NANO LETTERS

2007
Vol. 7, No. 10
3026−3030

Chemical Force Microscopy of Single Live Cells

Etienne Dague,[†,||] David Alsteens,[†,||] Jean-Paul Latgé,[‡] Claire Verbelen,[†] Dominique Raze,[§] Alain R. Baulard,[§] and Yves F. Dufrêne*,[†]

Unité de Chimie des Interfaces, Université Catholique de Louvain, Croix du Sud 2/18, B-1348 Louvain-la-Neuve, Belgium, Unité des Aspergillus, Institut Pasteur Paris France, 25 rue du Docteur Roux, 75724 Paris, France, INSERM U629, Lille, Mécanismes Moléculaires de la Pathogénie Microbienne, Institut Pasteur de Lille, IFR142, 1 rue du Professeur Calmette, F-59019 Lille Cedex, France

Received June 21, 2007; Revised Manuscript Received August 14, 2007

ABSTRACT

Traditionally, cell surface properties have been difficult to study at the subcellular level, especially on hydrated, live cells. Here, we demonstrate the ability of chemical force microscopy to map the hydrophobicity of single live cells with nanoscale resolution. After validating the technique on reference surfaces with known chemistry, we probe the local hydrophobic character of two medically important microorganisms, *Aspergillus fumigatus* and *Mycobacterium bovis*, in relation with function. Applicable to a wide variety of cells, the chemically sensitive imaging method presented here provides new opportunities for studying the nanoscale surface properties of live cells and for understanding their roles in mediating cellular events.

Hydrophobic interactions, which reflect the unusually strong attraction of hydrophobic groups in water, mediate various crucial biological events, including protein folding, membrane fusion, and cell adhesion.[1] Hydrophobic forces are of particular importance in microbiology because they are believed to be one of the driving forces for the adhesion of pathogens to surfaces and tissues.[2] During the past decades, a variety of methods have been developed for assessing the hydrophobic qualities of microorganisms, including water contact angle measurements, adhesion to hydrocarbons, partitioning in aqueous two-phase systems, and hydrophobic interaction chromatography.[3] So far, however, quantifying and imaging these characteristics at the subcellular level has been impossible.

Recent advances in atomic force microscopy (AFM) are revolutionizing our views of cell surfaces. While AFM imaging allows researchers to visualize membrane proteins[4,5] and live cells[6,7] in real-time with unprecedented resolution, force spectroscopy provides a means to detect and manipulate single molecules on cell surfaces.[8] Notably, modification of AFM tips with specific functional groups has enabled researchers to map the spatial arrangement of chemical groups and their interactions on organic surfaces, an approach known as chemical force microscopy (CFM).[9−11] Here, we demonstrate the ability of CFM to probe the hydrophobic character of live cells, on a scale of only ∼25 functional groups. This nanoscale, chemically sensitive imaging technique offers two major advantages over classical methods: (i) hydrophobic groups and their interactions are measured directly and quantitatively, and (ii) nanoscale variations of hydrophobicity are revealed on single live cells.

First, we validated the CFM technique using reference surfaces with defined hydrophobicity. AFM tips were modified with CH_3 groups while flat supports were functionalized with CH_3/OH groups mixed in different proportions (Figure 1a). Adhesion forces measured in water between CH_3 tips and CH_3/OH surfaces increased linearly with the CH_3 surface fraction, indicating the importance of hydrophobic forces in aqueous media (Figure 1b). Interestingly, the number of interacting groups contributing to the measured adhesion forces can be estimated by using the JKR model of adhesion mechanics.[10] For the interaction between pure CH_3/CH_3 surfaces, considering an effective tip radius of 20 nm, we found a contact area of 5 nm^2, which corresponds to only ∼25 chemical groups in interaction. Consistent with previous work,[10,11] these results demonstrate the sensitivity of the CFM method toward local surface hydrophobicity and provide a reference system for interpreting our cellular data.

