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▶ To cite this version:

Sofiane El-Kirat-Chatel, Audrey Beaussart. Probing Bacterial Adhesion at the Single-Molecule and Single-Cell Levels by AFM-Based Force Spectroscopy. Nanoscale Imaging: Methods and Protocols, 2018, pp.403-414. 10.1007/978-1-4939-8591-3_24. hal-03215692

HAL Id: hal-03215692 https://hal.science/hal-03215692

Submitted on 27 Jun 2022

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Probing bacterial adhesion at the single-molecule and single-cell

levels by AFM-based force spectroscopy

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i) SUMMARY/ABSTRACT

Functionalization of AFM tips with biomolecules or microorganisms allows for a better understanding of the interaction mechanisms driving microbial adhesion. Here we describe the most commonly-used protocols to graft molecules and bacteria to AFM cantilevers. The bioprobes obtained that way enable to measure forces down to the single-cell and single-molecule levels.

ii) KEY WORDS

Atomic force microscopy, functionalization, single-molecule, single-cell, interactions

Running title: AFM tip functionalization for force spectroscopy

1 INTRODUCTION

In the last two decades, atomic force microscopy (AFM) modes have evolved and now offer a wealth of new possibilities to address pertinent biological questions. As AFM is particularly suitable to determine sample surface topography at high resolution, the first adaptation to biological systems was to image cells under liquid, *i.e.* in physiological conditions. This approach allowed imaging the surface of live mammalian or microbial cells at the nanoscale. As non-exhaustive examples, the organisation of the bacterial peptidoglycan(1-5), the surface of macrophages(6, 7), the S-layer of *Corynebacterium glutamicum*(8), or the rodlets of *Aspergillus fumigatus*(9, 10) have been revealed with unprecedented resolution. Although powerful for topographical analysis, AFM is not limited to imaging but can also serve to probe interaction forces. Taking advantage of the sharpness of the AFM tip, several groups have developed approaches to detect single molecular interaction by the so-called single-molecule force spectroscopy (SMFS). In SMFS, AFM tips are functionalized with biomolecules and used to probe purified targets immobilized on abiotic surfaces or receptors expressed at the surface of living cells (Fig. 1a, b)(11-15). By mean of SMFS, biomolecules could be mapped on microbial surfaces to determine whether their distribution is random, spatially organized or can change upon external stimuli (Fig. 2 a, b) (16-18). Additionally, SMFS allows to decipher the intrinsic mechanical properties of biomolecules as they are manipulated and unfolded after detection (Fig. 1b, 2d). In that sense, fitting the unfolding curves with commonly-used models such as the worm-like chain (WLC) or free jointed chain (FJC) permits to quantitatively describe the elasticity of flexible molecules(19-23). Microbial adhesion is driven by a complex interplay of physico-chemical forces. Receptors-ligands interactions are often coupled to non-specific hydrophobic and electrostatic forces. Deciphering such component of cellular adhesion has been made possible with the development of chemical force microscopy (CFM), where AFM tips are decorated with specific functional groups, such as hydrophobic thiols(24-28).

Further understanding of how these different forces act together for microbial cells to attach to each other, to solid substrates or to host cell was not possible until the recent development of single-cell force spectroscopy (SCFS) adapted to microorganisms(29-31). Indeed, if the development of SCFS for large cells (mammalian cells, fungi) was quite straight forward with the use of tipless cantilever(30, 32, 33), probing interactions of smaller cells such as bacteria had needed a dedicated design that allows proper positioning of the cell. Here, we detailed how to proceed to perform force spectroscopy at the single-molecule and the single-cell levels and we give insights on the choice of functionalization procedures the best suitable for specific desired applications. The methods described hereafter are commonly used in bio-AFM studies but other ways to functionalize AFM probes with biomolecules or cells also exist, *e.g.* with dendritips for SMFS(34) or with microchanneled cantilevers for SCFS in the FluidFM mode(35).

2 MATERIALS

Prepare all solutions using ultrapure water and analytical grade reagents.

2.1 Microorganisms immobilization for force spectroscopy with functionalized tips

1. Polycarbonate porous membranes (it4ip or Millipore) of pore diameter similar to the bacterial size (if the goal is to image/probe the side of rod-shaped bacteria, Millipore is more suitable as these membranes present jointed pores making holes bigger and with adapted shapes).

- 2. Glass filtration unit (Sartorius).
- 3. AFM magnetic steel puck (Bruker).
- 4. Double-sided tape (3M).
- 5. Nanoscope VIII multimode atomic force microscope (Bruker).

6. Silicon nitride triangular-sharpened cantilevers with a nominal spring constant of 0.01 N m⁻¹ (MSCT, Bruker) or gold-coated cantilevers with a nominal spring constant of 0.02 N m⁻¹ (OMLC-TR400PB-1, AtomicForce, Olympus).

7. Piranha solution: 3:1 solution of concentrated H₂SO₄ and 30% H₂O₂.

2.2 Single-cell force spectroscopy

8. Bioscope catalyst atomic force microscope (Bruker) mounted on an inverted optical microscope (Zeiss Axio Observer ZI) equipped with a Hamamatsu C1000 camera with a 40X objective and a 100X oil-immersion objective.

