MEASURING MOLECULAR ADHESION WITH FORCE MICROSCOPY

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ABSTRACT. Local probe methods were used to investigate organic and biological molecules in order to characterize their surface density and to measure adhesion forces between individual molecular pairs. The unbinding force between a streptavidin functionalized flat surface and a biotinylated sensor tip was measured to be 1.25 nN indicating contributions of very few molecular pairs.

1. Introduction

The molecular interplay in biology is one of the most basic prerequisites of life in general. The ability of molecules and cells to specifically recognize each other relies on the existance of several binding interactions which are short-ranged, chemically and geometrically complementary and sometimes of remarkable strength.
In order to characterize the macroscopic properties of intermolecular adhesion, traditionally, binding energies, binding enthalpies and diffusion coefficients were used. Therefore, macroscopic techniques such as ELISA (enzyme linked immunosorbent assays) or indirectly crystallographic techniques have been successfully applied as analytical tools. In addition, some very interesting attempts have been undertaken to study intermolecular forces directly: 1) optical trapping

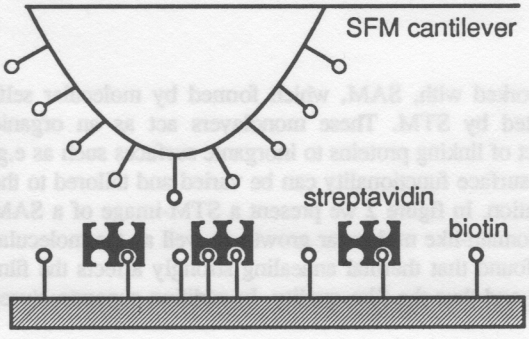

Fig.1:
Functionalized sensors are used for measuring adhesion forces between individual molecules

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techniques [1], 2) surface force apparatus measurements [2], 3) pipette suction techniques [3], and 4) local probe methods [4-6]. Whereas the first is limited to smaller forces (typically < 10 pN) the second and third are lacking spatial resolution. The conception of scanning force microscopy (SFM) allows investigation of surfaces from the micrometer down to the atomic scale [7]. Its spatial resolution capability in conjunction with its remarkable force sensitivity (< 1pN – 1 μN) allows to study biological molecules in direct space even under physiological buffer conditions.
In contrast to conventional scanning force microscopy where the interaction force between tip and sample is always kept as small as possible, especially tailored (functionalized) tips are used for measuring adhesion forces between molecules (figure 1). In order to probe specific molecular recognition events we have chosen biotin-streptavidin as an appropriate model system, since 1) it has an extremely high affinity (10^{-15}/mol, 88 kJ/mol), 2) it is extremely well and broadly characterized and last but not least 3) it is commercially available. Furthermore, immobilisation techniques for streptavidin are readily available and make it an ideal model system for this kind of experiments.

2. Experimental

All presented experimental data were either recorded with a homebuilt STM/SFM [8] or a commercial instrument [9]. For the STM and the SFM experiments we used mechanically prepared (cut) $Pt_{80}Ir_{20}$-tips and single cristalline Si-cantilevers (0.05 – 0.1 N/m) [10], respectively. Au(111)-films were epitaxially grown on mica under elevated temperatures (300° – 400° C) in high vacuum. Self-assembled monolayers (SAM) of dodecanethiols ($CH_3(CH_2)_{11}SH$) were formed by spontaneous molecular self-assembly upon immersing the freshly evaporated gold surfaces into a diluted (1 mM) ethanol solution for 48 h at room temperature followed by thermal annealing at 80°C for 2 h.
In order to derivatize a flat surface as well as the cantilever tip for measuring adhesion forces, a clean silicon wafer and the SFM cantilever tip were coated with 3 nm of chromium and in addition with 30 nm of gold at room temperature in high vacuum. Upon immersing the gold surfaces into a diluted (1 mM) solution of aminoundecanethiol ($NH_2(CH_2)_{11}SH$) in methanol for 12 h, a SAM of thiol molecules spontaneously forms [11]. After carefully rinsing in diluted HCl and in nanopure water, biotin was covalently attached to this organic surface [12] via an extended molecular spacer to allow for steric adjustments of bound molecules [13]. Just prior to the experiment, the substrate was incubated with streptavidin [14] to form a monolayer.

