (II) contact with its binding partner (either a surface, cell or receptor) is promoted and attachment between
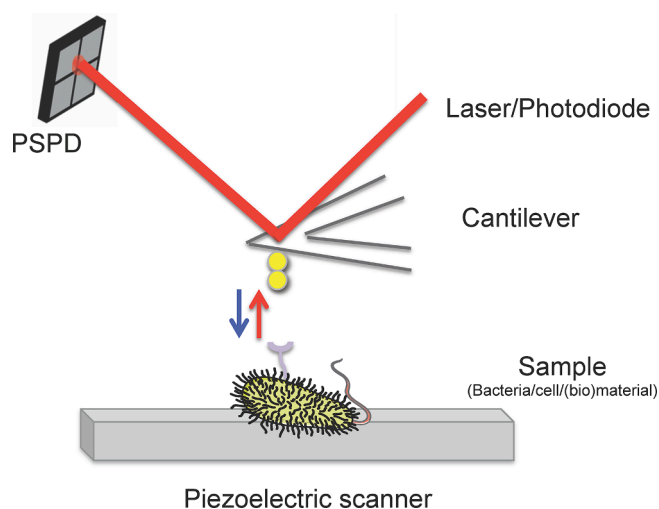
Fig. 2. AFM-based force spectroscopy general principle (schematic representation, not to scale). Adapted from (Maver et al., 2016).
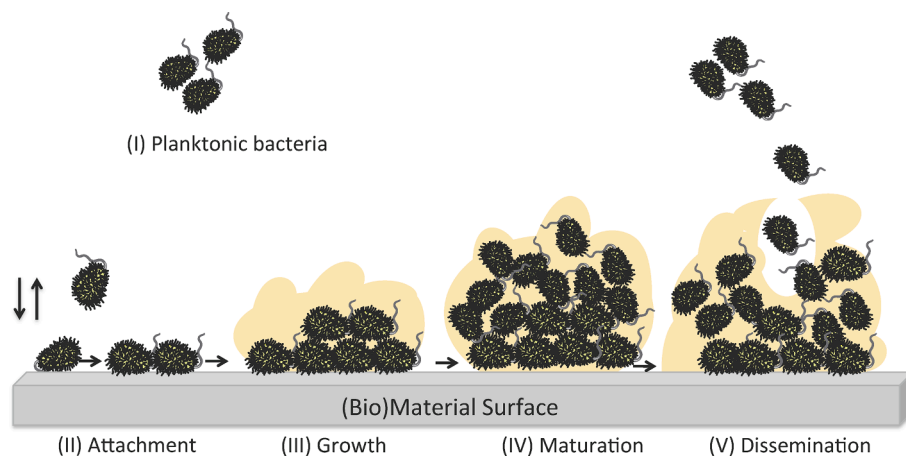
Fig. 1. Stages of biofilm formation on (bio)materials surfaces (schematic representation, not to scale). Adapted from (Vasudevan, 2014). (I) Planktonic bacteria; (II) Bacteria adhere to the surface in a dynamic process, mainly mediated by non-specific forces (hydrogen bonding, hydrophobic, van der Waals, electrostatic and macromolecular forces); (III) Cells aggregate, form microcolonies and start secreting extracellular polymeric substance. Simultaneously, bacterial attachment becomes irreversible; (IV) Cells form multi-layered clusters (3D growth) and further maturation of the biofilm occurs, which provides protection against host defense mechanisms and antibiotics; (V) Biofilm reaches a critical mass and disperses planktonic bacteria that may colonize other surfaces.
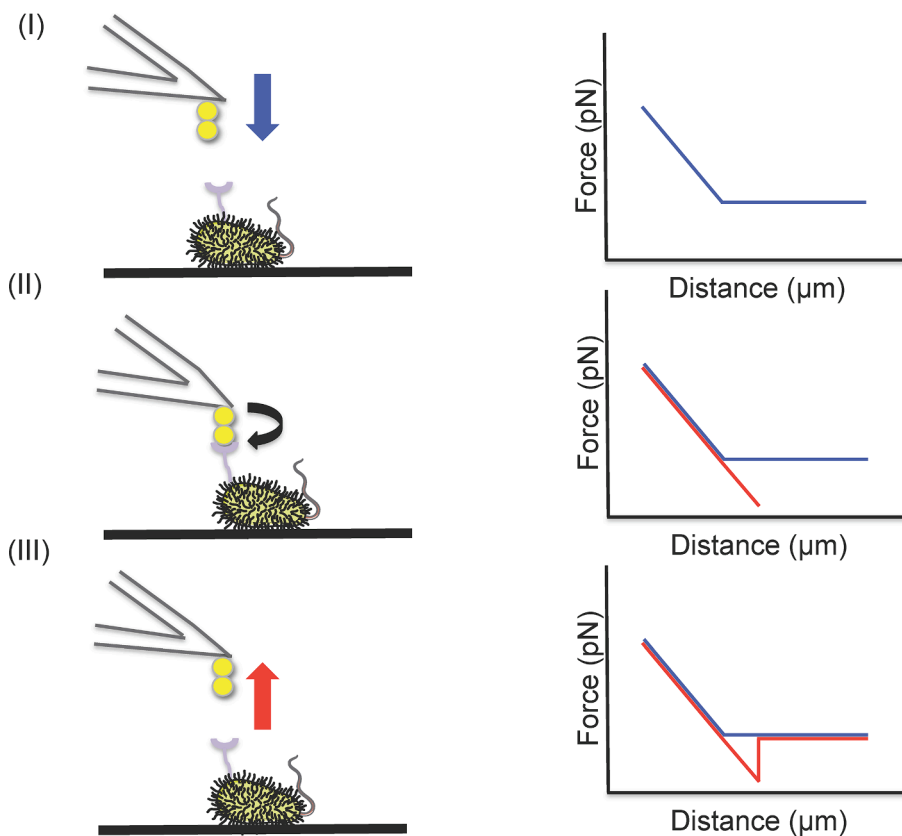
Fig. 3. (a) Approach-retract cycle (I) the AFM probe is approached to the sample with a controllable level of applied force; (II) contact and attachment between tip and sample surface is promoted, and (III) finally, the tip is retracted from the surface. (b) Typical force-distance curves showing the approach and retract parts of the cycle. (I) the tip is at a large distance and no interaction is measured; (II) the tip contacts with the ligand/cell/surface of interest; (III) an adhesive interaction between tip and surface is observed until the pull-off point is achieved. Adapted from (Maver et al., 2016; schematic representation, not to scale).

molecules is allowed, and (III) finally, the tip is retracted from the surface.

An approach-retract cycle may be performed at different locations to build a map of tip-surface interaction or can be repeated at the same time point to give a full statistical understanding of the interaction (Cappella and Dietler, 1999). Simultaneously, the tip-surface interaction is measured by recording the deflection of the cantilever, which yields a force–distance curve (Cappella and Dietler, 1999; Parreira et al., 2014) (Fig. 3b). As the AFM probe is pulled away, the established bonds between the two molecules are stretched until enough force is applied to rupture the bond. This process yields a force-distance curve, where the single peak represents the rupture event and the peak height indicates the magnitude of force required to disrupt the adhesion between the two molecules.

2.2.2. Rupture force, loading rate & bond strength

The rupture force associated to the binding event (F) can be calculated using the distance that the spring recoils after bond rupture (ΔD; deflection) and the spring constant (k_s), applying Hooke's law (1):

$$F = ks.\Delta D \qquad (1)$$

By definition, bond strength is the force that produces the most frequent failure in repeated tests of breakage, i.e., the peak in the distribution of rupture forces (Evans, 1999a, 1999b; Evans and Ritchie, 1997).

Evans and Ritchie established that the rupture forces measured will increase linearly with the logarithmically increase of the loading rate (the rate of force that is applied to a ligand-receptor complex for bond rupturing), an effect known as the "Bell-Evans effect" (Evans and Ritchie, 1997). Therefore, the bond strength is a dynamic property that is dependent upon the loading rate (Evans, 1999a, 1999b; Lo et al., 2002; Teulon et al., 2011).

