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COLLOIDS AND SURFACES A

Adhesive forces between ligand and receptor measured by AFM

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Abstract

Since its invention, the atomic force microscope (AFM) has been used to image a wide range of samples, including soft biological materials. Although various imaging modes have been developed in recent years, the images obtained by these techniques are primarily derived from the mechanical interactions between AFM tip and sample. An imaging mode based on the specific recognition of defined functional groups on the sample by receptor molecules attached to the AFM tip may prove to be beneficial, especially in complex specimens such as the cell membrane. A prerequisite towards this goal is the development of AFM tips functionalized with ligand-specific receptors. Here we report the fabrication of tips functionalized with either avidin or antibodies. These functionalized tips were characterized by force scan measurements on ligands cross-linked to agarose beads. The specificity of the observed adhesion between tip and sample was confirmed by specific blocking of the ligands or the receptors.

Keywords: Adhesive forces; Atomic force microscope; Ligand–receptor interactions

1. Introduction

The complex organization of life requires highly regulated specific interactions between molecules with binding energies on the order of a few kT. These interaction energies are typically measured under equilibrium thermodynamic conditions in calorimetric studies. However, in many processes such as cell–cell adhesion, it is the force between molecules rather than the binding energy that is the relevant parameter for characterizing the function of the system [1]. Despite the fact that ligand–receptor interactions are crucial in every aspect of life, little is known about the forces that govern these interactions. Experiments with the surface force apparatus [2], optical tweezers [3,4], magnetic beads [5] and micropipets [6] can provide measurements of forces between ligand and receptors, but these techniques typically involved a large, ill-defined number of interacting partners or lack lateral resolution.

In this paper, we demonstrate that the atomic force microscope (AFM) [7–9] can be used to measure the interaction between ligands and receptors when operated in the force scan mode [10,11]. AFM tips with curvature radius of about 10 nm can be manufactured [12,13], providing an interaction area that can only be occupied by a few molecules. The typical bond strength, based on an estimate of 150 pN, is within the optimal working sensitivity of the instrument (10 pN–5 nN) [14], making the AFM an ideal choice for these types of measurement. Direct measurements of the interaction forces between few and eventually individual molecules by AFM should therefore be possible.

A basic requirement for any binding experiment is the immobilization of receptors or ligands onto the AFM tip. We have chosen to investigate the

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biotin–avidin adhesion system, arguably the best characterized high affinity system available [2,15,16]. Furthermore, the biotin–avidin interaction forms a general linkage that can be used in the development of other types of functionalized tip. Tips functionalized with antigen-specific antibodies might have potential applications such as affinity imaging beyond force measurements. Our procedure for attachment of receptors to AFM tips is based on a series of simple adsorption and incubation steps rather than chemical reactions [17].

2. Experimental

2.1. Materials and methods

All chemical reagents including avidin and biotinylated agarose beads, except protein G Sepharose (Pharmacia, Freiburg, Germany), were obtained from Sigma (Deisenhofen, Germany). Agarose beads were washed with phosphate buffer saline (PBS; 20 mM PO_4^{2-}, 150 mM NaCl, Milli-Q H_2O, pH 7.2) and resuspended in the same buffer for all measurements. Typically, beads with 100 μm diameter were selected for measurements. The monoclonal antibody, M18 (rat IgG) was labeled with biotin, purified and used in the construction of the antibody functionalized tip. The home-built standalone AFM used in this study was described and characterized previously [18]. All force scan measurements were recorded using silicon nitride cantilevers with integrated tips (Digital Instruments, Santa Barbara, CA) from the same wafer. Cantilevers with a spring constant of 0.06 N m^{-1} were used for the work described here. The optical response of the cantilever to an external force was calibrated on a rigid surface. The slope of the deflection versus piezo displacement at the contact region provides the calibration between force and deflection signal. The sample compartment is formed by an aluminum ring and a circular glass coverslip (Fig. 1(a)) and is secured with a rubber O-ring. Both coverslip and O-ring were cleaned by sonication in 2% Hellmanex solution (Hellma, Müllheim, Germany) and rinsed extensively with Milli-Q water prior to experiments.

