Review

Microbial nanoscopy: a closer look at microbial cell surfaces

Vincent Dupres[1], David Alsteens[1], Guillaume Andre and Yves F. Dufrêne

Institute of Condensed Matter and Nanosciences, Université Catholique de Louvain, Croix du Sud 2/18, B-1348 Louvain-la-Neuve, Belgium

How cell envelope constituents are spatially organised and how they interact with the environment are key questions in microbiology. Unlike other bioimaging tools, atomic force microscopy (AFM) provides information about the nanoscale surface architecture of living cells and about the localization and interactions of their individual constituents. These past years have witnessed remarkable advances in our use of the AFM molecular toolbox to observe and force probe microbial cells. Recent milestones include the real-time imaging of the nanoscale organization of cell walls, the quantification of subcellular chemical heterogeneities, the mapping and functional analysis of individual cell wall constituents and the analysis of the mechanical properties of single receptors and sensors.

Microbes: small, yet heterogeneous

Cellular heterogeneity is a key feature of the microbial world. At the population level, microbial cells might widely differ from each other in terms of their genetic composition, physiology, biochemistry or behaviour, and it is believed that this heterogeneity influences various processes such as antibiotic resistance, fermentation productivity, efficacy of food preservatives and the potential of pathogens to cause disease [1]. At the cellular and subcellular levels microbes are highly organised and heterogeneous systems, and this cellular heterogeneity is used to achieve key functions [1–4]. In *Saccharomyces cerevisiae*, a ring of chitin is formed in the cell wall during the course of the division and ultimately forms part of the bud scar marking the division site on the mother cell (Figure 1). In bacteria, flagella and extracellular components can localise asymmetrically on the cell surface. Also, in rod-shaped bacteria a large number of proteins are localised to the cell poles and this asymmetry is important for many functions. But how exactly are cell envelope constituents organised at the molecular scale? Answering this question has thus far been hampered by the lack of suitable detection techniques in living cells.

Atomic force microscopy: feeling the force

Biological methods that are traditionally used to analyse microbial cell walls provide averaged information on large ensembles of cells and require solubilising and separating the cell wall constituents. By contrast, single-molecule techniques analyse individual molecules in complex systems, including single living cells, thereby revealing events and properties that would otherwise be inaccessible. Atomic force microscopy (AFM) is emerging as one of the most powerful single-cell and single-molecule tools of the modern cell envelope microbiologist, as is evident from the continuous growth of papers published in the field (for reviews, see [5,6]). Originally invented for topographic imaging, AFM has evolved into a multifunctional molecular toolkit, enabling researchers not only to observe structural details of cells but also to measure the localisation and properties of individual molecules [7,8].

Rather than using an incident beam as in classical microscopy, AFM senses tiny forces acting on the sample surface. Three-dimensional images are generated by scanning a sharp tip over the sample surface while sensing the interaction force between the tip and the surface. The sample is mounted on a piezoelectric scanner which ensures three-dimensional positioning with high accuracy. While the tip is being scanned laterally, the force interacting between the tip and specimen is monitored with piconewton sensitivity. This force is measured by the deflection of a soft cantilever which is detected by a laser beam focused on the free end of the cantilever and reflected into a photodiode. In addition to imaging, AFM can also localise and manipulate individual molecules, a modality known as single-molecule force spectroscopy (SMFS) [5–8]. Here, the cantilever deflection is recorded as a function of the vertical displacement of the scanner, that is as the sample is pushed towards the tip and retracted. This yields a force–distance curve which provides key information on the localisation, binding strength and mechanics of single molecules. Here, we review recent discoveries made in microbiology through the use of AFM imaging and force spectroscopy.

Cell wall architecture

Bacterial membranes and cell walls

Over the past few years, rapid progress has been made in applying AFM to resolve the supramolecular architecture of purified membranes and cell walls [9]. A striking example of cell wall study is the high-resolution imaging of peptidoglycan in purified *Bacillus subtilis* sacculi [10]. The inner surface of the cell wall had a regular macrostructure with 50 nm wide peptidoglycan cables running across the short axis of the cell. Cross-striations with an average periodicity of 25 nm along each cable were also present. It was suggested that during biosynthesis, small numbers of glycan strands are polymerised and cross-linked to form a peptidoglycan rope, which is then coiled

Corresponding author: Dufrêne, Y.F. (Yves.Dufrene@uclouvain.be)
[1] These authors contributed equally to this work.

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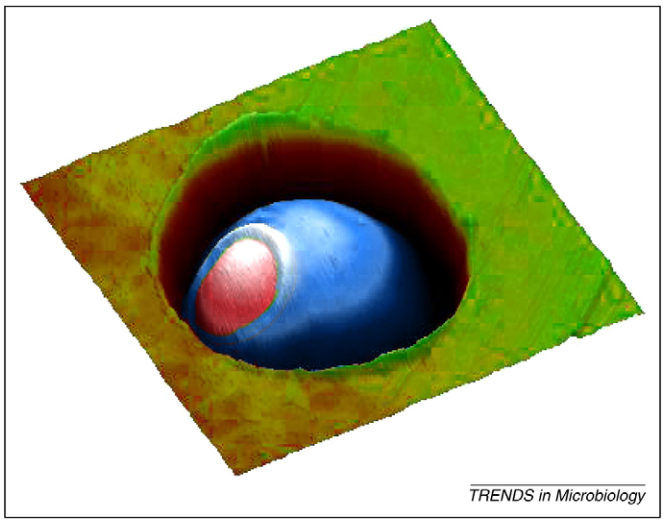

Figure 1. Microbes are heterogeneous. Three-dimensional AFM image (7 μm×7 μm; *z*-range=1500 nm) of a living *Saccharomyces cerevisiae* cell (blue colour) protruding from a porous polymer support (green colour). The cell shows cell wall heterogeneity in the form of a circular bud scar left after detachment of the daughter cell (red colour).

into a helix with a width of 50 nm to form the inner surface cable structures. The turgor pressure of the cell would cause the nascent cable helix to flatten, resulting in the characteristic 25 nm cross-striations within the nanocables.