We then used the method to probe the nanoscale structure and hydrophobicity of single microbial cells. As an example of a medically important microorganism, we examined the

* Corresponding author. E-mail: dufrene@cifa.ucl.ac.be. Telephone: (32) 10 47 36 00.
† Université Catholique de Louvain.
‡ Institut Pasteur Paris France.
§ Institut Pasteur de Lille.
|| E.D. and D.A. contributed equally to this work.

10.1021/nl071476k CCC: $37.00
Published on Web 09/13/2007

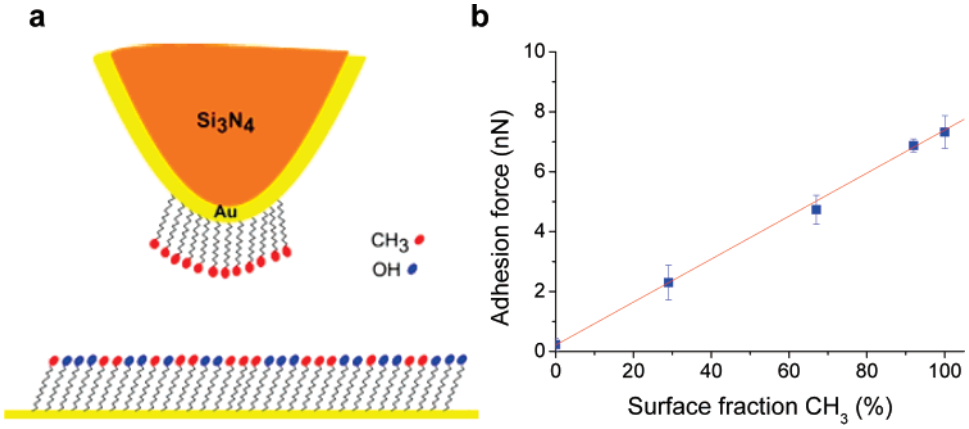

Figure 1. Chemical force microscopy (CFM): principle and application to reference surfaces. (a) Scheme for chemical modification of AFM tips and supports. Gold tips are functionalized with CH_3-terminated alkanethiols, while gold supports are modified with CH_3− and OH-terminated alkanethiols mixed in different proportions. (b) Adhesion force measured in water between hydrophobic tips and CH_3/OH surfaces as a function of the CH_3 surface fraction (determined by X-ray photoelectron spectroscopy). The adhesion force increases linearly with the CH_3 fraction, confirming the quality of hydrophobic tips and their sensitivity toward surface hydrophobicity.

human opportunistic pathogen *Aspergillus fumigatus*.[12] All conidia of aerial molds, including *A. fumigatus*, are covered by a layer of regularly arranged rodlets composed of hydrophobins, a family of small, moderately hydrophobic proteins that favor spore dispersion by air currents and mediate adherence to host cells.[12,13] Wild-type *A. fumigatus* conidia were immobilized on a polymer membrane, a method, which allows live cells to be imaged by AFM without using any drying or fixation step (Figure 2). High-resolution images revealed rodlet structures several hundreds of nanometers in length and 10 ± 1 nm in diameter (Figure 2a), consistent with earlier electron microscopy observations.[12] Force curves recorded across these surfaces with a hydrophobic tip showed large adhesion forces of 2982 ± 409 pN (n = 512) magnitude (Figure 2b,c). Comparison with the data obtained on reference surfaces (Figure 1b) indicates that the conidial surface has a marked hydrophobic character, corresponding to a surface composed of ∼10 CH_3 and ∼15 OH groups, which is fully consistent with the presence of an outermost surface layer of hydrophobins and provides direct indications as to their putative functions as dispersion and adherence structures. In agreement with the uniform surface structure, we also note that adhesion maps were rather homogeneous (Figure 2b), supporting the idea that the *A. fumigatus* conidial surface is homogeneously hydrophobic.