2.2.3. Experimental challenges

2.2.3.1. Loading rate. When pulling a Hookean spring, the loading rate is calculated by the product of the cantilever force constant (spring constant) times the tip retraction velocity (pulling speed) and is influenced by:

i. cantilever force sensitivity;
ii. hysteresis between the tip and the sample surface. In general, dissipation can be understood as a hysteresis of forces between approach and retraction of the tip, being the latter caused by hydrodynamic forces acting on the cantilever during the movement (Hinterdorfer and Dufrêne, 2006; Lange et al., 2012);
iii. the cantilever's resonance frequency. Even after considering hydrodynamic contributions, the resonance frequency of the cantilever and its viscoelastic response time still limit AFM force measurements. To ensure a proper response of the cantilever, the frequency of any repetitive feature in the force spectrum should be significantly lower than the resonance frequency of the cantilever (Hinterdorfer and Dufrêne, 2006; Janovjak et al., 2005)
iv. cantilever quality factor (Q). Q, or the number of oscillations before complete damping out, is proportional to the spring constant and inversely proportional to the viscous drag coefficient and the resonance frequency (Ashkin, 1999; Valotteau et al., 2019).

However, when subjected to an external source, cells and biomolecules often do not behave linearly and more complex models, namely a worm like chain model, must be employed (Kratky and Porod, 1949; Marantan and Mahadevan, 2018).

2.2.3.2. Spring constant. For single-molecule force measurements, best results are obtained using short cantilevers (<50 μm) with small spring constants (0.01–0.10 N/m), as they have lower force/noise ratio (te Riet et al., 2011).

Most cantilever suppliers state an approximate spring constant. This is estimated from the nominal cantilever shape (length, width, thickness) and is not very accurate. It is advisable to perform cantilever calibration, which is commonly done with the thermal noise method (Butt and Jaschke, 1995).

2.2.3.3. The complexity of biological bonds & achieving single molecule events. Although the Bell-Evans effect has been and still is the backbone of force spectroscopy data analysis, biological bonds are complex systems. In this context, many biological experimental data do not fit within the theoretical expectations, mainly because there are multiple molecule events, instead of truly single molecule data (Chang et al., 2015; Fuhrmann, 2015; Fuhrmann et al., 2012; Getfert and Reimann, 2012; Raible et al., 2006). This gives rise to another important aspect that is the number of bonds formed at each tip-sample contact, *i.e*, how to try to guarantee specific single bond events. Evans stated that if the probe tip is decorated with a very low density of reactive sites, then its contact with the sample surface would only create an occasional bond. Under controlled conditions of contact, a low frequency of attachments in repeated trials provides quantitative verification of the likelihood of rare, single bond events (probability >0.9 of being at single molecule level when 1 attachment occurs out of 10 touches) (Evans, 2001). Thus, in order to use attachment frequency as a statistical estimator for rare single-bond events, each touch to the surface must have the same magnitude and history of contact force (time in contact before the tip is retracted, i.e., duration of contact). Nonetheless, it is still possible that a reactive pair of molecules may unbind and rebind many times before final separation (Evans, 2001). Experimentally, one of the most convincing and straightforward approach to demonstrate single bond events is to perform blocking experiments, in which free ligands added in solution interact with the molecule of interest (cell receptors, cognate ligands, etc) thus inhibiting their specific recognition by the functionalized AFM-tips (Hinterdorfer and Dufrêne, 2006).

In practice and because the formation and forced rupture of bonds between the tip and the sample is a stochastic process, force spectroscopy experiments usually rely on thousands of force-extension curves that are collected to obtain a representative distribution of the data, where the number of bond ruptures at each pulling rate plotted against the frequency/probability, usually follows Poisson distribution. Then, detachment forces are cumulated into a histogram (Fig. 4a). The force spectra represent the plot of the most probable force for bond rupturing measured at loading rates with different orders of magnitude, being visible the "Bell-Evans" effect, i.e, force increases with the increase on the loading rate (Fig. 4b).

It is recommended to record several hundred of force curves on different locations of the sample and the histogram must reflect the distribution of at least 100 unbinding events (Evans, 2001; Hinterdorfer and Dufrêne, 2006). The reliability and reproducibility of the measured unbinding forces should be demonstrated by comparing data obtained using many independent tips and samples. Finally, unbinding force histograms should be generated while varying the loading rate to obtain the force spectra (the most probable force plotted against loading rates) associated with the molecular interaction under study (Fig. 4b).

Another approach that has been developed aiming to guarantee single bond events is the use of "fingerprint domains". This method allows filtering thousands of force curves towards those presenting single molecule events more efficiently (Ott et al., 2017). A straightforward approach is to add a specific immunoglobulin (Ig) domain at either end of the molecule being studied. Since these Ig domains unfold with very specific patterns, if present in a force trace, it indicates that the molecule of interest has been correctly stretched (Milles et al., 2018; Verdorfer and Gaub, 2018).

2.3. Single-molecule & Single-cell force spectroscopy

AFM allows to measure interactions at the single molecule (SMFS) and at single cell (SCFS) level, allowing to investigate the forces and motions associated with biological molecules and yielding valuable insights into the binding mechanisms of bacterial pathogens (Fig. 5) (Dupres et al., 2009, 2005; Helenius et al., 2008).

SMFS force distance curves are acquired between AFM tips decorated with ligands of interest and cognate ligand/cell surfaces. On a typical SMFS experiment, the unbinding force (i.e. rupture force) of substrate–ligand interactions can be measured by analyzing the force-distance curve of the AFM. Therefore, SMFS quantifies the interactions between two immobilized molecules as a function of the force applied to the surface (loading rate) (Fig. 5a). The first SMFS studies using functionalized AFM tips focused on the avidin/streptavidin–biotin interaction (Florin et al., 1994; Moy et al., 1994). On the other hand, in SCFS studies, a single cell is immobilized on the AFM tip (Benoit et al., 2000) (Fig. 5b). When performing SCFS studies, the force-distance curves recorded are usually more complex and used to extract biophysical parameters that quantitatively describe cellular mechanics and interactions, namely: the detachment force (peak adhesion force), adhesion rate (fraction of force-distance curves with at least one detected force step with a peak force greater than a reasonable threshold) and work of detachment (energy dissipated during a detachment experiment). The number of receptor–ligand bonds, the stretching of the cell body during retraction (deformation) and the number and length of membrane tethers contribute to the total amount of detachment work (Friedrichs et al., 2013). SCFS allows studying the extent to which single-molecule properties and interactions contribute to the behavior of whole cells. The maximum detachment force, the work of detachment and the adhesion rate characterize the overall
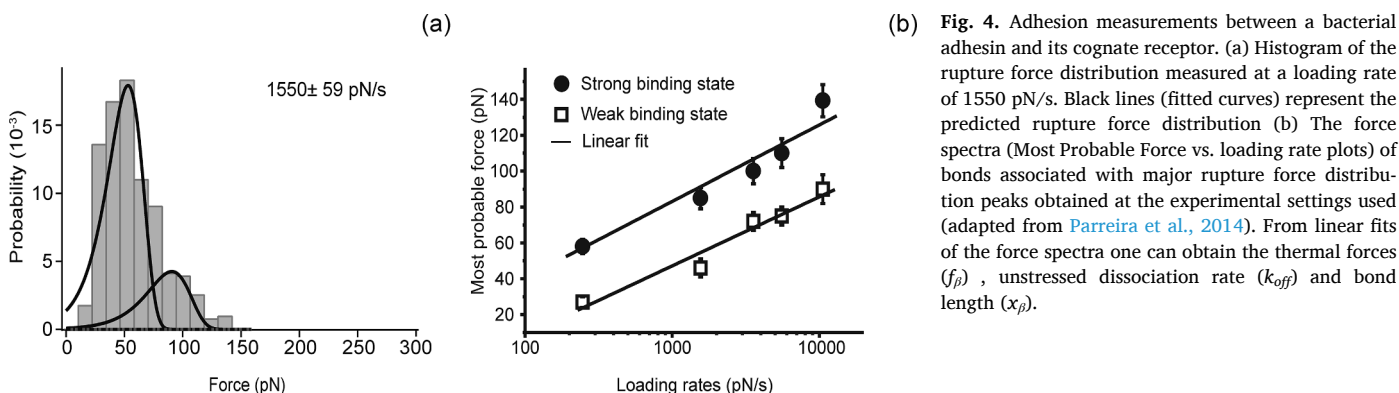