Purified bacterial membranes have also been imaged with unprecedented resolution. In a breakthrough paper, AFM revealed how the organisation of photosynthetic membranes is modulated in response to light [11]. Recently, AFM provided the first high-resolution views of isolated outer membranes from *Roseobacter denitrificans* [12]. Peptidoglycan remnants were removed using the AFM tip, making it possible to access the periplasmic porin surface. Outer membrane porins were found to be by far more densely packed than previously assumed. These porins covered approximately 70% of the membrane surface and formed locally regular lattices. The above high-resolution studies, which will not be extensively covered here, demonstrate the remarkable potential of AFM for understanding the organisation of isolated membranes and cell walls.

Live cells

Excitingly, images featuring a resolution of a few nanometres can be obtained on living microbial cells [13–20]. A classical example of such *in vivo* experiments is the observation of regularly arranged, 10 nm wide rodlets on fungal spores [13,17] (Figure 2a). Dramatic changes of cell surface structure were observed upon germination of *Aspergillus fumigatus* conidia, the rodlet layer changing into a layer of amorphous material reflecting the underlying polysaccharides [17]. Growth and division events in *Staphylococcus aureus* were monitored using AFM combined with electron microscopy [14]. Detailed images of the cell surface of dividing cells showed ring-like and honeycomb structures at 20 nm resolution [19]. The structural dynamics of single *Bacillus atrophaeus* spores was tracked during germination [15]. AFM images revealed previously unrecognised germination-induced alterations in spore coat architecture as well as the disassembly of outer spore coat rodlet structures. The nascent surface of the emerging germ cell showed a porous network of peptidoglycan fibres, consistent with a honeycomb model structure for synthetic peptidoglycan oligomers. In another study, spores of *Clostridium novyi* NT were surrounded by an amorphous layer that was intertwined with honeycomb parasporal layers [16]. During germination and outgrowth, the honeycomb layers, as well as the underlying spore coat and undercoat layers, sequentially dissolved until the vegetative cell was released. Two highly ordered layers were observed on the surface of *Corynebacterium glutamicum* (Figure 2b) [18]. In addition to the well-known hexagonal S-layer, AFM resolved a new inner layer with 11 nm periodicity, presumably made of mycolic acids. This layer was suggested to function as a molecular template that promotes the assembly and crystallisation of the S-layer monomers. Recently, AFM revealed the nanoscale organisation of cell wall peptidoglycan in living *Lactococcus lactis* bacteria [20]. The use of mutant strains impaired in the production of cell wall polysaccharides revealed that peptidoglycan forms periodic bands running parallel to the short cell axis (Figure 2c). Such bands were missing in purified sacculi, emphasising the importance of probing peptidoglycan directly on live cells rather than on isolated structures that have been subjected to aggressive treatments.

Cell–drug interactions

Also of interest is the possibility to investigate the activity of antibacterial agents on microbial cell walls [21–25]. Alsteens *et al.* [24] imaged the surface of mycobacteria prior to and after incubation with four antimycobacterial drugs. All drugs induced major ultrastructural alterations, reflecting the inhibition of the synthesis of three major cell wall constituents (i.e. mycolic acids, arabinans and proteins). Francius *et al.* [25] tracked over time the structural dynamics of single *S. aureus* cells exposed to lysostaphin, an enzyme that specifically cleaves the peptidoglycan crosslinking pentaglycine bridges, thereby hydrolysing the cell wall. Time-lapse images collected following addition of lysostaphin revealed major structural changes in the form of cell swelling, splitting of the septum and creation of nanoscale perforations. These structural changes were correlated with major differences in cell wall nanomechanical properties and were attributed to the digestion of peptidoglycan, leading eventually to the formation of osmotically fragile cells. In summary, owing to its ability to track the surface of single live cells while they grow or interact with drugs, real-time AFM imaging opens up new possibilities for studying the assembly and remodelling of cell walls, and for understanding the mode of action of antibiotics.

Nanoscale variations of chemical properties

Although powerful, AFM topographic imaging lacks chemical and biological specificity. However, advanced AFM modalities now make it possible to identify and probe specific chemical groups and biological constituents on living cells [7,8]. In chemical force microscopy (CFM), AFM tips are modified with specific groups to map the