3. Results

In order to characterize the surfaces we worked with, SAM, which formed by molecular self-assembly on Au(111) were first investigated by STM. These monolayers act as an organic interlayer and are very important with respect of linking proteins to inorganic surfaces such as e.g. silicon or gold. Their length, chemistry and surface functionality can be varied and tailored to the demands of an optimized protein immobilization. In figure 2 we present a STM-image of a SAM ($CH_3(CH_2)_{11}SH$) on Au(111) showing the domain-like molecular growth as well as the molecular ordering within individual domains. It was found that thermal annealing strongly affects the film homogeneity, increases the domain size [15] and thus the film quality. In addition superstructures

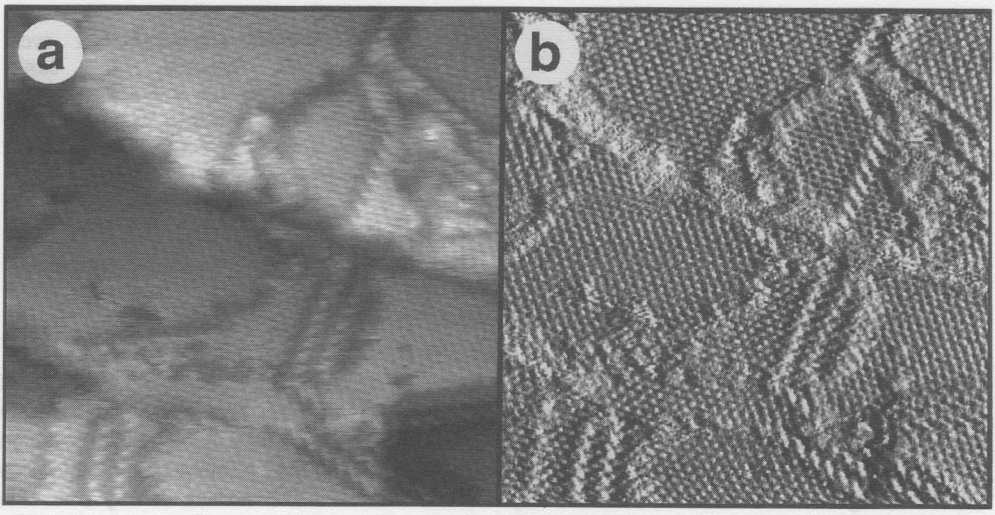

Fig. 2:
STM constant current topograph (47 nm × 47 nm, 3 pA, 1.2 V) of a SAM on Au(111). The domain-like molecular growth as well as the molecular structure can clearly be identified in the (a) topview and (b) differentiated representation.

superimposed on the regular ($\sqrt{3} \times \sqrt{3}$) molecular lattice could be identified suggesting two inequivalent molecules per unit cell caused by a molecular twisting [16].
In order to immobilise proteins, slightly different SAM were used. Streptavidin functionalized surfaces were prepared either by directly coupling the proteins to a N-hydroxysuccinimide terminated SAM or, which is even more convenient, by simply incubating biotinylated surfaces with streptavidin.

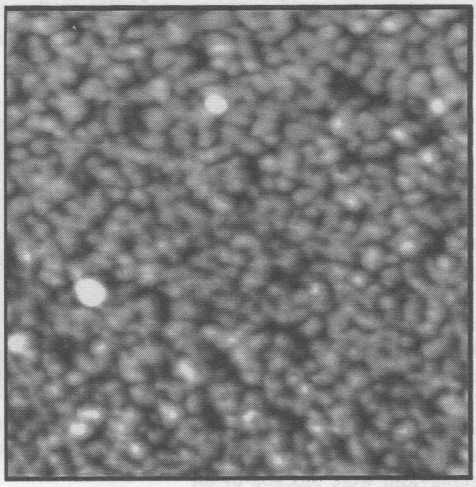

Fig. 3:
SFM topograph (500 nm × 500 nm, tapping mode), showing the streptavidin surface density as used in the adhesion experiments. The measured surface density agrees well with values determined from optical surface analysis techniques.