(a)

(b) **Fig. 4.** Adhesion measurements between a bacterial adhesin and its cognate receptor. (a) Histogram of the rupture force distribution measured at a loading rate of 1550 pN/s. Black lines (fitted curves) represent the predicted rupture force distribution (b) The force spectra (Most Probable Force vs. loading rate plots) of bonds associated with major rupture force distribution peaks obtained at the experimental settings used (adapted from Parreira et al., 2014). From linear fits of the force spectra one can obtain the thermal forces (f_β), unstressed dissociation rate (k_{off}) and bond length (x_β).
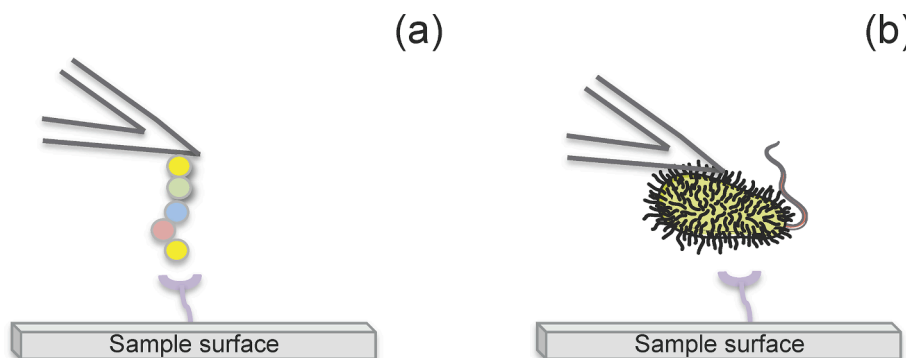
Fig. 5. (a) Single molecule force spectroscopy (SMFS) and (b) Single cell force spectroscopy (SCFS) experiments (schematic representation, not to scale).

adhesion of a cell.

The main challenge associated to SMFS/SCFS is how to attach a single molecule/cell to the cantilever and ensure that both cell viability and functionality are maintained and avoid changes in bioactivity. For that, a wide range of functionalizing chemistries have been developed to attach either a ligand, protein or even live cells to the AFM tip (Barattin and Voyer, 2008; Bowers et al., 2012; Neuman and Nagy, 2008; Xiao and Dufrêne, 2016). For instance, to attach bacterial cells, a straightforward approach is to use tipless AFM cantilevers coated with polyethylenimine (Razatos et al., 1998). Other approaches comprise the use of charged polymers (Le et al., 2011), silanes (Lower et al., 2001), hydrophobic alkanethiols (Emerson et al., 2006) or bioinspired wet adhesives (Kang and Elimelech, 2009). When only one cell is required on the tip, a micrometer-sized colloidal particle may be first bound to the cantilever, since the bacterial cell is much smaller than the cantilever. Then, the colloidal particle is coated with a wet adhesive, such as polydopamine, which allows adhesion of a single live cell to it (El-Kirat-Chatel et al., 2014). The different tip functionalization strategies are outside the scope of this mini-review (for more details see (Formosa-Dague et al., 2018)) but in common, all the (bio)functionalization chemistries have the use of non-adhesive spacer molecules (such as polyethylene glycol chains) and cross-linker molecules exhibiting the desired functional groups to anchor the desired ligands firmly at low density, while allowing flexibility and functionality (Hinterdorfer et al., 1996; Parreira et al., 2014; Xiao and Dufrêne, 2016).

In both SMFS and SCFS, a permanent challenge is how to distinguish in the retraction force curves the signatures originating from specific and non-specific interactions, which may simultaneously occur. Still, despite the experimental challenges associated, the combination of SMFS and SCFS is increasingly used in microbiology to decipher forces driving bacteria–bacteria, bacteria–host cell and bacteria–(bio)material surface interactions. Unraveling the binding mechanics (e.g., specificity, binding strength, among others) of bacteria may allow identifying potential novel drug targets and contribute for the design of alternative infection therapies.

On the next sections, the use of AFM-based force spectroscopy as a tool for designing and developing newer targeted therapies against some of the most prominent bacterial pathogens is presented.

3. *Staphylococcus aureus* & *Staphylococcus epidermidis*: Biofilm divas

Biofilms may form in different settings and surfaces, but here only biofilms-associated infections that develop on indwelling medical devices will be highlighted due to its massive negative impact in modern society. Nowadays, biofilm-associated infections represent a tremendous burden on the healthcare system, accounting for more than 65% of nosocomial infections (CDC, 2013). Biofilms are frequently refractory to antimicrobial therapy, leading to device removal/revision surgery with consequent societal and economical repercussion (Grainger et al., 2013;

Zander and Becker, 2018).

Biofilms begin with the adhesion of a few live planktonic cells that evolve to multicellular microbial communities (Kolter and Greenberg, 2006; Berne et al., 2018) (Fig. 1). This multifactorial process is determined by biological, chemical and physical properties from the environment, the surface and bacteria (Berne et al., 2018). Therefore, understanding the binding mechanisms and the forces driving initial adhesion steps to biomedical implants (endovascular prostheses, orthopedic prostheses, central venous catheters, artificial heart valves, etc…) may be the core question to answer.

AFM-based force spectroscopy, both SMFS and SCFS, have been used to understand and clarify cell adhesion and biofilm formation. In the first stage of biofilm formation, cells weakly attach to the surface/substrate through physical forces (e.g. van der Waals forces and electrostatic interactions). This process is facilitated by pili and flagella, which are found on bacterial surface and allow them to be surface-tethered and become less susceptible to being washed away by natural cleansing mechanisms of the organism (Berne et al., 2018; Kiedrowski and Horswill, 2011). However, depending on the implant site, implanted devices are rapidly coated with a layer of host plasma proteins to which bacteria can specifically and strongly bind via adhesin recognition. Surface adsorbed proteins, like fibrinogen and fibronectin, are specifically recognized by staphylococci adhesins, enabling the formation of strong bounds (Pacelli et al., 2016). Also, biofilm formation is dependent upon a family of adhesive proteins (Microbial Surface Components Recognizing Adhesive Matrix Molecules: MSCRAMM) (Foster et al., 2014; Nilsson et al., 1998; Otto, 2008).

The Gram-positive bacteria *Staphylococcus aureus (S. aureus)* and *Staphylococcus epidermidis (S. epidermidis)* are the ones responsible for the largest proportion of biofilm related infections on medical devices, resulting in significant morbidity and mortality (Percival et al., 2015; Kong et al., 2018; Jamal et al., 2018).

3.1. Staphylococcus epidermidis

Although there was already a considerable amount of structural and biochemical information regarding *S. epidermidis* adhesins, the force and dynamics that guide these interactions were poorly understood prior to the studies of Herman et al. (2014). Here, both SCFS and SMFS were used to study the interaction between *S. epidermidis* SdrG adhesin, part of the serine-aspartate repeat (Sdr) protein family, with fibrinogen (Herman et al., 2014), one of the host proteins commonly encountered recovering biomaterials (Foster et al., 2014). Using SCFS, a single bacterial cell was bound to a colloidal probe previously coated with polydopamine. Backlight LIVE/DEAD™ staining demonstrated that the immobilized cell was kept viable. Then, the adhesion forces between bacterium and fibrinogen-coated substrates were measured revealing an adhesion force in the range of ~2 nN, which is equivalent to the strength of a covalent bond. This is a much stronger bond than previously characterized adhesion-ligand pairs, which are usually in the 50–400 pN

range (Alsteens et al., 2010; Grandbois et al., 1999; Herman et al., 2014).