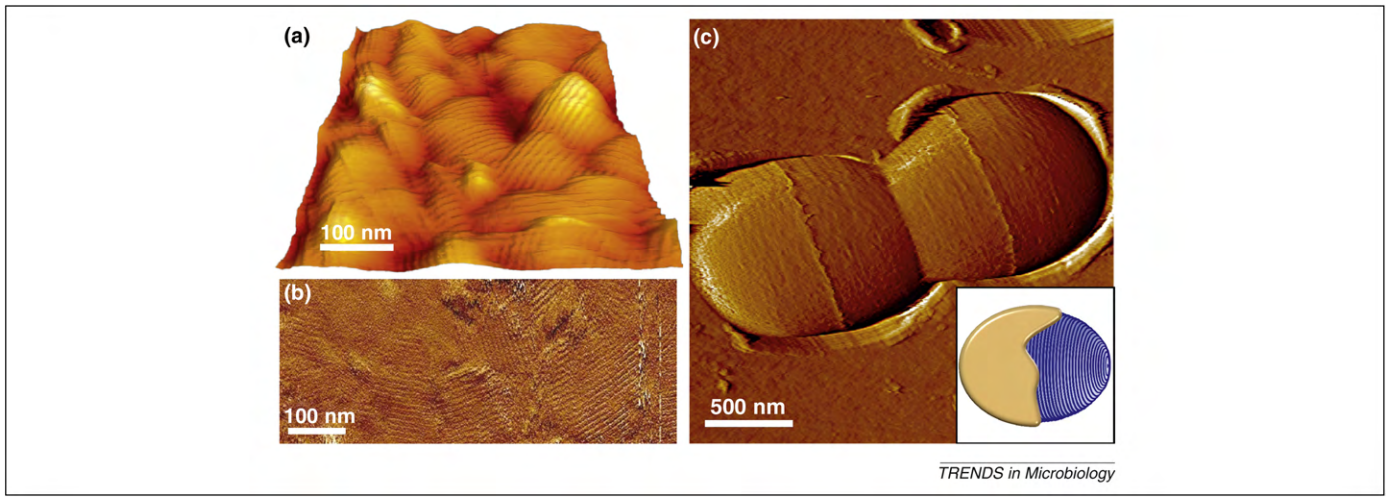

Figure 2. High-resolution imaging of living microbial cells. **(a)** AFM image of the surface of an *Aspergillus fumigatus* spore documenting the presence of ordered rodlets. **(b)** Image of an unexpected highly ordered surface layer recorded on *Corynebacterium glutamicum*. **(c)** The surface of a *Lactococcus lactis* mutant cell lacking cell wall exopolysaccharides. This AFM image shows peptidoglycan bands running parallel to the short cell axis. The sketch in the inset emphasises the outermost surface layers of *L. lactis*, that is cell wall polysaccharides in wild-type cells (brown colour), and peptidoglycan bands in the mutant (blue colour). (b) and (c) Reprinted with permission from Refs. [18] and [20], respectively.

spatial arrangement of chemical properties [26,27]. CFM with hydrophobic tips demonstrated large adhesion forces on the surface of *A. fumigatus* conidia, reflecting strong hydrophobic properties [27] (Figure 3). This finding was consistent with the presence of hydrophobins in the outer rodlet layer and with the role these proteins play in mediating dispersion and adhesion. Interestingly, this method could also resolve nanoscale variations of hydrophobicity on a single cell (Figure 3). Rodlet and polysaccharide regions displayed contrasting hydrophobic and hydrophilic characteristics. In another study, *Acinetobacter venetianus* and *Rhodococcus erythropolis* strains showing different macroscopic surface hydrophobicity were probed with chemically functionalised tips [28]. Topological differences between the two bacterial species were directly correlated with major differences in adhesion forces, as revealed by retraction force curves and were consistent with contact angle measurements. Multiple adhesion force measurements over the microbial surfaces confirmed their nanoscale heterogeneity. These characteristics could have significance for bioremediation of hydrophobic contaminants in the environment because differences in cell surface properties could modulate cell attachment to hydrophobic oil droplets in water. CFM circumvents the limitations of macroscopic assays currently used for assessing surface properties, such as hydrophobicity and charge, and allows resolving nanoscale variations of such properties.

Spatial arrangement of cell surface constituents

SMFS with tips functionalised with cognate bioligands offers a powerful means for mapping the distribution of individual cell surface constituents [29]. In the context of tuberculosis, SMFS was used to map the surface distribution of mycobacterial heparin-binding haemagglutinin (HBHA) engaged in host–microbe interactions [30]. Recognition maps revealed that the adhesin was concentrated into nanodomains, which might promote the recruitment of receptors in host cells. SMFS was also used to map the surface distribution of fibronectin-attachment proteins in mycobacteria yielding that the proteins were widely exposed on the mycobacterial surface [31]. Treatment of the cells with enzymes or antibiotics led to a substantial reduction of the protein surface density, confirming they were surface-associated. AFM tips functionalised with specific antibodies were used to map the distribution of outer membrane cytochromes OmcA and MtrC on living *Shewanella oneidensis* bacteria grown on hematite [32].