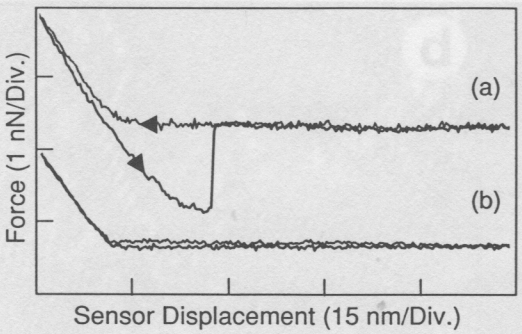

Fig. 4:
(a) Typical approach/retract cycle measured between a biotin functionalized tip and a streptavidin derivatized surface (scan rate: 0.1–0.2 Hz). The unbinding or adhesion force can be extracted from the hysteresis of the retracting branch. (b) Upon blocking the receptor sites the adhesion peak disappeared.

To check for the streptavidin surface density we imaged the surfaces with SFM. In figure 3 a SFM topograph is shown, significantly different from that of the SAM (figure 2), where homogeneously distributed objects, 10-15 nm in size, can be identified. From their size they agree with an assumed diameter of ~5 nm for streptavidin, taking convolution effects from the tip into account, which can lead to a significant increase in observed object sizes. The estimated surface density from this image corresponds to about 1 monolayer which nicely agrees with results from optical surface analysis techniques like surface plasmon resonance methods. After having determined the surface density of streptavidin we investigated the unbinding force between this streptavidin functionalized flat surface and a biotinylated cantilever tip prepared in the way as described above. Both surfaces, streptavidin functionalized sample and biotinylated cantilever were directly transferred and kept in the liquid cell of our SFM under phosphate buffered saline (PBS).
To measure adhesion forces the cantilever deflection was monitored during approach/retract cycles (force vs. distance curves [17]). Upon retracting, the cantilever has to compensate for adhesion forces between tip and sample, visible as a force hysteresis with a maximum attractive force. This unbinding, rupture or adhesion force strongly depends on the surface chemistry, the area of contact and the experimental environment. In the case of biotin and streptavidin a typical approach/retract cycle is given in figure 4a where the measured adhesion force equals 1.2 nN. The measured approach/retract cycles are very similar to those of a previous work [4] but do not exhibit any subfeatures as reported elsewhere [5]. To account for statistical uncertainties we recorded 300

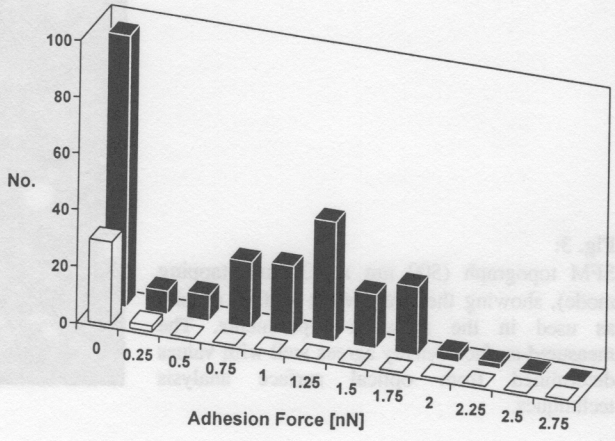

Fig. 5:
Distribution graph representing the adhesion forces of ~300 approach/retract cycles between a biotin functionalized tip and a streptavidin derivatized surface. The black columns clearly indicate a mean value of ~1.25 nN. White columns represent the control experiment where all free receptor sites of streptavidin were blocked by an excess of biotin.

force cycles, determined the unbinding force and plotted the results as dark columns in the distribution graph of figure 5. Clearly, a mean value of 1.25 nN with a standard deviation of ±0.25 nN can be extracted. 30 % of the recorded experiments yielded no or only very little adhesion. To check for the significance of this experiment, we incubated during 20 min with an excess of biotin in order to block all empty receptor sites. Afterwards we thoroughly rinsed sample and cantilever several times with PBS and measured again the apparent adhesion forces represented by figure 4b and the white columns of figure 5. It is obvious that the large adhesion peak disappeared, indicating a complete surface blocking of biotin as expected for a specific recognition experiment. Even after hours and several washing steps with PBS the surface remained irreversibly blocked. However, new sets of sample and cantilever again exhibited the reported effects.