Using SMFS, the binding strength between SdrG adhesin and fibrinogen (Table 1), as well as the cell surface localization of the SdrG at molecular resolution, was identified (Herman et al., 2014). For that, fibrinogen-coated AFM tips were used. To maximize the chances of single-adhesin detection, the tip was functionalized with a PEG-benzaldehyde linker. Spatially resolved SMFS of live cells immobilized onto porous membranes allowed measuring the localization and binding strength of single adhesins. It was shown that SdrG forms nanoscale domains on the cell surface, which ultimately may contribute to strengthen cell adhesion and withstand shear forces, contributing for the *S. epidermidis* success in colonizing indwelling devices (Vanzieleghem et al., 2015). It was also observed that by increasing the interaction time, the adhesion probability also increased. This suggests that stable cell adhesion requires time-dependent conformational changes, which will ultimately impact the fate of bacterial adhesion onto fibrinogen-coated surfaces (Herman et al., 2014, 2013). Also, *S. epidermidis* strains that displayed higher amounts of the SdrG protein on its surface had enhanced adhesion to surfaces coated with fibrinogen (Vanzieleghem et al., 2015). In SCFS experiments, surfaces coated with poly-dimethylsiloxane (PDMS), fibrinogen and bovine-serum albumin (BSA) were prepared. Then, the binding force between the different surfaces and single *S. epidermidis* cells, which were bound to polydopamine-coated colloidal probe cantilevers, was measured. The obtained force

curves demonstrated high bacterial adhesion to the PDMS substrate, which is explained by a non-specific adhesion between the bacterium surface and the hydrophobic surface. These adhesion forces ranged from ~250 to 1500 pN depending on the *S. epidermidis* strain. Adhesion to fibrinogen-coated surfaces demonstrated some strain variability but the adhesion forces reported were around 2 nN in accordance to previous findings (Herman et al., 2014; Vanzieleghem et al., 2015). On the contrary, low adhesion was reported for BSA-coated surfaces (50–100 pN and in<20% of the curves). This was expected since BSA coatings are recognized for inhibiting cell − surface interactions.

SdrF is a collagen-binding cell surface protein that like SdrG belongs to the serine-aspartate repeat family of the *S. epidermidis* surface proteins (Bowden et al., 2005). Interestingly, collagen was the main extracellular protein found to be coating the surfaces of ventricular assist devices and the ability of *S. epidermidis* to adhere to type I collagen arose as pivotal in the infection pathogenesis (Arrecubieta et al., 2009, 2007). SdrF-collagen binding forces were measured at the whole cell level with SCFS studies, where one bacterium was attached to a cantilever coated with polydopamine and force-distance curves were recorded between cells and the collagen substrate (Herman-Bausier and Dufrêne, 2016). The force profile showed adhesion events ranging from ≈100 to ≈3000 pN and a rupture length in the range of ≈25–1000 nm. This study unraveled two distinct binding forces: a weak (≈362 pN) and a strong (≈1264 pN) binding force. The authors stated that these results reflected specific interactions between a single SdrF adhesin and collagen

Table 1
Bacterial adhesion strategies analyzed using AFM-based force spectroscopy and main findings.

Bacteria	Molecule studied	Main Findings	Reference
S. epidermidis	SdrG(fibrinogen-binding)	• 2 nN binding strength; • Low dissociation rate (steady cell adhesion) • Multistep binding model "dock, lock and latch" with dynamic conformational changes; • Higher surface SdrG amount enhances adhesion to fibrinogen-coated surfaces; • High adhesion to hydrophobic surfaces (250-1500pN) and low adhesion to BSA-coated surfaces (50-100pN); • SdrG protein mapped on bacterial surface.	(Herman et al., 2014) (Vanzieleghem et al., 2015)
	SdrF(collagen-binding)	• Dual-ligand-binding to collagen-coated substrates (strong: 1264 pN and weak: 362pN); • High dissociation rates; • SdrF protein mapped on bacterial surface.	(Herman-Bausier and Dufrêne, 2016)
S. aureus	Fibronectin-binding proteins	• Specific single-amino-acid polymorphisms give distinct binding force signature; • Mediate cell–cell adhesion via multiple low-affinity homophilic bonds.	(Lower et al., 2011)
	SasG protein	• Cell-cell adhesion during the accumulation phase of biofilm formation in a Zn^{2+} dependent manner; • 500pN maximum adhesion force; • 180 nm rupture length; • Establishes homophilic bonds with the Aap protein of *S. epidermidis* (multispecies biofilms).	(Formosa-Dague et al., 2016)
P. aeruginosa	Type IV pili	• Strong binding to hydrophobic surfaces in a time dependent manner (contact time increases adhesion strength); • Mechanical and shear stress resistance at the mucosal surfaces; • Adhesion to host epithelial cells depends on pili formation of membrane tethers from host cells; • 95pN adhesion force (mica surface)	(Touhami et al., 2006; Beaussart et al., 2014; Sullan et al., 2014)
	LecA	• Glycoclusters hinder interaction of the bacterial LecA with the Gb3 receptor.	(Zuttion et al., 2018)
E. coli	Type 1 and P pili	• Respond to external mechanical force (unwinding helical quaternary structure, pili 2–4 four times longer); • Steady state measurements, P and type 1 pili have unfolding force under equilibrium conditions similar ≈ 30 pN; • Dynamic force measurements (w/ surface): plateau region for P pili is 35 pN ; type 1 pili 60 pN; • Type 1 pili unraveling were determined to be fully reversible; • Type 1 pili is more rigid than P pili.	(Miller et al., 2006; Andersson et al., 2007)
	FimH(Type 1 pili)	• "Catch bond"; • Forces loaded quickly: FimH-surface rupture forces in the range of 140-180pN ( ) • Forces loaded slowly: FimH-surface bond rupture forces < 60pN	(Yakovenko et al., 2008)
	Curli(CsgA protein)	• 50pN rupture force for CsgA protein-fibronectin binding; • Multiple bonds with high tensile strength with fibronectin.	(Oh et al., 2016)
H. pylori	BabA	• Forms different adhesive attachments to the Le[b] determinant (enhances the efficiency and stability); • Bacteria specifically recognize and bind to synthetic immobilized receptors.	(Parreira et al., 2014)

molecules, since (i) this force pattern was not observed on cells that did not express SdrF and (ii) the addition of soluble collagen decreased the binding forces. Moreover, the long ruptures reported match the collagen structure, which is known to form 300-nm filaments that tend to self-associate (Herman-Bausier and Dufrêne, 2016). Furthermore, SMFS studies allowed the mapping of SdrF proteins on live bacteria and to quantify the strength of single SdrF-collagen bonds. It was unveiled that SdrF is exposed throughout the cell surface and that both weak and strong binding forces were randomly distributed. To ensure single molecule level detection, several factors were taken in account, namely: replicates were performed with different tips and the same force distribution profile was observed; collagen was attached in low density to the tip and both the weak and strong binding events between SdrF and collagen were observed on different cells (Ebner et al., 2007; Herman-Bausier et al., 2016). In summary and unlike the SdrG adhesin, the collagen-binding protein SdrF features a dual-ligand-binding activity to collagen-coated substrates, with a strong and weak bond involving two distinct regions of the protein (Table 1). The high dissociation rates of these bonds suggested that they were less stable than the SdrG-fibrinogen (Herman-Bausier and Dufrêne, 2016).

Altogether, the results obtained with AFM-based spectroscopy (SMFS and SCFS) demonstrated that both SdrG and SdrF are important for *S. epidermidis* success in establishing infection. SdrG mediates a specific, strong (2 nN) and stable (low dissociation rate) binding to fibrinogen, enabling steady cell adhesion. A multistep binding model using a "dock, lock and latch" approach with associated dynamic conformational changes was proposed (Herman et al., 2014). Another adhesin, SdrF mediates *S. epidermidis* adhesion to collagen-presenting substrates, although in a rather less stable interaction than the one recorded for SdrG. This knowledge is of high biomedical relevance as it allows understanding *S. epidermidis* ability for colonization of implanted biomaterials and highlights the importance of improving the chemistry of implanted surfaces to reduce the risk of infection.