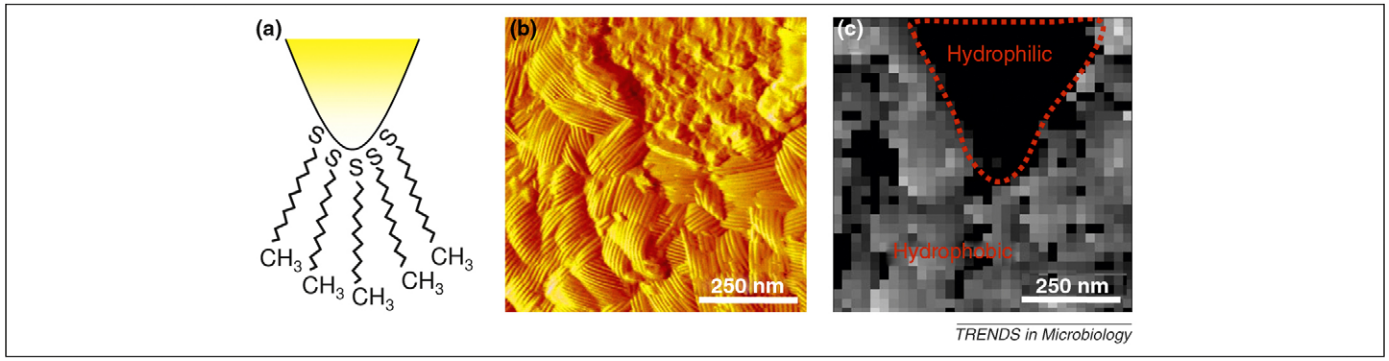

Figure 3. AFM reveals chemical heterogeneities on single cells. **(a)** Chemical modification of an AFM tip enables to quantify and map hydrophobic properties. **(b)** High-resolution image and **(c)** adhesion force map (*z*-range=6 nN) recorded on a sodium dodecyl sulfate-treated *A. fumigatus* spore. Structural and hydrophobic heterogeneities are highly correlated: the rodlet surface is globally hydrophobic, whereas the polysaccharide patch is hydrophilic. Reprinted with permission from Ref. [27].

The main findings were (i) that OmcA and MtrC were expressed on the outer cell surface when Fe(III), including solid-phase hematite, was the terminal electron acceptor; and (ii) that MtrC displayed a more uniform distribution than OmcA. SMFS could also map single peptidoglycan chains on living *L. lactis* cells, revealing that they localise as bands running parallel to the short cell axis [20]. Consistent with the structural images, the data make a case for an architectural feature in the plane perpendicular to the long axis of the cell. In the yeast sensor context, SMFS recently resolved the lateral clustering of the cell integrity sensor Wsc1 in living *S. cerevisiae* cells [33]. Individual wild-type sensors were localised on the cell surface and shown to form clusters of approximately 200 nm size, for which the term 'sensosome' was proposed. Analyses of mutants indicated that the cysteine-rich domain of Wsc1 has a crucial, unanticipated function in sensor clustering and signalling. Clustering of Wsc1 was strongly enhanced in deionised water or at elevated temperature, suggesting its relevance in proper stress response. SMFS can also be used to study how antibiotics bind to cell wall constituents. For instance, the distribution of D-Ala–D-Ala sites on living bacteria was imaged using vancomycin-modified tips [34]. Consistent with fluorescence images, AFM recognition maps revealed that the drug binding sites were highly localised.

Alternatively, immunogold labels can be used as cell surface recognition markers, as is traditionally used in electron microscopy. Cells are first incubated with monoclonal antibodies directed against specific cell wall constituents, then further incubated with the corresponding gold-conjugated secondary antibodies and finally imaged using topographic imaging. Immunogold AFM imaging was used to detect and localise lipoarabinomannan (LAM) on the surface of hydrated mycobacteria, prior to and after treatment with the antibiotics isoniazid and ethambutol [24]. Whereas the surface of native cells showed essentially no labelling, drug-treated cells revealed a large coverage of gold particles, indicating that LAM was exposed. These observations provided direct evidence that the two drugs lead to the massive exposure of LAM at the cell surface. A similar AFM-based immunolabelling technique was applied for the proteomic mapping of macromolecular structures on *Bacillus* spore surfaces [35]. The immunospecificity of this labelling method was established through the utilisation of specific polyclonal and monoclonal antibodies that target spore coat and exosporium epitopes of *B. atrophaeus* and *Bacillus anthracis* spores. Accordingly, these studies suggest that recognition imaging by AFM should become an important tool for mapping the molecular composition of cell surfaces.

Forces that drive cell adhesion

A current challenge in microbiology is understanding the molecular interactions that drive the adhesion and aggregation of microbial cells [36,37]. These interactions result from a complex interplay of fundamental physicochemical forces that can be either specific (receptor–ligand interactions) or nonspecific (hydrophobic and electrostatic interactions). Traditionally, macroscopic assays have been used to investigate the mechanisms of microbial adhesion and biofilm formation. AFM force spectroscopy complements these approaches by providing quantitative information on the fundamental forces driving adhesion processes. Interestingly, the use of cellular probes in which AFM cantilevers are functionalised with microbial cells offers a means to measure cell surface interactions on a single-cell basis [38–40]. For instance, by immobilising metabolically active yeast cells on the apex of cantilevers, Bowen *et al.* could measure the adhesion forces between the cells and various solid surfaces [39]. In an environmental context, bacteria-coated cantilevers were used to probe the forces between living *S. oneidensis* bacteria and goethite [40].

Force spectroscopy experiments have shown to be particularly useful in quantifying microbial adhesion forces that are of biomedical importance. Examples include the nanoscale characterisation of the adhesion forces of *Pseudomonas aeruginosa* pili [41], the measurement of the interaction forces of *Candida parapsilosis* and *P. aeruginosa* to surfaces [42], the study of the influence of support surface properties on the adhesion strength of *Staphylococcus epidermidis* [43], the investigation of the role of cranberry on *Escherichia coli* adhesion forces and its implications for uroepithelial cell attachment [44] and the probing of the adhesion forces between *S. epidermidis* and self-assembled monolayers in the presence of model proteins [45]. Note that a complete understanding of the interaction forces of biomedical relevance requires the use of actual clinical isolates of bacteria rather than laboratory strains. An interesting attempt towards this direction was a study where the forces between fibronectin and a collection of *S. aureus* isolates were measured [46]. There was a strong distinction in the binding force signature observed for the invasive versus control populations.