To confirm that the measured hydrophobic properties are associated with hydrophobins, we recorded topographic images and spatially resolved adhesion maps (i) on wild-type conidia treated with NaOH, a procedure that removes all cell wall proteins including rodlet proteins (Figure 2d−f), and (ii) on the ΔrodA ΔrodB double mutant (Figure 2g−i).[12] In both cases, the conidial surface was devoid of any rodlet, revealing an underlying layer of cell wall polysaccharides (chitin, glucan, and galactomannan).[14] These structural changes were correlated with profound modifications of the cell surface hydrophobicity, the adhesion force toward the hydrophobic tip being only 333 ± 160 pN (n = 512) and 443 ± 168 pN (n = 512) for NaOH-treated and mutant conidia, respectively. In the light of our reference surfaces (Figure 1b), we conclude that the two conidial surfaces are purely hydrophilic, corresponding to a pure OH-terminated surface, which agrees well with the exposure of cell wall carbohydrates. These data illustrate the potential of CFM in microbial genetic studies for assessing the phenotypic characteristics of mutants altered in cell wall constituents.

Notably, we also resolved nanoscale variations of hydrophobic properties across *A. fumigatus* conidia. To this end, heterogeneous conidial surfaces were generated by using gentle SDS treatments (Figure 2j−l). Topographic images of SDS-treated conidia showed that, in some regions, rodlet patches had been removed, revealing the underlying cell wall polysaccharides. These nanoscale structural heterogeneities were directly correlated with differences in hydrophobicity, the rodlet and polysaccharide regions displaying contrasted hydrophobic and hydrophilic characters, respectively. Accordingly, CFM is a valuable method for resolving local variations of cell surface properties on a scale that was not accessible before.

Next, we explored the surface properties of mycobacteria, which include some very devastating pathogens such as *Mycobacterium tuberculosis* and *M. leprae*, and their modifications upon incubation with antimycobacterial drugs (Figure 3). Currently, there is much interest in studying the properties of mycobacterial cell walls because they offer excellent targets for novel antibiotics.[15] In this context, mycolic acids are particularly important because these hydrophobic constituents are thought to represent an important permeation barrier to common antibacterial agents. Therefore, we investigated the surface of *M. bovis* prior to and after incubation with isoniazid, an antibiotic known to inhibit the synthesis of mycolic acids. The surface morphology of native cells was very smooth and homogeneous (Figure 3a) and showed only subtle modifications upon treatment with isoniazid (Figure 3d). By contrast, isoniazid caused a dramatic change of surface hydrophobicity: the adhesion force toward the hydrophobic tip was 3032 ± 102 pN for native cells (Figure 3b,c), consistent with the presence of mycolic acids, while it decreased to only 137 ±