3.2. Staphylococcus aureus

Like *S. epidermidis*, *S. aureus* also expresses a variety of cell surface proteins that enable binding to host extracellular matrix proteins and bacterial colonization of biomedical devices (Herman-Bausier et al., 2016; Nilsson et al., 1998a, 1998b) The binding forces between *S. aureus* and fibronectin proteins were measured using AFM-based force spectroscopy and *S. aureus* clinical isolates obtained from patients with an infected device. Distinct binding force signature as well as specific single-amino-acid polymorphisms were reported (Table 1) (Lower et al., 2011). The data, using *S. aureus* isolates and a fibronectin-coated AFM tip, disclosed that polymorphisms in fibronectin binding protein A and B lead to stronger and more resilient binding mechanisms. Herman-Bausier et al. (2015) used methicillin resistant *S. aureus* (MRSA) and underlined that its fibronectin-binding protein A mediates adhesion to neighboring cells via multiple low-affinity homophilic bonds, hence contributing to the biofilm dynamics. The low-affinity binding may be of important biological significance, as it provides resources that enable the bacteria to detach and colonize new sites during biofilm formation. Such homophilic cell–cell interactions are a widespread strategy among staphylococci to support biofilm accumulation. Hence, fibronectin binding protein-mediated homophilic interactions emerge as an attractive target to overcome MRSA biofilms (Feuillie et al., 2017). In practice, this knowledge may allow identifying and selecting isolates of *S. aureus* strains more prone to development of infectious biofilms and explore a more personalized and aggressive treatment.

Another *S. aureus* surface protein that mediates cell–cell adhesion is SasG. This protein, that was identified and characterized by SCFS, promotes bacterial attachment to adjacent cells during the accumulation phase of biofilm formation in a Zn^{2+} dependent manner (Formosa-Dague et al., 2016). SCFS measurements were performed between two *S. aureus* cells attached to colloidal probes via dopamine. In the absence

of Zn^{2+}, the adhesion frequency between cells, *i.e* cells that adhered to each other, was very low (5–31%) but when $ZnCl_2$ was added, adhesion frequency increased to 80–100%. It was also established that the force required to unfold individual domains was very strong, with a maximum adhesion force of ≈500 pN and a rupture length around 180 nm. This accounts for the ability of the SasG protein to withstand the high physiological shear forces and contributes to *S. aureus* success in colonizing and establishing infection. In addition, the work by Formosa-Dague *et al.* showed that SasG forms homophilic bonds with the structurally related accumulation-associated protein (Aap) of *S. epidermidis*, suggesting the possibility of formation of multispecies biofilms during host colonization and infection. The zinc dependent nature of this adhesion process, the strong nature of those homophilic bonds, as well as the hypothesis that under mechanical stress the SasG domains will unfold and expose extended conformations with enhanced adhesive properties were established (Formosa-Dague et al., 2016).

Virtually all staphylococci infections in indwelling medical devices would be prevented by avoiding the initiating bond in the early adhesion stages. With the identification of novel targets to hinder binding and the biophysical characterization of staphylococcal proteins binding mechanisms this is now a more tangible goal. Undoubtedly, the "smart bioengineering of biomedical devices" using the knowledge obtained with AFM, which provides the identification of new therapeutic targets and/or mechanisms to avoid/disrupt specific binding events/adhesins activity, is an extremely interesting path to follow on the quest to diminish the rate of staphylococci infections.

4. Pseudomonas aeruginosa: Pili are the key to success

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative bacteria that evolved from being an environmental species to one of the most prominent opportunistic human pathogens (De Bentzmann and Plésiat, 2011). The overuse and misuse of antibiotics and consequent selection of resistant strains has also largely contributed to *P. aeruginosa* success as an infectious agent (Moradali et al., 2017). Generally speaking, *P. aeruginosa* mainly targets the urinary and pulmonary mucosa due to its ability to bind to ligands exposed on mucins and to develop biofilms within the mucosal barrier that also shield bacteria from the antibiotics action (Landry et al., 2006; Zuttion et al., 2018). Regarding the pulmonary mucosa, *P. aeruginosa* is mostly associated with chronic infection of the lungs of cystic fibrosis patients in particular and immune-compromised people in general and is also associated to biofilm formation on respiratory stents (Gellatly and Hancock, 2013; Cole et al., 2014; Moradali et al., 2017). *P. aeruginosa* can also cause severe acute infections (bacteremia), when bacteria cross the mucosa and reach distant organs (Golovkine et al., 2018). Moreover, this notable pathogen is recognized by the ability to colonize the urinary tract, either by binding to the urinary epithelium or by forming biofilms on urinary catheters (catheter-associated urinary tract infection (UTIs)) (Moradali et al., 2017; CDC, 2013). These UTIs are associated with increased morbidity and mortality and are the most common cause of secondary bloodstream infections (Flores-Mireles et al., 2015).

However, how is *P. aeruginosa* able to sustain its infection status? The answer to this question might rely on Type IV pili (pili), one of the main virulence factors that contribute to success in such adverse settings (Leighton et al., 2015). Numerous studies have provided evidence that host glyco-conjugates, such as the glycolipid asialo-GM1 and asialo-GM2, are a common target for several bacterial adhesins that are present on pili and fimbriae, helping bacteria to bind to the epithelial cell surface, aggregate and start biofilm formation (Beaussart et al., 2014; Craig et al., 2004; Formosa-Dague et al., 2018; Golovkine et al., 2018; Krivan, 1988). Moreover, pili provide swimming and twitching motions, which enable the bacteria to explore the mucosa and to overcome the host's natural flushing systems (Lepanto et al., 2011; Golovkine et al., 2018). Hence, pili present an appealing target for the development of a vaccine, as specific antibodies could potentially block the attachment of

P. aeruginosa to the host cell receptors (Beaussart et al., 2014; Faezi et al., 2017). For that, it is critical to perform a thorough characterization of the nanoscale forces that drive *P. aeruginosa* type IV pili binding to either abiotic (medical devices) or biotic (epithelial cells) surfaces and endure attachment when subjected to high shear forces.

Touhami et al. (2006) applied AFM-based spectroscopy measurements to study *P. aeruginosa* type IV pili and its interaction with a mica substrate. For that, *P. aeruginosa* was attached to AFM cantilevers coated with poly-L-lysine. Then, to engage pili with the mica surface, the tip was brought in contact with the underlying mica surface and contact was made for 1 s, in order to allow pili filaments to spread and adhere. The contact between bacteria and the buffer-immersed mica surfaces was made with gentle forces (<200 pN) so as not to break or deform pili. In order to validate the obtained results, control experiments were performed using naked poly-L-lysine-coated AFM tips (no bacteria) or using a mutant strain for pili expression (without pili). The histogram of the rupture forces obtained with constant pulling speed displayed a single maximum and a mean magnitude of 95 pN. Assays using bare poly-L-lysine-coated probes or probes with a mutant *P. aeruginosa* strain yielded higher adhesion forces (~500 pN), indicating that the measurements with lower magnitude corresponded to specific interactions between the *P. aeruginosa* pili and the mica surface.

Adhesion forces between an individual *P. aeruginosa* cell and hydrophobic (methyl-terminated; mimicking catheters) and hydrophilic (hydroxyl-terminated; mimicking metal implants) substrates were also studied using SCFS (Beaussart et al., 2014). For that, *P. aeruginosa* cells were picked up with colloidal probe cantilevers coated with polydopamine as previously reported (Beaussart et al., 2014, 2013). These assays demonstrated that *P. aeruginosa* pili were able to strongly bind to hydrophobic surfaces in a time dependent manner, while they weakly bound to hydrophilic surfaces (Table 1). The obtained force curves regarding adhesion to hydrophobic surfaces showed well-defined single or multiple force peaks of 250–750 pN and rupture lengths between 50 and 70 nm. If the contact time between cells and surface was increased (1.1 s versus 100 ms) cell adhesion was strengthened (El-Kirat-Chatel et al., 2014; Helenius et al., 2008). Nonetheless, despite the mean adhesion force increased up to 3000 pN, the rupture lengths were not altered.