As a complement, biospecific tips can identify individual polysaccharides and proteins on cells, and measure their adhesion and conformational properties [6,30]. Using SMFS and tips modified with lectins, polysaccharides involved in biofilm formation on the surface of *Lactobacillus rhamnosus* GG were analysed [47]. The method demonstrated the coexistence of two polysaccharides of different natures. The measured polysaccharide properties (i.e. distribution, adhesion and extension) of the wild-type bacterium were markedly different from those of a mutant strain impaired in adherence to gut epithelium, biofilm formation and exopolysaccharide production, suggesting that these molecules play a role in bacterial adhesion and in promoting beneficial health effects. SMFS has also been useful in quantifying the specific binding strength of cell adhesion proteins. Mitchell *et al.* [48] measured the molecular strength of a fibronectin–*S. aureus* interaction and explored the genetic regulation of the binding process on the surface of living bacteria. The results suggested that the fibronectin interaction plays a role in the formation of a mechanically resistant adhesion of *S. aureus* to host tissues. The average rupture force between the mycobacterial HBHA adhesin and its heparin receptor was quantified [30]. Prolonged contact time was required to establish strong HBHA–heparin interaction, reflecting the time necessary for conformational changes within both molecules to allow an optimal fitting between the positive charges of the HBHA heparin-binding domain and the

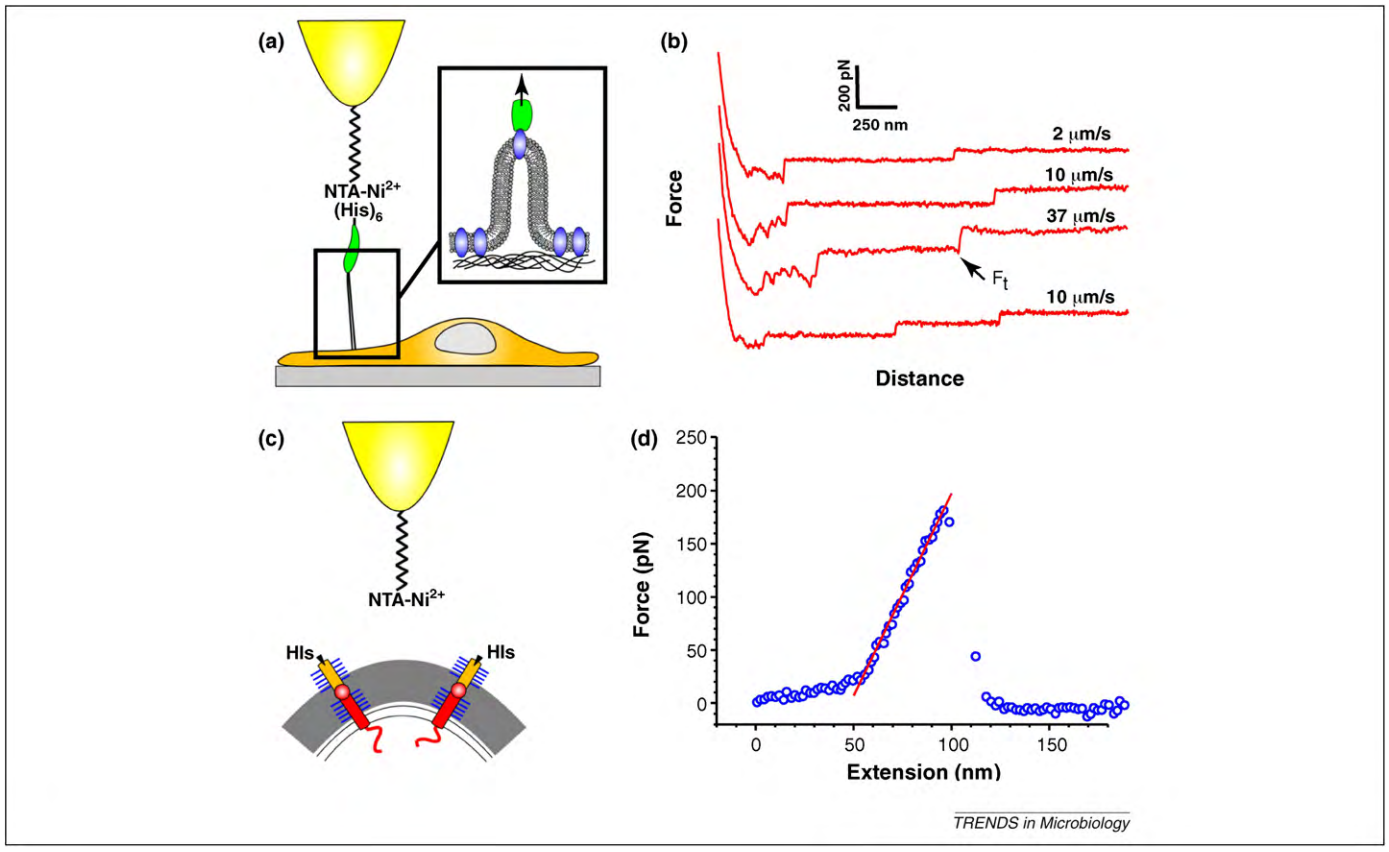

Figure 4. AFM measures the binding strength and mechanical behaviour of cellular proteins. **(a)** Measuring the forces between the mycobacterial adhesin HBHA and A549 pneumocyte cells. **(b)** Force curves showing discrete rupture forces, attributed to single adhesin bonds, as well as constant force plateaus, reflecting the extraction of membrane tethers schematically shown in the close-up box in (a). **(c)** Stretching single His-tagged Wsc1 sensors using an AFM tip functionalised with Ni^{++}–NTA groups. **(d)** Representative force extension revealing that Wsc1 behaves as a linear nanospring. (a) and (b) Reprinted with permission from Ref. [49]. (c) and (d) Reprinted with permission from Ref. [58].