2.2 Functionalization of AFM tips

Prior to any surface modification, the Si_3N_4 tip/cantilever showed no measurable affinity towards biotin-derivatized, avidin-derivatized and regular agarose beads. As illustrated in Fig. 2, the initial step in the construction of the avidin tip is the adsorption of biotinylated BSA (bovine serum albumin) onto the surface of the Si_3N_4 tip. This step consisted of an overnight incubation of the cantilevers at 37°C in a biotin–BSA solution (5 mg ml^{-1} in PBS). This resulting biotin-labeled intermediate was stored at 4°C until needed and exhibited affinity towards avidin beads, but not biotin beads. Avidin-functionalized tips were prepared freshly for each measurement from biotin-functionalized tips. After a 5 min incubation period

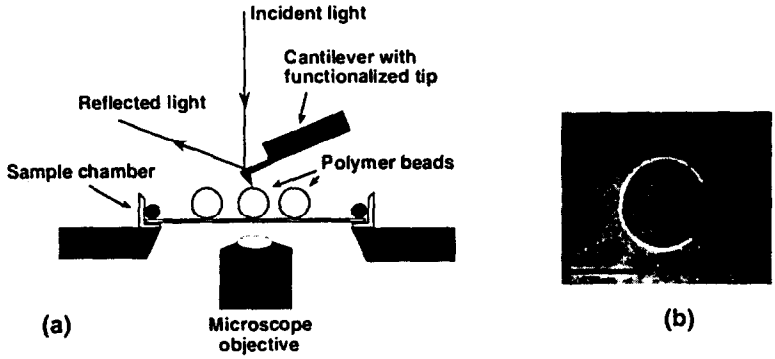

Fig. 1. (a) Schematic diagram of the scanned stylus atomic force/light microscope used in force scan measurements of polymer beads, (b) Light micrograph of the AFM cantilever on top of an agarose bead.

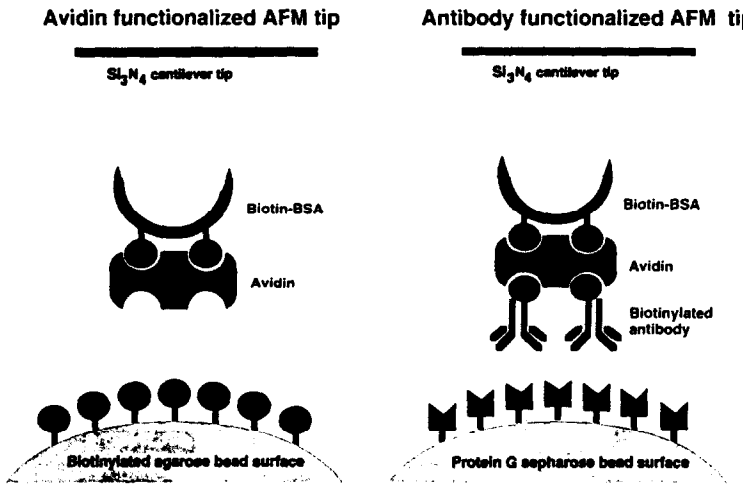

Fig. 2. Schematics of the functionalized tips.

in avidin at 2 mg ml^{-1}, the tips were used immediately. Antibody-functionalized tips were prepared from avidin-functionalized tips after incubation in biotinylated antibodies (1 mg ml^{-1} in PBS, 5 min). Between all steps in this process, the tips were rinsed five times with PBS.

3. Results

The force scan measurement, consisting of an approach trace, followed by a retraction trace, characterizes the interaction between sample and AFM tip. During the approach trace, the surfaces, initially separated, approach each other and make contact. In the contact region, the cantilever pushes against the sample, causing the cantilever to bend away from the sample. The retraction trace takes place immediately following the approach trace with the tip and sample in contact. During retraction, the system reaches a point where there is no tension on the cantilever. Upon further separation, contact is broken in the absence of adhesion between the surfaces. Alternatively, the tip and sample can remain in contact, causing the cantilever to deflect in the direction of the sample until the tension on the cantilever exceeds the adhesion force between the surfaces. The maximum deflection of the cantilever provides a measurement of the adhesion force between the surfaces.

Force scan measurements were carried out to characterized the functionalized tips using ligands covalently immobilized to elastic polymer beads as samples. Functionalized agarose beads were localized under an inverted microscope (Zeiss Axiomat) vibrationally isolated on an optical table (see Fig. 1(a)). Measurements were carried out using a stand-alone AFM positioned so that its cantilever tip was directly over the center of the bead. Fig. 3 shows force scans on (A) a glass surface and (B) a biotin agarose bead obtained with a biotin-derivatized tip in PBS solution. As shown, the force scan on glass recorded with the derivatized tip is qualitatively identical to the scans recorded with standard Si_3N_4 tips [10]. The lateral displacement between approach and retraction traces has been attributed to frictional effects [19]. The qualitative differences between the scans on beads and glass surfaces are in the slopes of the deflection of the cantilever and in the deviation from linearity at low forces observed with the beads. Both effects can be attributed to the elastic properties of the beads [20]. From these scans, we can estimate the deformation on the beads induced by the AFM tip from the difference in the piezo displacement between beads and rigid samples for a given applied force. The deformation includes both the indentation of the tip into the bead and the compression of the bead. All measurements were carried out at a maximum applied force of approxi-