Concerning bacteria-host cell binding interactions, SCFS studies performed with *P. aeruginosa* and pneumocytes revealed that adhesion to the host pulmonary epithelial cells relies on the extension of pili and on the formation of membrane tethers from host cells (Beaussart et al., 2014; Sullan et al., 2014). These events enhance the adhesion lifetime of bacteria to host cells, ultimately contributing to *P. aeruginosa* success as an infectious agent.

Another virulence factor that enables *P. aeruginosa* colonization of human lungs is the lectin LecA. It is a homotetrameric surface adhesin that favors epithelium invasion by interacting with the globotriaosylceramide (Gb3) cell receptor. Moreover, the two adjacent LecA binding site pairs face opposite directions, which besides enabling adhesion to epithelial cells, also contributes to linkages between bacteria and lead to biofilm formation (Worstell et al., 2018). Synthetic multivalent molecules (glycoclusters) targeting LecA with higher affinity than its natural ligand have been developed as new therapeutic approaches. Zuttion *et al.* used SCFS to study the effect of glycoclusters on *P. aeruginosa* binding to human lung epithelial cells at the single cell level. Since this technique does not require cell labeling or purification, it mimics the *in vivo* system more closely than molecular and conventional *in vitro* studies (Zuttion et al., 2018). To demonstrate the anti-adhesive properties of a mannose-based glycocluster, *P. aeruginosa* cells were adhered to a glass dish and maintained under buffer. Human bronchial epithelial cells (16HBE) were bound to a tipless cantilever using the commercial cell adhesive Cell-Tak®. Force distance curves were acquired in the absence and presence of the mannose based-glycocluster, using 1.0 s of contact time between 16HBE cells and the bacterial monolayer. Control experiments were performed using bare

glass (no bacteria) to display the specificity of the cell-bacteria interaction. The anti-adhesive properties of the glycocluster were demonstrated at the single cell level during cell-bacteria contact by comparing the adhesion parameters of each cell-bacteria system, namely the detachment force (the maximum adhesion between cell and the bacterial monolayer) and detachment work (the dissipated energy of the cell bacteria detachment process). After the addition of the mannose-based glycocluster, a 21% decrease in the detachment force (from 0.67 ± 0.11 nN to 0.53 ± 0.08 nN) and a 66% reduction in the detachment work (from 4.14 ± 0.69 fJ to 1.39 ± 0.19 fJ) were observed. The obtained results demonstrated that this glycocluster can be further explored as an anti-adhesive strategy against *P. aeruginosa* infections (Zuttion et al., 2018).

AFM-based force spectroscopy studies have unlocked key information about *P. aeruginosa* pili-related proteins and their impact onto infection establishment.

This can be translated onto an improved biomaterials design, namely urinary catheters and respiratory stents in what concerns *P. aeruginosa* infections (Smith et al., 2017). By making these biomedical devices highly hydrophilic and capable of inducing a higher shear stress at the mucosal surfaces, it is possible to reduce the incidence and prevalence of associated infections (Percival et al., 2015). Moreover, SCFS AFM-based studies provided insights for the development of inventive bioengineering approaches aiming to block adhesion to host epithelial cells (Zuttion et al., 2018).

5. *Escherichia coli*: Living on the stream

Escherichia coli (E. coli) is by far the most usual species infecting the urinary tract, one of the most common sites of bacterial infection (Spurbeck and Mobley, 2013). Uropathogenic *E. coli* has a plethora of structural (fimbriae, pili, flagella, curli, …) and secreted (toxins, iron-acquisition systems) virulence factors that contribute to cause disease. On the other hand, the kidney/bladder epithelium presents intrinsic host defenses, such as urine flow and secretion of antimicrobial substances. Therefore, *E. coli* skill to adhere to the host epithelial cells in the urinary tract is the most important determinant of pathogenicity (Terlizzi et al., 2017).

E. coli bacteria harbor on their surface a network of long polysaccharide chains and other biopolymers, called the polymeric brush layer (Berne et al., 2018). This layer creates a long-range of steric forces that are responsible for achieving proximity and to begin interaction with the surface, a fundamental requirement to initiate attachment (Berne et al., 2018; Camesano and Logan, 2000). Virtually, all uropathogenic strains of *E. coli* encode filamentous surface adhesive organelles anchored in the bacterial pili, which can bind to a wide range of nonspecific substrates and are important for numerous functions including colonization, invasion, biofilm formation and withstanding of shear forces (Capitani et al., 2006; Berne et al., 2018; Formosa-Dague et al., 2018; Liang et al., 2000; Pedersen et al., 2018; Thomas et al., 2004). AFM experiments that measured interaction forces between a single bacterium and the tip of the AFM cantilever revealed that these steric interactions can be more important than van der Waals and electrostatic forces for bacterial adhesion, which is related with the initial events of bacterial adhesion onto biomedical devices (Berne et al., 2018).

Regarding specific binding, uropathogenic strains of *E. coli* use type 1 pili and P pili to colonize the bladder and kidney, respectively (Miller et al., 2006). The type 1 pili binds to mannose receptors in the surface of epithelial bladder cells, while the P pili adheres to the glycolipid receptors of human epithelial kidney cells (Abraham et al., 1988; Sauvonnet et al., 2000). Miller et al. used SMFS to characterize the mechanical properties of type 1 and P pili. For that, purified pili (both type 1 and P pili) were adsorbed to a glass coverslip, followed by random adsorption to the cantilever tip and then stretched for several micrometers. Single molecule level was guaranteed by keeping the number of

pili adsorbed to the coverslip low (1 binding out of 50). It was obtained a force curve with 3 distinct regions: a first region that was firstly attributed to nonspecific interactions; a second region corresponding to unwinding of the pilus and a third region where the stretching of the unfolded pilus chain (region with higher force) occurred (Miller et al., 2006). However, further studies highlighted that the first region actually corresponds to the extension of the layer-to-layer bonds in a pili shaft (Andersson et al., 2007).

In the second region, the ≈60 pN forces observed were reported as being within physiological levels of shear flow (up to ≈ 90 pN/bacterium) and it was suggested that type 1 pili can elongate several times their unstretched length (Thomas et al., 2004). The extensibility of individual type 1 pili was estimated using a worm-like chain model (semiflexible) (Bustamante et al., 1994) to predict the relationship between extension and the entropic restoring force generated. In the third region, it was observed that a larger force was required to further stretch the chain and therefore, this was considered to be the rupture force (detachment from the tip or substrate). P pili structures have mechanical properties similar to type I pili structures yet not identical (Miller et al., 2006). In SMFS dynamic force measurements, the plateau region for P pili is set at lower forces than the one for type 1 pili (35 pN vs. 60 pN, respectively), suggesting a weaker interaction, which may represent a specific adaptation to the biological niche that each structure targets (Table 1). Work performed with optical tweezers (steady state) also highlighted that the unfolding force of the quaternary structures under equilibrium conditions was similar for P pili and type 1 pili (28 ± 2 and 30 ± 2 pN, respectively) (Andersson et al., 2007). However, differences in rigidity were reported, being type 1 pili more rigid than P pili and further SMFS studies demonstrated that type 1 pili untangling is fully reversible, with helical rewinding taking place under considerable forces (≈60 pN), which corroborates previous work with optical tweezers (Andersson et al., 2007; Miller et al., 2006). Overall, type 1 pili are highly extensible, dynamic structures with spring-like properties under applied forces. This elastic behavior provides a lifeline to *E. coli* cells, as it allows extending the lifetime of the interaction bacteria-bladder epithelium under shear forces.