sulfate groups of heparin. Similar interactions were measured between HBHA and heparin sulfate proteoglycan (HSPG) receptors on living A549 pneumocyte cells [49] (Figure 4a,b). Specific binding forces between single HBHA–HSPG pairs were measured at moderate pulling velocity (1 μm s^{-1}). At large pulling velocities (>2 μm s^{-1}), however, constant force plateaus were observed in the force curves (Figure 4b). Presumably, stressed HSPG receptors detached from the cytoskeleton, therefore leading to the extraction of membrane tethers or nanotubes. Tether formation could play a role in pathogen–host interactions because the invasion mechanisms of pathogens such as *Salmonella* and *Shigella* involve the production of large membrane projections and the formation of membrane-bound vacuoles. Combined with macroscopic adhesion assays, the above micro- and nanoscale analyses provide key insights into the molecular bases of microbial adhesion processes, particularly pathogen–host adhesion, and, in the future, might serve as a powerful platform for the development of new therapies based on antiadhesion molecules.

How do cellular proteins respond to force?

SMFS has also been established as a powerful tool to study how membrane proteins respond to force, providing insight into their unfolding pathways and the forces that anchor them into the membrane [50]. This approach has recently enabled researchers to characterise the mechanical unfolding pathways of the β-barrel-forming outer membrane protein OmpG from *E. coli* in reconstituted membranes [51]. An important breakthrough is the demonstration that such nanomechanical measurements can be performed directly on living microbial cells in relation to their function (such as adhesion and sensing).

Protein unfolding and cell adhesion

Studying the cellular 'unfoldome' [52], that is the set of cellular proteins that can be unfolded as part of their physiological function, has recently emerged as an important issue in cell biology. In animal cells, force-induced conformational changes in cell adhesion proteins such as integrins are known to increase the protein binding strength [52–54]. Whether such force-dependent mechanisms also occur in the microbial world remains largely unknown, but recent AFM experiments suggest that this is indeed the case. The *E. coli* fimbrial adhesive protein FimH mediates weak adhesion at low flow but strong adhesion at high flow. By applying force to single isolated FimH bonds with an AFM, Yakovenko *et al.* [55] demonstrated that the protein mediates binding to mannosylated surfaces via so-called 'catch bonds', that is receptor–ligand bonds that are strengthened by tensile mechanical force. Tensile force induces an allosteric switch to the high affinity, strong binding conformation of the adhesin. Because catch bonds are believed to be widespread among adhesive proteins [56], AFM will be very useful in the

future to identify such mechanisms in other microbial adhesins.

Another example is the force-induced unfolding of the agglutinin-like sequence (Als) proteins from *Candida albicans*, a family of cell surface proteins that mediate adhesion to host tissues [57]. AFM force extension curves obtained on soluble Als fragments showed sawtooth patterns with well-defined force peaks. Each peak corresponded to the force-induced unfolding of the secondary structures of individual tandem repeats engaged in cell–cell aggregation. Single Als proteins were also unfolded directly on living cells. The unfolding probability increased with the number of tandem repeats expressed by the cells and was correlated with the level of cell–cell adhesion, suggesting these modular domains might play a role in fungal adhesion. Presumably, the force-induced unfolding of Als proteins leads to extend conformations in which hydrophobic groups are freshly exposed, thus favouring hydrophobic interactions between opposing cells.

Protein nanosprings and mechanosensing

Mechanosensors in living cells convert mechanical forces into biochemical signals. In yeast, the membrane sensor Wsc1 is thought to function as a mechanosensor capable of feeling mechanical forces acting on the cell wall, and, in turn, activating the intracellular cell wall integrity signalling cascade. Using a combination of SMFS with genetic manipulation, Dupres *et al.* measured the mechanical behaviour of single Wsc1 proteins in living *S. cerevisiae* cells [58,59] (Figure 4c,d). His-tagged sensors were picked up with an AFM tip carrying nitrilotriacetate (NTA) groups. Remarkably, force extension curves displayed a linear region where force is directly proportional to extension, thus characteristic of a Hookean spring. From these data, the sensor spring constant was estimated to be 5 pN nm^{-1}, which is very close to the behaviour of ankyrin repeats. The use of mutants with reduced glycosylation resulted in severe alterations in protein spring properties, indicating the important role of glycosylation at the extracellular serine/threonine-rich region. At low salt concentration or elevated temperature, the sensor spring constant was substantially reduced, demonstrating that Wsc1 is sensitive to cell surface stress.

Concluding remarks and future directions

The experiments reviewed here demonstrate that AFM has now taken root in the microbiological community. There is a strong indication that the technique is evolving