- 9. UV-curable glue NOA 63 (Norland, Edmund Optics).
- 10. LIVE/DEAD Baclight viability kit (Invitrogen).
- 11. Silica microspheres, 6.1 µm diameter (Bangs laboratories).

12. Triangular-shaped tipless cantilever with a nominal spring constant of 0.06 N m⁻¹ (NP-010, Microlevers, Bruker).

13. Dopamine hydrochloride, 99% (Sigma).

14. Tips and gold surface cleaning: UV-ozone treatment (Jetlight)

15. Glass-bottomed Petri dishes (WillCo-dish).

16. UV lamp, 365 nm, 6W (handheld UV lamp UVL-56; UVP).

3 METHODS

Carry out all procedures at room temperature unless otherwise stated

3.1 Force measurements at the surface of microorganisms with (bio)-molecules grafted tips.

1. Grow bacterial cell cultures in the recommended conditions (stationary or exponential phase; with or without shaking for aerobic and anaerobic conditions, respectively; at appropriate temperature; with the proper antibiotics).

2. Transfer 20 mL of the bacterial suspension in a 50 mL falcon and rinse twice or 3 times by centrifugation (2 min at 5000 g or other recommended parameters for harvesting) in the experimental buffer.

3. To properly immobilize mechanically the microorganisms, filter 1 mL of a 10⁶ cells mL⁻¹ suspension of the rinsed suspension through a polycarbonate membrane whose pore size approximate the bacterial size, using a filtration unit.

4. Cut a 1 cm x 1 cm square in the porous membrane, gently rinse to remove untrapped cells and stick it to an AFM magnetic steel puck using double-sided tape. (see **Note 1**)

5. Immediately cover the membrane with a 100 μ L drop of buffer and transfer to the AFM liquid set-up without dewetting.

6. Image the membrane under low applied force (~100 pN) using a bare soft cantilever (MSCT, Bruker Corporation, are recommended).

7. Once bacteria are identify and located on the image, remove the bare AFM tip and place the functionalized tip (see details below) exactly at the same position above the membrane.

8. Several methodologies can be chosen to decorate the tip with molecules. The most common ones are described here:

8.A. Covalent attachment of single protein using PEG-benzaldehyde linkers as described by Ebner *et al.* (36-38) (see Note 2)

Briefly, cleaned MSCT (silicon nitride) cantilevers are decorated by amine groups *via* overnight immersion in an ethanolamine solution. The ethanolamine-coated cantilevers are then dipped in an acetal-PEG-NHS solution and the acetal groups are further transformed into aldehyde functions by immersion in a citric acid solution. The tips are covered with a droplet of buffer containing 0.2 mg mL⁻¹ of the proteins to

be attached (see **Note 3**). Coated cantilevers are then immersed in ethanolamine solution in order to passivate unreacted aldehyde groups, washed with and stored in buffer.

8.B. **Covalent attachment of single protein using NHS-EDC.** (see **Note 4**) Goldcoated cantilevers are rinsed with ethanol, cleaned for 15 min with UV-ozone, rinsed again with ethanol, dried with a gentle flow of N₂, immersed overnight in a 1 mM solution of 10% HS(CH₂)₁₆COOH (Sigma-Aldrich) and 90% HS(CH₂)₁₁OH (Sigma-Aldrich) and rinsed with ethanol. They are then immersed for 30 min in a solution containing 10 mg mL⁻¹ *N*-hydrosuccinimide (NHS) (Sigma-Aldrich) and 25 mg mL⁻¹ 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Sigma-Aldrich), and rinsed with Ultrapure water. The activated cantilevers are incubated with 0.2 mg mL⁻¹ of protein solution, followed by rinsing and storage in buffer.

8.C. Oriented attachment of His-tagged-protein. (see Note 5) Gold-coated cantilevers are rinsed with ethanol, cleaned for 15 min with UV-ozone, rinsed again with ethanol, dried with a gentle flow of N₂, and immersed overnight in a 0.1 mM solution of 90% HS-C11-(EG)₃-OH thiols (ProChimia) and 10% HS-C11-(EG)₃-NTA thiols (ProChimia). The tips are then rinsed with ethanol, dried with N₂, and immersed in a 40 mM aqueous solution of NiSO₄ (pH 7.2) for 30 min. They are then incubated in a 200 μ L droplet of a 0.2 mg mL⁻¹ His₆-tagged proteins solution for 1h, rinsed and stored in buffer until use(39) (see Note 6).