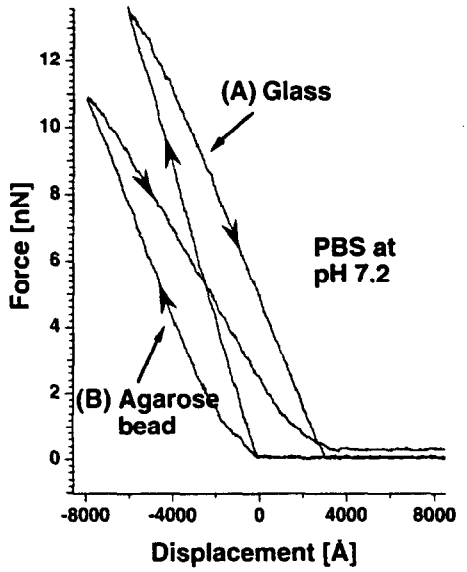

Fig. 3. Force scans on glass (A) and biotin bead (B) using a biotin functionalized tip.

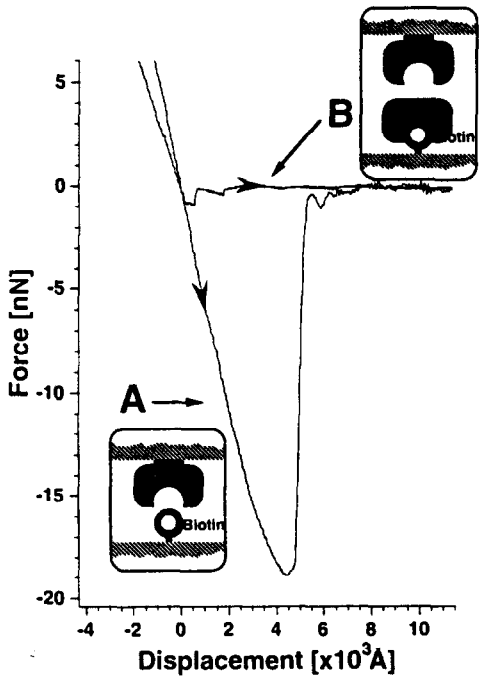

Fig. 4. Force scan (retraction trace only) on biotin bead using an avidin functionalized tip (A). Inhibition of the adhesion with free avidin (B). The inserts illustrate the surface chemistry of the interacting surfaces.

mately 10 nN resulting in an elastic deformation of 300 nm. The geometry of this deformation can not be determined directly from the force scans without further assumptions, however it is clear that the contact area is enlarged by the use of the polymer beads when compared to rigid substrates.

Fig. 4 shows the retraction traces of the force scans carried out on a biotinylated agarose bead using avidin-functionalized AFM tips. With an applied force of 10 nN, measurements using different tips and on different beads revealed an adhesion force of 15–20 nN for the interaction between biotin and avidin surfaces. To confirm the specificity of this interaction, blocking experiments were carried out. Free avidin (Fig. 4, trace B) or free biotin (data not shown), but not BSA inhibited the adhesion. Furthermore, tips coated with just biotin–BSA, but not avidin, did not exhibit adhesion to the biotin beads (Fig. 3, trace B). It should be pointed out that not all traces are as simple as the one presented in Fig. 4, trace A. In some instances, the trace reveals a de-adhesion process that occurs in steps. In inhibition experiments with free avidin, 85% of the adhesive interaction was blocked after 15 min and 95% inhibition was detected 60 min after addition of avidin. The remaining interaction seemed quantized in steps of a few hundred piconewtons (see Fig. 4, trace B). Whether these steps reflect a few remaining specific interactions or whether they are caused by frictional effects cannot be determined with this limited set of data. All measurements shown in Fig. 4 were obtained using the same tip. The approach traces of force scans presented in Figs. 4 and 5 are not relevant in our discussion and were omitted from the figures.

To demonstrate that these measurements can be extended to systems of wider interest, we also measured the interaction between antibody functionalized tips and protein G agarose beads [21]. We designed the experiment in a manner that would permit comparison with the adhesion strength of the biotin–avidin interaction. First, the adhesion force between an avidin tip and a biotin bead was recorded as shown in Fig. 5, trace A. Subsequently, the same tip was incubated with biotinylated antibody and the resulting antibody