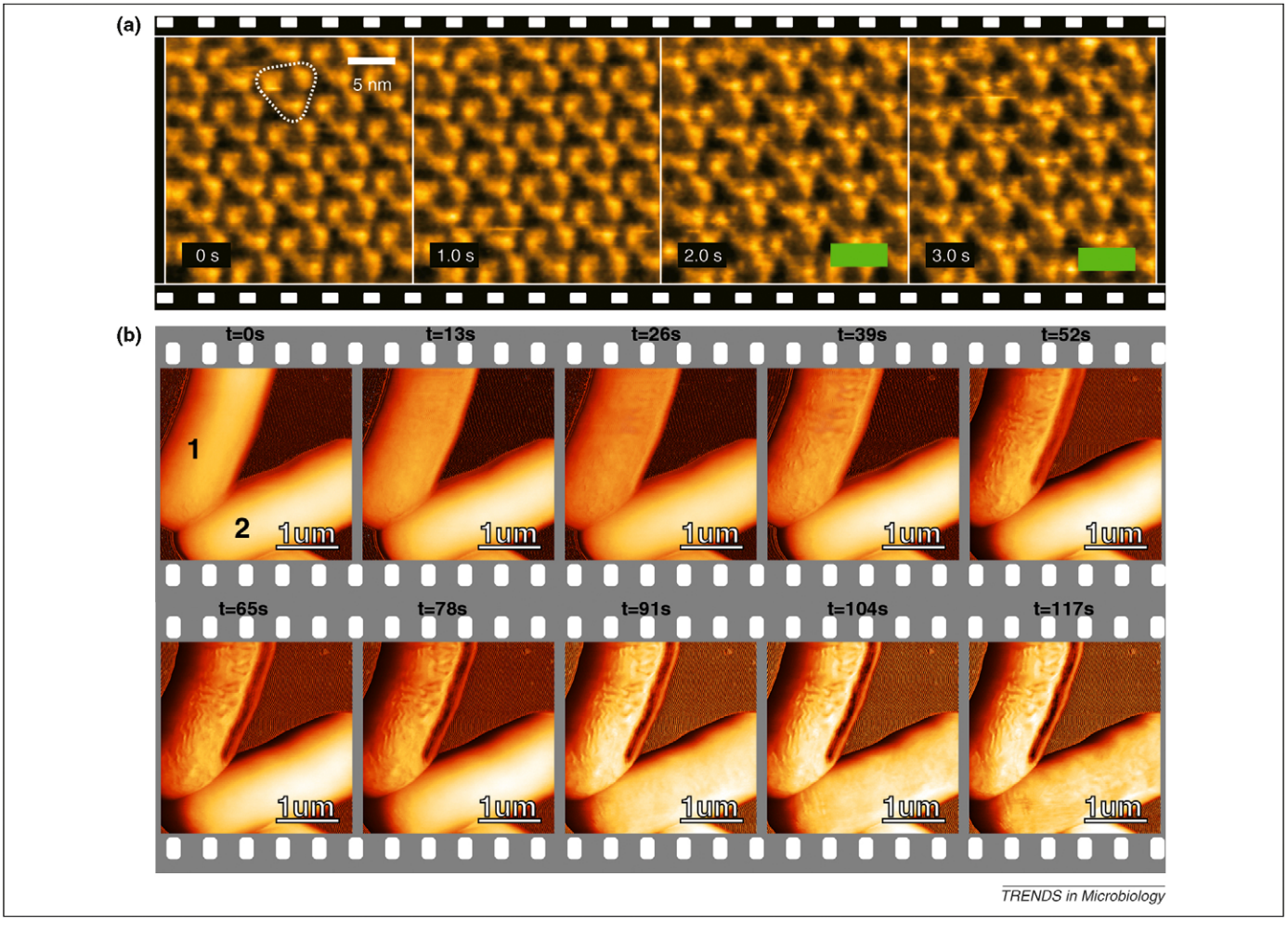

Figure 5. High-speed AFM reveals protein and cell dynamics. **(a)** Successive AFM images of bacteriorhodopsin captured at 1 frame per second and revealing major structural changes upon illumination (compare images at 1 and 2 s). Bacteriorhodopsin is a light-driven proton pump which is most efficient at absorbing green light. The green bars indicate illumination with green light. A bacteriorhodopsin trimer is highlighted by the white triangle. **(b)** High-speed imaging of *E. coli* cell disruption induced by CM15. Images recorded every 13 s. The surface of the upper bacterium **(1)** starts changing within 13 s. The lower bacterium **(2)** resists changing for 78 s. (a) Reprinted with permission from Ref. [64]. (b) Reprinted with permission from Ref. [65].

Box 1. Outstanding questions

- What is the nanoscale organisation of peptidoglycan and other cell wall constituents in Gram-positive and Gram-negative species?
- How does the cell wall of microbes remodel upon growth or in the presence of external stimuli such as pH, temperature, drug binding or mechanical stress?
- What are the molecular forces driving cell surface interactions?
- What are the adhesion mechanisms underlying pathogen–host interactions and how can these be modulated for therapy?
- Are cell surface receptors clustered or evenly distributed? Does the receptor distribution change in response to environmental changes?
- How do surface properties and interactions vary across a single cell?
- What are the nanomechanical properties of cell surface proteins? How are these properties related to functions such as adhesion and mechanosensing?
- What are the concentrations of toxins or pathogens in a medical sample?

from a qualitative imaging tool to a quantitative molecular toolbox, enabling researchers not only to image cell wall architecture but also to force probe their individual constituents. These single-cell and single-molecule experiments complement traditional macroscopic methods used to analyse microbial envelopes and will contribute to answering many outstanding microbiological questions (Box 1).

From a technology perspective, although there has been rapid progress in improving the AFM instrumentation and methodology for analysis of living cells (advanced procedures for sample preparation and tip functionalisation, high-resolution imaging, chemical and recognition imaging, quantification of biomolecular interactions, single-molecule detection and manipulations), there are still key technological issues to address. We expect that the next stage of AFM evolution will involve (i) the design of high speed instruments for imaging dynamic events with unprecedented time resolution; (ii) the use of AFM-based biosensors for ultrasensitive detection applications; and (iii) the integration of AFM with other advanced imaging techniques, such as stimulated emission depletion microscopy [60]. As discussed below, advances in these directions are already occurring.