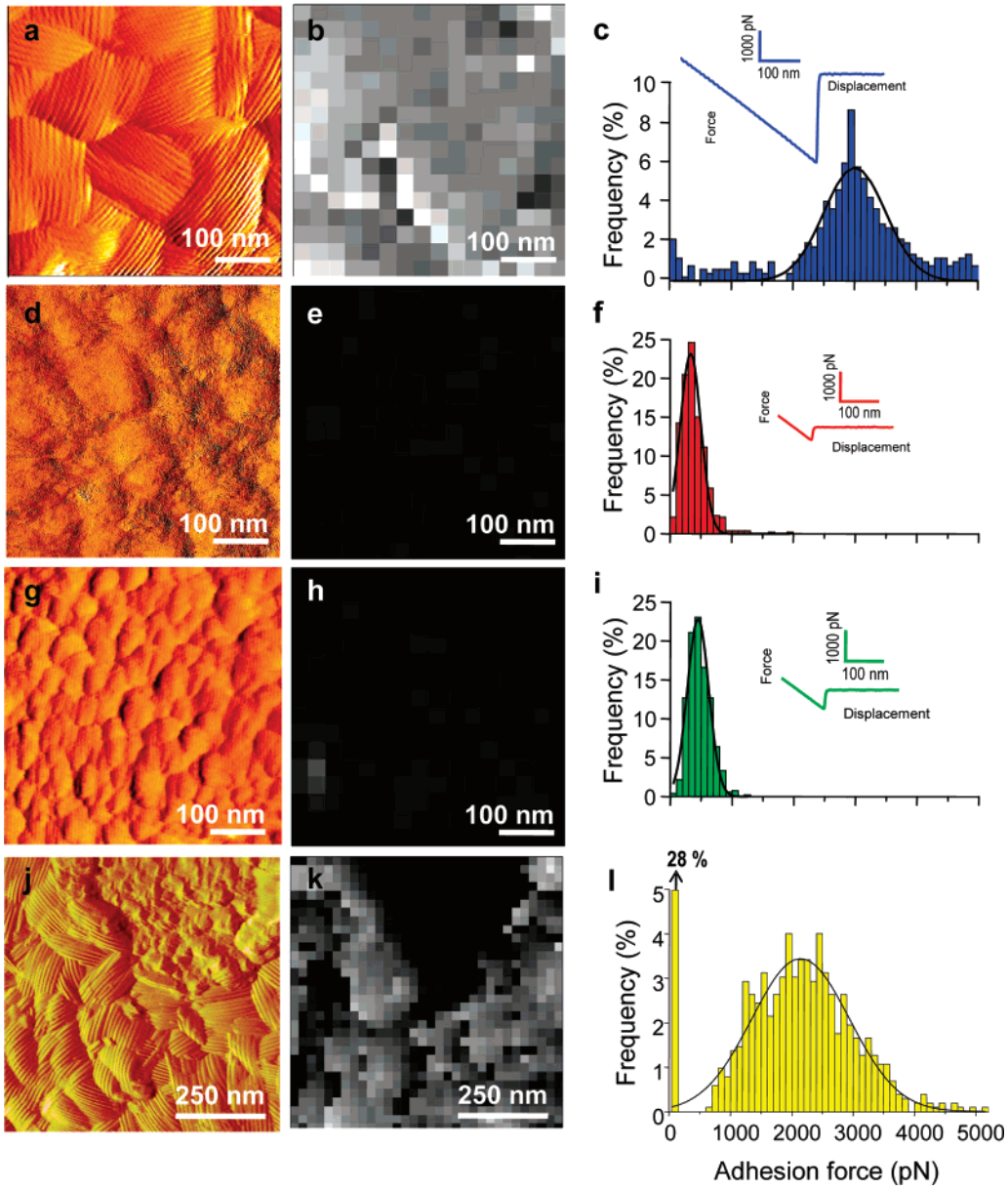

Figure 2. Nanoscale structure and hydrophobicity of *Aspergillus fumigatus*. (a) High-resolution image of a wild type conidial surface in aqueous solution revealing rodlets. (b) Adhesion force map (z-range: 6 nN) and (c) adhesion force histogram ($n = 512$) recorded with a hydrophobic tip, indicating that the rodlet surface is uniformly hydrophobic. (d−i) High-resolution images, adhesion force maps, and histograms ($n = 512$) obtained on NaOH-treated conidia (d−f) and on the ΔrodA ΔrodB double mutant (g−i). The purely hydrophilic surface is attributed to cell wall polysaccharides. (j−l) High-resolution image, adhesion force map, and histogram obtained on SDS-treated conidia, revealing highly correlated structural and hydrophobic heterogeneities.

31 pN for isoniazid-treated cells (Figure 3e,f), confirming that hydrophobicity is conferred by mycolic acids and that the latter are exposed at the cell surface. These results represent the first direct, quantitative measurement of the impact of an antimycobacterial drug on the hydrophobic character of single mycobacterial cells, which may be of great relevance in future research. For instance, it may contribute to refine our understanding of the macromolecular cascade of events leading to bacterial death upon treatment with specific cell wall antibiotics. Last, our finding that native cells show uniform distribution of hydrophobicity (see homogeneous adhesion map in Figure 3b) may help to draw a more integrated view of the cell wall architecture, as it confirms the idea that mycolic acids form an external layer and suggests the absence of hydrophilic polysaccharides, particularly lipoarabinomannans, embedded in this layer.