Yakovenko et al. established that the E. coli type 1 pili adhesion to the urinary tract is based on a shear-enhanced bond (catch bond) (Yakovenko et al., 2008). In contrary to "slip bonds" that are weakened by tensile force as the receptor and ligand are pulled apart, "catch bonds" have its strength/dissociation lifetime increased when a tensile force is applied (Dembo et al., 1988; Rakshit et al., 2012). SMFS experiments were performed between FimH (type 1 *E. coli* adhesin) and mannose functionalized tips, as FimH recognizes terminal mannoses on epithelial glycoproteins (Zhou et al., 2001). Briefly, FimH was immobilized onto a polystyrene tissue culture dish followed by BSA blocking to avoid non-specific interactions. The cantilever tips were coated with mannose-BSA and further blocking was performed. Then, the functionalized tip was pressed to the surface (1 s and 100 pN of force) and withdrawn at a constant velocity. This caused the tip to deflect with a constantly increasing force until the formed bond dissociated. To ensure single molecule level, the concentration of the mannose on the tips was chosen so that the probability of a binding event was on average 22%. Nonspecific interactions between tip-surface were measured by adding 4% α-methyl mannose to a PBS-BSA solution to prevent specific bonds from forming. The spring constant of the fimbrial tip-mannose bonds was estimated to be 8.75 ± 1.91 pN/nm. If force was loaded slowly, most of the bonds broke up at low force (<60 pN of rupture force). However, when force was loaded rapidly, all bonds survived until much higher force (140–180 pN of rupture force), a behavior indicative of a catch bond (Yakovenko et al., 2008). This spring-like change provides a meaningful mechanism for absorbing physiological shear forces on bacteria to facilitate adhesion and colonization during urinary tract infection. *In vivo* experiments showed that mutated type I pili have altered ability to withstand shear forces, with detrimental effects on pilus function and pathogenesis (Spaulding et al., 2018).

Due to its vital key in promoting adhesion in the host under adverse conditions, molecules targeting this and other lectin-carbohydrate interaction have come to the spotlight in what concerns antiadhesion/anti-infective strategies towards this Gram-negative pathogen, being unquestionable the input that AFM-based force spectroscopy brought to it (Sharon, 2006).

Another *E. coli* virulence factor are extracellular amyloid fibres called curli that are important molecular players in the adhesion to host surface fibres, such as fibronectin and are crucial in promoting biofilm formation (Casillas-Ituarte et al., 2012). Curli also confer protection from host innate immune factors, namely by the ability to neutralize the human cathelicin (LL-37) and resistance to antibiotics (Hung et al., 2013; Tursi et al., 2017). SMFS studies revealed essential details on the curli-fibronectin binding (Oh et al., 2016). For the mentioned spectroscopy studies, curli in its isolated monomeric form (CgsA monomers) and on the oligomeric state, *i.e*, as expressed on bacterial surfaces, were used. AFM cantilevers were functionalized with fibronectin constructs (either full-length fibronectin, fibronectin isolated domain III or a peptide with the core RGD sequence), using a 3-aminopropyltriethoxysilane (APTES) and a Maleimide (MAL)-PEG-N-hydroxysuccinimide (NHS) binding chemistry (Ebner et al., 2007). Briefly, tips were coated with APTES (for introduction of amino groups) and then a PEG linker was bound to the APTES-coated cantilever via the NHS-group. The MAL group on the free-end of the PEG was then used for the coupling of fibronectin constructs via its lysine residues. SMFS experiments were done between the functionalized tips and CgsA monomers that were tethered to silicon chip surfaces via a flexible PEG. The values for unbinding forces were similar between the fibronectin construct: for RGD it was 51 ± 19 pN; for the fibronectin III 43 ± 16 pN and for fibronectin 57 ± 23 pN. This suggested that the binding to CsgA might occur through the same binding epitope. Specificity of the binding events was demonstrated by performing blocking experiments: RGD peptide was added in solution and was observed a decrease in the binding probability for all the 3-fibronectin constructs tested. The kinetic off-rate constant (K_{off}) and the length scale (X_β), the parameters that characterize the molecular transition during dissociation, were estimated by varying the pulling rate and plotting the unbinding force as a function of the loading rate. As expected and in accordance with the Bell-Evans model, the rupture force linearly increased with the logarithmically increase in the loading rate (Bell, 1978; Evans and Ritchie, 1997). The K_{off} was similar for all three interactions (~1s^{-1}) but the X_β varied, which might be related with the accessibility of the RGD binding sites and the different lengths of the fibronectin constructs. Bond lifetimes were directly calculated from the kinetic off-rates and revealed a relatively short bond survival of about 0.85 s (Oh et al., 2016). For SMFS studies between the different fibronectin constructs and whole curli expressing- *E. coli* cells, live *E. coli* cells were immobilized on gelatine-coated mica surfaces (Doktycz et al., 2003). 3 *E. coli* strains were used: wild type (CsgA(wt)); a CsgA knockout (CsgA(-)) and an over expressing CsgA(CsgA(+)) strain. The wild type and the overexpressing strains showed high binding probabilities in their interactions with the fibronectin constructs but the knock out strain, which did not have the CsgA protein on its bacterial membrane, had very little binding probability. The unbinding forces between *E. coli* wild type and CsgA (+) strain were in the same range of those observed in studies using the monomeric protein (45–60 pN), demonstrating that the RGD-CsgA interaction drives bacterial adhesion between curli and fibronectin. Oh et al. also resourced to SCFS to further explore *E. coli* adhesion to fibronectin-coated surfaces. *E. coli* cells were attached to AFM tipless cantilevers via poly-L-lysine and glutaraldehyde protocol. This yields sparse bacterial coverage on the tip and reduces the probability of two or more cells to touch the surface simultaneously under controlled moderate force (~400 pN) (Gultekinoglu et al., 2016). The force-distance curves between *E. coli* CsgA(+) and fibronectin-coated surfaces showed multiple force peaks, a final large unbinding event of 413 ± 102 pN and unbinding length of 568 ± 152 nm. The authors assumed that the first force spikes indicate step-by-step ruptures

(molecular unbind events or tether-like structures) while the last unbinding event may occur as result of multi-bond breakage (Dufrêne, 2015). On the other hand, for the CsgA(−) and CsgA(wt) strains, lower forces were detected (113 ± 58 pN and 55 ± 29 pN, respectively) as well as shorter unbinding lengths (47 ± 28 nm for CsgA(−) and 310 ± 207 nm for CsgA(wt)). In a simple bond analysis model it was shown that the simultaneous breakage of N number of bonds occurs at a force less equal than N-times the force for breaking a single bond (Williams, 2003). Thus, the ratio of the force required to dissociate CsgA (+) from a fibronectin-coated surface to the unbinding force of a single CsgA/RGD bond is 413 pN/51 pN = 8.1. This implied that at least 9 bonds were broken simultaneously. In addition, several bonds may have disrupted sequentially before the final unbinding occurred (Oh et al., 2016). With this, it was established that "curliated" *E. coli* strains are able to form multiple bonds with high tensile strength with fibronectin through specific RGD/CsgA binding. This prevents that shear forces detach *E. coli* from host cells (Oh et al., 2016).

Overall, *E. coli* success in infection and biofilm formation will depend on the complex interplay of specific and non-specific adhesion forces that mediate the initial adhesion events. It is critical to unravel these *E. coli* binding mechanisms, as they play a crucial role in *E. coli* associated-disease initiation. Also, pili are an attractive therapeutic target for development of innovative therapeutics and AFM-based force spectroscopy techniques may allow to better engineer strategies targeting these structures.

6. *Helicobacter pylori*: Expert in colonizing the harsh gastric mucosa

Helicobacter pylori (*H. pylori)* is a gastric pathogen that can be seen as an example of the importance of bacterial specific binding via adhesion receptor pair for establishment of chronic infection.