An important limitation of AFM imaging is its rather poor temporal resolution. The time required to record a high-resolution image with a commercial instrument is on the order of 30–60 s, thus too slow to address many dynamic processes. Hopefully, new high-speed instruments are now capable of providing millisecond time resolution [61–63]. Recently, the use of high-speed AFM to observe dynamic molecular processes in photoactivated bacteriorhodopsin was reported [64] (Figure 5a). High-resolution movies of this light-driven proton pump showed that illumination induces major structural changes within 1 s. A cytoplasmic portion of each bacteriorhodopsin monomer was brought into contact with adjacent trimers. As suggested by the authors, the direct visualisation of the dynamically changing structure of stimulated proteins provides a straightforward way of elucidating how protein molecules function. In another recent report, high-speed AFM could measure the kinetics of antimicrobial peptide activity on individual bacterial cells [65] (Figure 5b). The increased time resolution allowed the researchers to characterise the initial stages of the action of the antimicrobial peptide CM15 on individual *E. coli* cells with nanometre resolution. Bacterial killing by CM15 was suggested to be a two-stage process consisting of an incubation phase, lasting from seconds to minutes, followed by an execution phase in which most of the damage is completed in less than a minute. These two recent reports indicate that fast AFM instruments open up fascinating new perspectives to explore membrane and cellular dynamics.

Another fast-moving area is the use of AFM cantilever arrays for biosensing applications. AFM-based sensors enable the rapid, ultrasensitive detection of bioanalytes and cells without any need for labelling or external probes [66]. The general principle is to directly translate specific biomolecular recognition into nanomechanical motion. Cantilevers are functionalised with receptor

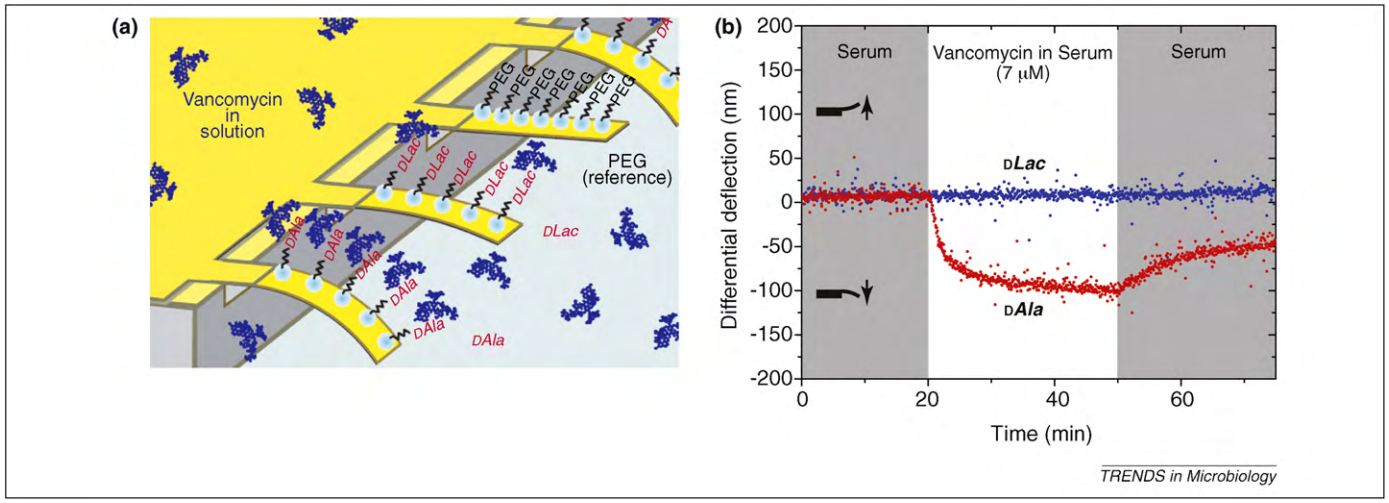

Figure 6. AFM-based sensors explore the binding mechanisms of antibiotics **(a)** Schematic showing cantilevers coated with D-Ala–D-Ala (vancomycin-sensitive) or D-Ala–D-Lac (vancomycin-resistant) surface groups. Vancomycin is injected in solution and binds specifically to the D-Ala–D-Ala groups, causing the cantilever to bend downwards due to a compressive surface stress. **(b)** Detection of antibiotics in blood serum at clinically relevant concentrations. Differential bending signal of D-Ala–D-Ala (red) and D-Ala–D-Lac (blue) upon injection of 7 mM vancomycin. Reprinted with permission from Ref. [68].

molecules (e.g. antibodies), incubated with the cognate ligands, and the resulting specific biomolecular recognition events are detected by monitoring either the cantilever bending or the resonance frequency shift. Two recent studies have demonstrated the potential of AFM sensors in microbiology. Burg *et al.* [67] used fluid filled-microcantilevers to weigh single bacterial cells in water with sub-femtogram resolution. Accordingly, the sensor could measure the mass of individual live *E. coli* and *B. subtilis* bacteria, making this tool very promising for the rapid detection of pathogens. Ndieyira *et al.* [68] used microcantilever arrays to explore the binding mechanisms of antibiotics with bacterial cell wall polymers, down to a sensitivity of 10 nM, and at clinically relevant concentrations in blood serum (Figure 6). The results suggested that changes in the surface stress cause mechanical disruption of both the bacterial membrane and the cell wall, which eventually leads to the destruction of the bacteria.

In conclusion, with its ability to observe single microbial cells at nanometre resolution, to monitor structural dynamics in response to environmental changes or drugs, and to detect and manipulate single-cell surface constituents, AFM provides new insight into the structure–function relationships of cell envelopes. This emerging new field of microbial nanoscopy should have an important impact on many disciplines of microbiology, including cellular and molecular microbiology, pathogenesis, diagnosis, antimicrobial therapy and environmental microbiology.

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