AFM Imaging and Force Spectroscopy of Individual Bacterial Adhesins

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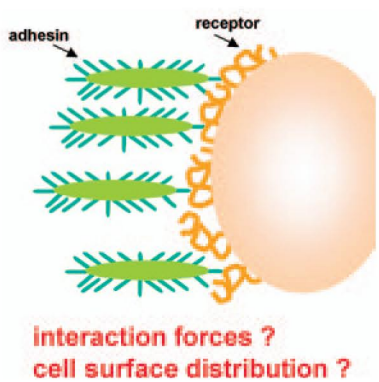

Figure 1. Bacterial pathogens adhere to host cells via the specific molecular recognition between surface adhesins and host surface receptors. In the case of *Mycobacterium tuberculosis*, adhesion to epithelial cells is mediated by the heparin-binding haemagglutinin adhesin (HBHA), which binds heparan sulphate receptors. In this context, two crucial questions remain unanswered, namely, *"What are the forces driving the adhesin-receptor interaction?"* and *"How do the adhesins distribute across the bacterial surface?"*

Introduction

Because microbial surfaces are in direct contact with the external environment, they are vital to organisms. Microbial surfaces play key roles in determining cellular shape and growth, enabling organisms to resist turgor pressure, acting as molecular sieves, and mediating molecular recognition and cellular interactions. Therefore, studying the structure-function relationships of these surfaces represents a very exciting and challenging field of microbiology.

A striking example of bacterial surface interaction is found during pathogenesis, where the infectious process is generally initiated by the interaction between cell adhesion molecules on the bacterial surface, referred to as adhesins, and specific receptors on the host cell surface (see Figure 1). Despite the vast body of literature accumulating on bacterial adhesins and their great medical significance, their functions remain poorly understood at the molecular level. For instance, what are the molecular forces driving the interaction of bacterial adhesins with their receptors and how can they be modulated? Also, does the distribution of adhesins vary across the bacterial surface? Until now, these questions could not be addressed due to a lack of *in-situ* nanoscale imaging techniques.

During the past decade, atomic force microscopy (AFM) has been increasingly used to image and manipulate biomolecules and cell surfaces in their native environment[1,2]. In this application note, we show how the power of AFM force spectroscopy with tips bearing biologically active molecules can be utilized to explore the dynamics of the interaction between individual bacterial adhesins and their receptors as well as to map single adhesins directly on living bacteria. This approach was applied to *Mycobacterium tuberculosis*, the etiologic agent of tuberculosis, which adheres to heparan sulphate receptors on epithelial cells via the heparin-binding haemagglutinin adhesin (HBHA)[3].

Modification of AFM Tips and Substrates with Adhesins and Receptors

The key element in biological AFM force spectroscopy is the design of tips bearing biologically active molecules. Several issues need to be considered in order to achieve reliable, single-molecule force measurements[4]: (1) the binding of the molecules should be stronger than the intermolecular force being studied; (2) the attached molecules should retain sufficient mobility so that they can freely interact with complementary molecules;

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** We dedicate this application note to Franco D. Menozzi, who passed away in July 2005.*

(3) unspecific adsorption on the modified surfaces should be minimized; (4) molecules should be attached at low density to ensure single-molecule recognition; and (5) for oriented systems, the best results are usually obtained when the molecules are attached in a defined orientation.

To fulfill these requirements, HBHA was engineered with a His-tag at its N-terminal end and coupled onto an AFM tip modified with mixed, self-assembled monolayers (SAMs) of nitrilotriacetic acid (NTA) and tri(ethylene glycol) (EG) terminated alkanethiols (see Figure 2). The primary benefit of employing this site-directed strategy is that it uniformly exposes the C-terminal binding domains of the adhesins. Biotinylated heparin, used as a model receptor, was attached on gold substrates via a classical sandwich layer of streptavidin and biotinylated bovine serum albumin (BBSA).

Force Spectroscopy of the HBHA/heparin Interaction

Force-distance curves were recorded between the HBHA tip and the heparin-coated substrate at a loading rate of 10,000 pN/s. As shown in Figure 3a, 32% of a total of 1,000 curves displayed adhesion forces; the remaining curves exhibited no adhesion. The adhesion force histogram revealed a bimodal distribution with average rupture forces of 50 ± 23 pN and 117 ± 18 pN, attributed to one and two binding events between HBHA and heparin. The ~50 pN binding force may correspond to the interaction strength quantum between heparin and a monomeric form of HBHA, while the ~117 pN force may be due either to the simultaneous interaction of two single HBHA molecules or to the binding of a HBHA dimer.

Since adhesion forces measured between receptors and ligands usually depend on the rate at which the load is applied[5], force curves between the HBHA tip and the heparin surface were recorded at various loading rates. Surprisingly, we found that the mean adhesion force did not depend on the loading rate, indicating that the force measurements were done close to thermodynamic equilibrium. By contrast, both the adhesion frequency and adhesion force increased dramatically with contact time (see Figures 3b and 3c), a finding that suggests the formation of multiple intermolecular bridges between HBHA and its receptor. Presumably, the prolonged contact time required to establish a strong HBHA-heparin interaction reflects the time necessary for conformational changes to allow an optimal fitting between the positive charges of the HBHA heparin-binding domain and the negative sulphate groups of heparin.