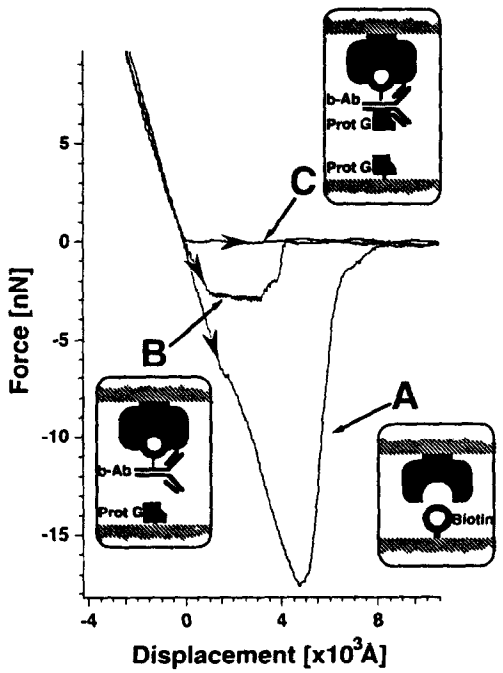

Fig. 5. Force scan (retraction trace only) on biotin bead using an avidin functionalized tip (A). Force scan of the same tip after incubation with a biotin labeled IgG antibody on a protein G agarose bead (B). Inhibition of the antibody–protein G adhesion with free protein G (C). The inserts illustrate the surface chemistry of the interacting surfaces. b-Ab and prot G are the abbreviations for biotin-labeled antibody and protein G respectively.

functionalized tip was used in the force scan on protein G agarose beads. The adhesion force measured in this system was 3–4 nN (Fig. 5, trace B), approximately fivefold weaker than the avidin–biotin interaction, and could be inhibited with soluble protein G (Fig. 5, trace C).

4. Discussion

Although there was variability in cantilevers from different wafers and between preparations, the avidin-functionalized tips described in this work were functionally stable for hundreds of force scans and on different beads. These observations indicate that the contact breaking bond is between the biotin on the bead and the avidin on the tip. Since this is not intuitive, it is necessary to examine the construction in some detail, beginning with the adsorption of biotin–BSA onto the oxidized Si_3N_4 tip. The process is analogous to adsorption of BSA on glass which has a binding energy of -200 kJ mol^{-1} [22]. The binding energy of biotin to avidin is -88 kJ mol^{-1} [23]. If we assume equal gradients of the potential in both cases, this difference in binding enthalpies would explain the stability of the adsorbed BSA film. The second linkage that requires examination is coupling of avidin to the tip via biotin–BSA. Since this interaction is identical to the interaction under consideration, one would expect that this linkage should break with equal frequency. However, it is likely that the tetrameric avidin molecule is attached to the tip via two or even three biotin-binding sites as a result of the long incubation time. In our experiments, the scan cycle was 1 Hz with surface contact time of a few hundred milliseconds. It is therefore unlikely that an avidin molecule can form more than a single bond with the biotin bead even if multiple biotin sites were available. The antibody-functionalized tips were less stable than the avidin tip. We attributed this observation to the greater complexity of the antibody tip and the greater inherent mechanical stability of avidin [18]. The finite durability may limit the use of antibodies in imaging or may require frequent regeneration of the tips.

The principal objective of this work is the development of functionalized tips. In designing a system that optimizes the detection of specific interactions between ligand and receptor, the ability to measure discrete interaction was compromised. Because the binding force of the individual molecular pairs is not known, the number of interacting molecules cannot be determined exactly. However, if we approximate the adhesion force as binding energy divided by the binding length, about 8 Å [24], the estimated binding force per avidin–biotin pair is on the order of 175 pN. Under this assumption, the adhesion between the avidin tip and the biotin bead in our experiments corresponds to a contribution from about 100 molecular pairs. Furthermore, if we assume a dense coverage of the tip with avidin (30 nm^2 per molecule of avidin), then the interaction area is equivalent to half the area of a sphere with a radius of

22 nm. This would mean that in our experiment the entire curvature of the tip contributes to the adhesion.

5. Conclusions

Our experiments clearly demonstrate that the AFM can be utilized to investigate specific ligand–receptor interaction. The geometry used in our force scan experiments favored the overall interaction strength over the discrimination of single events. The necessity for the latter measurements became obvious during the course of our data analysis. Although it was our intention to be able to compare adhesion strengths between the biotin–avidin and the antibody–protein G interactions, this was not feasible since the number of interacting molecules in neither case can be determined or set to equal value. Single molecular interactions should become detectable by a reduction of the interaction area in combination with a lateral dilution of the binding partners. Minor modification of our existing system should allow us to measure forces with 10 pN sensitivity, which is an order of magnitude smaller than the estimated strength of the avidin–biotin interaction. Measurements of the interaction forces between single ligand–receptor pairs will provide new insight in the basic mechanisms of molecular recognition, not accessible with conventional calorimetric studies.

Applications for functionalized tips beyond the force measurements have been implied earlier. The feasibility of chemical identification and antigen-specific affinity imaging will be reported in future communications. The manipulations of surface structure with enzyme-functionalized tips might also be possible in the not too distant future.

Acknowledgments

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