8.D. **Tip coating with thiols molecules.** (see **Note 7**) Gold-coated cantilevers are rinsed with ethanol, cleaned for 15 min with UV-ozone, rinsed again with ethanol, dried with a gentle flow of N₂, and immersed overnight in a 0.1 mM solution of HS-(CH₂)₁₁-CH₃ or HS(CH₂)₁₆COOH thiols (Sigma-Aldrich), for hydrophobic or negatively-charged probes, respectively.(24, 26, 27)

8.E. **Grafting of nanoparticles.** Given the wild range of NPs chemical compositions that exist, no systematic protocol can be given. Here, one example is detailled for the attachment of mannofullerene NPs containing one thiol group. Briefly, thiol-mannofullerenes (disulfide-containing bis-mannofullerene displaying 20 peripheral mannose residues) were synthetized from a [5:1] heterovalent fullerene scaffold.(40) Prior to tip immersion, 1 mM TCEP (tris(2-carboxyethyl)phosphine hydrochloride)) solution is added to the thiol solution (50% vol) for reduction of the disulfide bond of the mannofullerenes. Gold-coated cantilevers are rinsed with ethanol, cleaned for 15 min with UV-ozone, rinsed again with ethanol, dried with a gentle flow of N₂, and immersed overnight in a 1 mM solution of thiol-mannofullerenes, rinsed with ethanol and dried with N₂.

9. Choose an appropriate size of the surface area to be mapped, depending on the size of the cell (for instance 500 nm x 500 nm for bacteria; $1 \mu m \times 1 \mu m$ for yeast cells) (Fig. 1a)

10. Record force-distance curves on the selected area using the force-volume mode (Fig. 1a, b), with a maximum applied force of 250 pN and approach and retract velocities of around 1 μ m/s. (See **note 8**)

11. To generate adhesion force and rupture length histograms (Fig. 1c), use the AFM software to calculate the adhesion force and rupture distance for each curve. Color-scaled map of the adhesion values can be recreated using the Matlab software (Fig. 1d).

3.2 Single-cell force spectroscopy

1. Deposit a small amount of UV-curable glue on a glass slide stuck to a magnetic steel puck using double-sided tape. (see **Note 9**).

2. Transfer the puck to an AFM set-up equipped with an optical microscope. Dip an AFM soft tipless cantilever into the glue by manual engagement (Fig. 3a).

3. Deposit a small amount of silica microsphere on another glass slide stuck to a magnetic puck.

4. Using the AFM, bring the glue-covered cantilever down to a single microsphere (Fig. 3b).

5. After a short contact of few seconds, disengage the cantilever and cure the glue by putting the colloidal probe under the UV lamp for 10 min.

6. Prepare a 10 mM Tris-HCl buffer solution at pH 8.5 containing 4 mg mL⁻¹ of polydopamine hydrochloride and immediately immerse the colloid probe for 1h in the solution (Fig. 3c).

7. Rinse the probe by immersion in Ultrapure water and dry under gentle flow of nitrogen.

8. For attaching a single-bacterium, first deposit 50 μ L of diluted bacterial suspension (cultured as in step 1 of 3.1) on a glass-bottom Petri-dish and let the bacteria to settle.

9. Mark the bacteria viability using a LIVE/DEAD kit and complete the volume of the Petri dish on settled-stained bacteria.

10. Prepare the sample whose interactions with the bacteria will be measured and insert it in the AFM liquid set-up.

11. Using an AFM mounted on an inverted fluorescence microscope, bring the dopamine-coated colloidal probe into contact with an isolated bacteria marked in green for 1 to 3 minutes (Fig. 3d, e).

12. Withdraw the colloidal probe from the glass Petri-dish and ensure that the bacteria is properly attached by taking an optical/fluorescent image (Fig. 3f).

13. Engage the cell-probe on the substrate of interest and record consecutive forcedistances curves on different spot of the surface of interest. 14. To generate adhesion force and rupture length histograms, use the AFM software to calculate the maximum adhesion force and rupture distance for each curve.

4 NOTES

 In order to facilitate the sticking of the wet membrane to the double-sided tape, the membrane can be very gently deposited briefly on a wiper (for instance Kimwipes, KimTeck Science) before adhesion to the tape. In this case ensure that the top surface layer of the membrane remains moist.

2. This chemistry allows grafting covalently a single-molecule to the apex of the AFM tip. The use of a PEG linker lowers unspecific interactions and ensures the flexibility of the attached protein for a better accessibility to the target.

3. Addition of sodium cyanoborohydride (NaCNBH₃) is required for this step. Care should be taken given the toxicity of the cyanide compound, in particular as dangerous vapor may emanate in case of contact with acid.

4. This protocol allows to covalently attach the protein to the tip, and to modulate the grafting density by varying the ratio of COOH/OH terminated thiols. The NHS and EDC have to be mixed just before use.

5. The main advantage here is that grafting the protein *via* a tag permits to control its orientation. In case of enzyme for instance, the tag would be located outside the catalytic domain for it to remain active.

6. Given the weakness of the His-tag/NTA bond, we recommend to use the tip directly after functionalization and to avoid storage.

7. Decorating the tip with thiol molecules presents the advantage to obtain a probe whose chemistry can be fully controlled, to decipher the surface physical-chemistry properties (for instance hydrophobicity(26, 27) or charges distribution (24)).

8. In case of ligand/receptor interactions, the contact time between the tip and the cell surface may be varied from 0 to 1 s. When analyzing the adhesion properties of different strains for instance, the contact time should be equivalent for comparison to be possible.

9. To obtain a small quantity of glue on the surface, spread the glue droplet using another glass slide or a pointed tweezer.

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