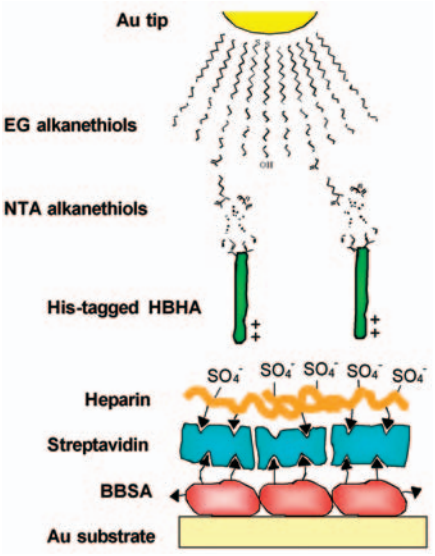

Figure 2. Functionalization of AFM tips and substrates with HBHA and heparin. HBHA was engineered with a His-tag for site-directed immobilization onto a NTA/EG-terminated tip. This strategy allows the adhesins to be attached at a low surface density, thereby ensuring single-molecule binding events. Biotinylated heparin was bound to gold substrates via streptavidin and BBSA layers. Adapted with permission from Nature Methods[3].

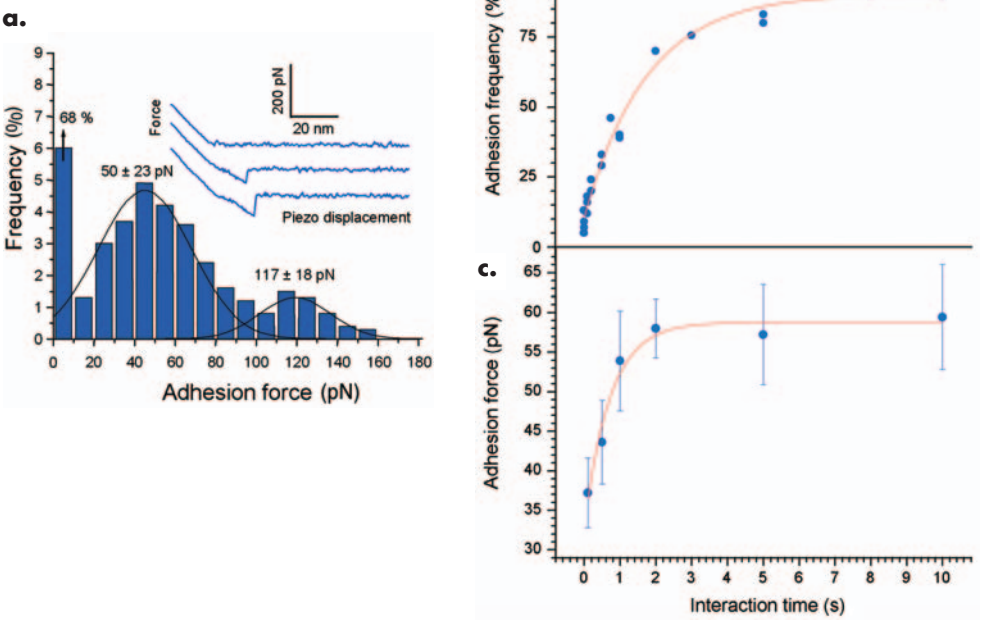

Figure 3. (a) Representative force-distance curves and adhesion force histogram (n = 1,000) obtained in PBS between a HBHA tip and a heparin substrate. A significant fraction of the curves showed adhesion forces, with a mean magnitude of 50 ± 23 pN and 117 ± 18 pN, which reflected the occurrence of one and two unbinding events between HBHA and heparin. (b) The adhesion frequency and (c) adhesion force depended strongly on the interaction time(s) (constant loading rate of 10,000 pN/s). Adapted with permission from Nature Methods[3].