In conclusion, we have shown that CFM with hydrophobic tips enables researchers to quantify and map the surface hydrophobicity of single live cells in relation with function (i.e., dispersion, adherence, and interaction with drugs). This chemically sensitive imaging method circumvents the main limitations of methods currently available for assessing surface hydrophobicity and, for the first time, allows resolving nanoscale variations of surface properties. Also, it has often been argued that AFM is limited by its depth resolution in that it only analyzes the outermost cell surface. Here, we

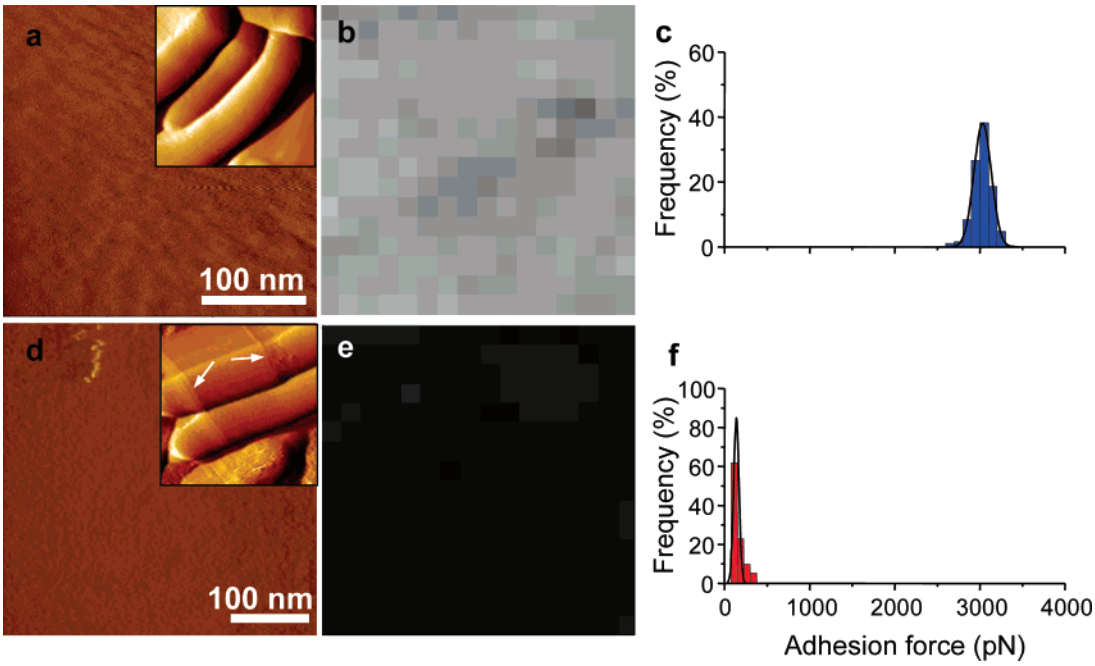

Figure 3. Nanoscale structure and hydrophobicity of *Mycobacterium bovis*. (**a**) Low-resolution (inset image; 2.5 μm × 2.5 μm) and high-resolution images, (**b**) adhesion force map (z-range : 4 nN) and (**c**) adhesion force histogram (n = 1024) obtained on *M. bovis* with a hydrophobic tip. The smooth, hydrophobic surface is attributed to the outer layer of mycolic acids. (**d**) Low-resolution (inset image; 2.5 μm × 2.5 μm) and high-resolution images, (**e**) adhesion force map (z-range : 4 nN) and (**f**) adhesion force histogram (n = 512) obtained following treatment of the cells with isoniazid. While only slight structural alterations are seen (arrows), a dramatic decrease of hydrophobicity is noted, reflecting the inhibition of mycolic acid synthesis.

show that combining the technique with chemical or drug treatments allows microscopists to explore inner cell wall layers. Applicable to prokaryotic, plant, and animal cells, as well as to the probing of surface charge, the CFM methodology provides new avenues for understanding the structure−function relationships of cell surfaces, particularly in relation with pathogen−drug and pathogen−host interactions.

Methods. Wild-type conidia of *A. fumigatus* Dal CBS144-89 were selected because of their clinical importance. Conidia were harvested from a one-week-old culture grown at 25 °C on 2% malt extract agar. They were rinsed 3 times in Tweened water and 5 times in deionized water. For some experiments, conidia were boiled 2 × 1 h in 1 M NaOH or in a 50 mM Tris-buffered solution containing 2% SDS, 50 mM EDTA, and 40 mM β-mercaptoethanol and then rinsed again to eliminate any contaminants. The ΔrodA ΔrodB double mutant was obtained as described elsewhere.[12] *Mycobacterium bovis* BCG was grown in Sauton medium at 37 °C for about 3 weeks in static conditions using 75 cm^2 Roux flasks. For some experiments, cells were resuspended for 24 h in Sauton medium containing isoniazid at 0.02 μg/mL, corresponding to the MIC. All cells were harvested by centrifugation and washed three times with deionized water.