The "discovery" of *H. pylori* by Warren and Marshall in 1982 and its impact on gastric disorders was a major event in Gastroenterology, which was awarded with the Medicine Nobel Prize in 2005 (Mégraud, 2005; Piazuelo et al., 2010). *H. pylori* is a Gram-negative bacteria considered as one the most successful human pathogens, being estimated to infect the stomach of more than 50% of the worldwide population (Malfertheiner et al., 2017). It is the etiologic agent of gastro-duodenal diseases, such as chronic gastritis, and 1–3% of the individuals will develop gastric adenocarcinoma as a consequence of infection (Moss, 2017; Piazuelo et al., 2010). In fact, 75% of the global gastric cancer burden (Amieva and Peek, 2016; Malfertheiner et al., 2017), the 5th most common and 3rd deadliest cancer worldwide, is attributed to *H. pylori* infection (Dang and Graham, 2017; Ferlay et al., 2013). The currently available antibiotic-based treatment fails in 10–40% of the cases (Ermis and Senocak Tasci, 2015; Vakil and Vaira, 2013), being high resistance rates the key players accounting for treatment failure (Malfertheiner et al., 2017, 2012; Vakil, 2006). Currently, *H. pylori* is listed among the 16 antibiotic-resistant bacteria that pose the greatest threat to human health according to the World Health Organization (Dang and Graham, 2017). Therefore, innovative strategies to counteract *H. pylori* infection are urgently required.

Adhesion of *H. pylori* to the gastric mucosa is a key event for creating chronic inflammation status and long-term infection (Mahdavi et al., 2003, 2002; Magalhães et al., 2015). To achieve this, *H. pylori* cells have a large family of outer membrane proteins, of which some are adhesins (Ilver et al., 1998; Kao et al., 2016; Parreira et al., 2013). In particular, the Blood group Antigen-Binding Adhesin (BabA) has high affinity (5×10^{11} M^{-1}) for the Lewis b (Le^b) determinant, a fucosylated blood group antigen expressed in the human gastro-intestinal epithelium (Ilver, 1998; Aspholm et al., 2006). It is known that individuals infected with strains expressing the BabA adhesin are at higher risk for duodenal ulcer and gastric cancer development (Figura et al., 2016). Therefore, anti-adhesive therapies that block or diminish this bacterial interaction to the host gastric mucosa are of particular relevance.

The molecular interaction between the purified bacterial BabA adhesin and Le^b antigen was characterized with SMFS (Table 1) (Parreira et al., 2014). For that, a glycosylated Le^b was immobilized onto biotinylated model surfaces (self-assembled monolayers on gold; SAMs) via a neutravidin bridge, a configuration previously used to promote *H. pylori* recognition and binding to immobilized ligands (Parreira et al., 2013). Gold-coated AFM tips were used for BabA immobilization. After gold deposition onto tips, a monolayer of 1,8-octanedithiol and 6-mercapto-1-hexanol was self-assembled onto the gold-coated cantilever. The BabA density on the cantilever surface was controlled by changing the thiol ratio and optimized to achieve optimum binding frequency (10–30% binding events) that generally ensures that bond rupture refers to single binding (Evans, 1999a, 1999b) The monolayers were then activated with a heterobifunctional PEG-linker (NHS–PEG–MAL). The MAL group reacted with 1,8-octanedithiol monolayer on the tip, while the NHS group on the other side of the PEG-linker bound to the free amines on the BabA protein. As previously mentioned, the use of a flexible PEG linker allowed for rapid reorientation of the protein when the AFM tip approached the surface and also enabled to reduce non-specific binding between the tip and substrate. The bond rupture forces between BabA-Le^b were measured in liquid (phosphate buffer saline), in order to mimic the neutral environment of the gastric epithelium where the interaction occurs (Ilver et al., 1998; Nogueira et al., 2012; Parreira et al., 2014).

The functionalized tip cantilever was brought into contact with the Le^b-presenting surface, interaction was allowed and then, the tip was retracted at a constant velocity. Force curves were taken at different loading rates and bond kinetics were accessed using the Evans-Ritchie model (Evans and Ritchie, 1997). In these SMFS experiments, impingement force was kept at <30 pN to further minimize occurrence of non-specific binding. Also, control measurements evaluated the binding specificity, namely measurements between neutravidin–SAMs without Le^b and Le^b–SAMs assayed with a naked cantilever (without BabA protein) were performed. In these controls, the non-specific binding frequency (number of adhesion events/number of tip–surface contacts) was <2–3%, compared with the binding frequency of 10–20% obtained when BabA protein was bound to the tip. Furthermore, the non-specific forces were low and randomly distributed. The obtained results highlighted a two-state model, in which two independent bond rupture events occurred. Therefore, Parreira *et al.* established that the BabA adhesin is capable of forming different adhesive attachments to the gastric mucosa in ways that enhance the efficiency and stability of bacterial adhesion. In the gastric environment, where shear stress at the gastric wall, constant cell renewal, and mucus shedding make it difficult for bacteria to adhere, different bonds with different kinetics and strengths play an important role in initiating and maintaining a chronic bacterial infection (Krachler et al., 2011; Parreira et al., 2014). These SMFS measurements also identified additional biophysical properties of the BabA-Le^b interaction, namely the dissociation rates of 3 ± 0.7 s^{-1} and 0.6 ± 0.1 s^{-1}, for the weak and strong bond respectively and bond length of 0.26 ± 0.03 and 0.23 ± 0.01 nm (weak and strong bonds, respectively). The BabA-Le^b interaction had been previously characterized with optical tweezers using whole cells and a single-state binding model with a single slip bond model was proposed by Björnham et al. (Björnham et al., 2009). However, the obtained results with AFM-based spectroscopy presented different conclusions. The main reasons for that were stated as the use of a two-binding state model to fit the obtained data, since one of the two bonds observed in the AFM measurements could form more slowly than the short dwell time used in the optical tweezers experiments, and therefore, may not have been as prevalent in the previously reported force histograms. Alternatively, the recombinant, purified protein could be more conformationally heterogeneous than the membrane-bound form. In this case, the purified protein might adopt conformations with slightly different bond properties, whereas the membrane bound BabA population could be more uniform (Parreira et al., 2014). Nonetheless, the AFM-based data was in agreement with

another study that used surface plasmon resonance to study this interaction and where it was also suggested a two-step conformational-change model (Younson et al., 2009). The strong, specific binding mediated by BabA suggested that translational applications by use of synthetic Le[b] structures as nanoadhesives for *H. pylori* were appropriate for development of new therapeutic strategies for infection management (Parreira et al., 2014). This knowledge further validated the rational that bioengineered bacterial decoys using synthetic receptors (mimicking the naturally expressed ones on the host gastric epithelium) could be an appealing alternative to counteract infection that was previously described in model surfaces (Parreira et al., 2013). This knowledge was translated onto biocompatible polymers by development of chitosan microspheres functionalized with receptors for the BabA adhesin and provided promising results (Gonçalves et al., 2016). Once translated onto clinical "real world" applications, this strategy is expected to allow binding and removal of this gastric pathogen from infected hosts.

7. Conclusion: From single bacterial cells to life-threatening infections: Can adhesion be the gold target we are seeking?

Bacterial adhesion to surfaces or to other cells is the first step for bacterial colonization/biofilm formation and infection, which may be the golden target that we are looking for.

In this short review, specific examples of bacterial adhesion strategies during host infection and how AFM-based force spectroscopy was used to unravel their infection biophysics were presented. The main findings are summarized in Table 1.

Currently, AFM is the only technique well suited for probing forces on microbial cells, both at single cell and single molecule levels. Examples are glycoclusters as anti-adhesive molecules for *P. aeuruginosa* infection or the development of bioengineered decoys to hinder *H. pylori* adhesion to gastric mucosa. In both cases, AFM-based force spectroscopy studies provided insightful information that further supported development of each rational. Nonetheless, other studies can be done to gather "biophysical biological information" to fight infection, such as studying the dynamics of antibiotic–ligand and the identification of crucial adhesion proteins to which vaccines can be developed (Foster et al., 2014).

In conclusion, the future for fighting bacterial infections and finding innovative strategies once antibiotics fade will undoubtedly benefit from the deeper understanding of the "biophysics of infection" and, for that, AFM-based force spectroscopy technique is a powerful ally for researchers for the design and development of novel drugs/therapies.

CRediT authorship contribution statement

Paula Parreira: Conceptualization, Writing - original draft, Writing - review & editing. **M. Cristina L. Martins:** Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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