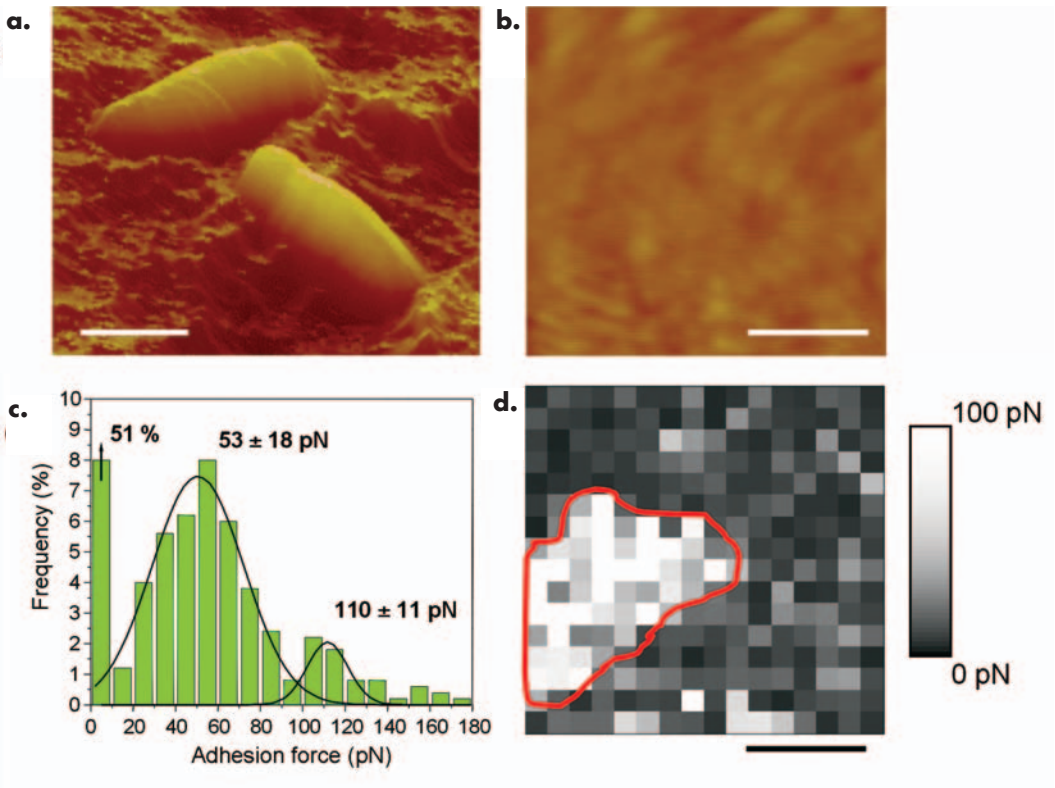

Figure 4. (a) AFM topographic image recorded in PBS showing two *M. bovis* BCG cells on a polymer substrate (scale bar: 2 µm). (b) Higher magnification image of the cell surface (scale bar: 100 nm). (c) Histogram and (d) spatially resolved map of adhesion forces recorded with a heparin tip (scale bar: 100 nm). Single HBHA adhesins were detected and found to be organized in nanodomains (red line). Adapted with permission from Nature Methods[3].

Imaging Single Adhesins on Living Bacteria

Having explored the HBHA-heparin interaction, we then mapped the spatial distribution of individual HBHA on the surface of living mycobacteria using heparin-modified tips. For imaging native cells, we mechanically immobilized the cells onto porous polymer membranes, an approach that does not require chemical fixation or drying of the specimen[3]. Figure 4a shows a topographic image of two *M. bovis* BCG cells, in buffer solution, immobilized on a membrane. As shown in Figure 4b, the cell surface was fairly smooth and homogeneous, in agreement with the expected cell wall architecture. In an attempt to detect HBHA molecules on the cell surface, multiple force-distance curves were recorded in the force-volume mode over 300nm x 300nm areas. Importantly, the curves showed adhesion forces with mean values centered at 53 ± 18 pN and 110 ± 11 pN (see Figure 4c), which matched remarkably well those obtained for the model HBHA-heparin interaction (see Figure 3a). This finding strongly suggests that the ~50 pN binding force corresponds to the detection of single HBHA, a conclusion further supported by the observation that a mutant *M. bovis* BCG strain lacking HBHA did not bind the heparin tip.

To address the question of how the adhesins distribute across the cell surface, quantitative adhesion maps were generated from the force-volume data sets. As shown in Figure 4d, adhesion events (clear pixels) were observed in about half of the locations, which again were attributed to the detection of single HBHA since these events were not observed on the mutant strain. Interestingly, the HBHA distribution was not homogeneous, but apparently concentrated into nanodomains for which we have proposed the term *adherosomes*. These nanostructures may play an important biological function in that they may promote adhesion to target cells by inducing the recruitment of receptors within membrane rafts.

Conclusion

AFM imaging and force spectroscopy with biologically modified tips provides a powerful tool for exploring the molecular bases of pathogen-host interactions. For the first time, microscopists can measure the interaction forces between adhesins and receptors on a single-molecule basis and investigate the dynamics of the interaction by varying the loading rate and contact time during the measurements. In addition, modified tips can be used to image the distribution of single adhesins directly at the surface of native bacteria. The benefits of utilizing this AFM-based approach are twofold. First, the investigations are noninvasive and performed directly in aqueous solution without any cell pretreatment, thus preserving the native organization and conformation of the surface molecules. Second, the piconewton force sensitivity of the atomic force microscope (we used a Digital Instruments MultiMode from Veeco) permits a functional analysis of individual adhesins. In the future, we believe this type of nanoscale cell imaging study may be of biomedical relevance by serving to facilitate the development of new drugs capable of blocking adhesin-receptor interactions.

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