AFM contact mode images and force−distance curves were obtained in deionized water using Nanoscope III and IV Multimode AFMs (Veeco Metrology Group, Santa Barbara, CA). Oxide-sharpened microfabricated Si_3N_4 cantilevers with spring constants of 0.01 N/m (Microlevers, Veeco Metrology Group, Santa Barbara, CA) and silicon wafers (Siltronix, France) were coated by electron beam thermal evaporation with a 5 nm thick Cr layer followed by a 30 nm thick Au layer. Gold-coated cantilevers were immersed for 14 h in 1 mM solutions of $HS(CH_2)_{11}CH_3$ in ethanol and then rinsed with ethanol. Gold-coated supports were immersed for 14 h in ethanol solutions containing 1 mM $HS(CH_2)_{11}CH_3$ and $HS(CH_2)_{11}OH$ in various proportions and then rinsed with ethanol.

Acknowledgment. This work was supported by the National Foundation for Scientific Research (FNRS), the Université Catholique de Louvain (Fonds Spéciaux de Recherche), the Région Wallonne, the Federal Office for Scientific, Technical, and Cultural Affairs (Interuniversity Poles of Attraction Programme), and the Research Department of the Communauté Française de Belgique (Concerted Research Action). Y.F.D. is a Research Associate of the FNRS.

References

(1) Meyer, E. E.; Rosenberg, K. J.; Israelachvili, J. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 15739.
(2) Doyle, R. J. *Microbes Infect.* **2000**, *2*, 391.
(3) Doyle, R.J., Rosenberg, M., Eds. *Microbial Cell Surface Hydrophobicity*; American Society for Microbiology: Washington, DC, 1990.
(4) Engel, A.; Müller, D. J. *Nat. Struct. Biol.* **2000**, *7*, 715.
(5) Scheuring, S.; Sturgis, J. N. *Science* **2005**, *309*, 484.
(6) Dufrêne, Y. F. *Nat. Rev. Microbiol.* **2004**, *2*, 451.
(7) Touhami, A.; Jericho, M.; Beveridge, T. J. *J. Bacteriol.* **2004**, *186*, 3286.
(8) Hinterdorfer, P.; Dufrêne, Y. F. *Nat. Methods* **2006**, *3*, 347.
(9) Frisbie, C. D.; Rozsnyai, L. F.; Noy, A.; Wrighton, M. S.; Lieber, C. M. *Science* **1994**, *265*, 2071.
(10) Noy, A.; Frisbie, C. D.; Rozsnyai, L. F.; Wrighton, M. S.; Lieber, C. M. *J. Am. Chem. Soc.* **1995**, *117*, 7943.

(11) Vezenov, D. V.; Noy, A.; Rozsnyai, L. F.; Lieber, C. M. *J. Am. Chem. Soc.***1995**, *119*, 2006.
(12) Paris, S.; Debeaupuis, J.-P.; Crameri, R.; Carey, M.; Charlès, F.; Prévost, M. C.; Schmitt, C.; Philippe, B.; Latgé, J. P. *Appl. Environ. Microbiol.* **2003**, *69*, 1581.
(13) Wessels, J. G. H. *Adv. Microbiol. Physiol.* **1997**, *38*, 1.
(14) Fontaine, T.; Simenel, C.; Dubreucq, G.; Adam, O.; Delepierre, M.; Lemoine, J.; Vorgias, C. E.; Diaquin, M.; Latgé, J. P. *J. Biol. Chem.* **2000**, *275*, 27594.
(15) Brennan, P. J.; Nikaido, H. *Annu. Rev. Biochem.* **1995**, *64*, 29.

NL071476K