4. Discussion

In order to use a versatile and well defined system, we have chosen covalently linked biotin-streptavidin surfaces, which we characterized with STM and SFM at every preparation step. Since similar studies appeared recently [4,5], it is worth discussing the results we obtained in these adhesion experiments with respect of similarities or discrepancies. By verifying that we worked with a well defined monolayer of streptavidin and by our control experiment where we blocked the specific surface binding sites with biotin, we showed that the measured adhesion forces are due to specific molecular recognition mechanisms between biotin and streptavidin. The measured adhesion force value of 1.25 nN in our experiments is somewhat larger compared to the values recently reported [4,5], indicating either multiple molecular binding, a badly calibrated cantilever force constant, or non-specific force background contributions. Since the adhesion forces completely vanish upon blocking the free binding sites with biotin and the force constants of the used cantilevers were found to be off by 10 % (max.) from the values specified by the manufacturer [18], multiple bonding mechanisms have therefore to be considered. In this case tip geometry effects such as the unknown effective contact area of the tip, which directly relates to the number of possible interacting molecules, have to be taken into account as well. Due to parallel batch processing, all cantilever tips on a wafer have very similar tip geometries leading to a more or less uniform contact area distribution, which might partly explain discrepancies in absolute measured force numbers obtained in experiments of different research groups. Although we and others [4] to date do not observe any force quantization, this phenomenon was recently observed [5] indicating detection and discrimination of single binding events upon using ultrasoft functionalized agarose beads. The authors claim that this approach is crucial for the experiment, since the soft beads allow for force parallelization of inequivalent binding site positions as well as relaxation of lateral stress built up by non-orthogonal binding geometries. However, the role of the agarose beads and the bovine serum albumin (BSA) as used for streptavidin immobilisation is not completely evident.
Since in force microscopy only a single macroscopic force can be measured (deduced from the deflection of a macroscopic cantilever) it will be very important in the future to tailor the experimental system to be as simple as possible to rule out parasitous shunt interactions which could lead to misinterpretations [19].
In order to estimate the forces involved in molecular binding experiments, deduction from molecular equilibrium constants or bond energies is not directly applicable since the distance dependence of the interaction potential is unknown. In a simplified model, however, it is possible to give an estimate via binding energies [20,4,5]. By assuming l_0 = 1.5 Å as the length of the molecular interaction (corresponding to the length of a hydrogen bond), U = 88 kJ/mol for the

binding energies in the streptavidin-biotin system [21] and that the interaction force can be represented by $F = - U_{(per\ molecule)}/l_o$, we deduce an adhesion force of 1 nN.

5. Conclusion and Outlook

At present, force microscopy has successfully demonstrated the possibility of investigating molecular recognition mechanisms between functionalized sensor tips and sample surfaces. Its present force sensitivity is limited by the cantilever spring constant, the thermal cantilever fluctuations and the capabilities of the cantilever deflection readout system. Since state-of-the-art microscopes have a force resolution of 0.1 – 1 pN [22], many other molecular recognition systems such as ligand-receptor -, antibody-antigen systems and/or systems with complementary strands of DNA can further be accessed and studied with this promising technique. However, in order to use this technique in the future as an absolut standard method within pharmaceutical and medical research, a number of prerequisites have to be established and fullfilled: 1) a well understood sample and sensor preparation which includes reliable molecule and cell immobilisation techniques, 2) well-calibrated force sensors and piezoelectric transducers, 3) sharp and very well defined force sensor geometries in order to locally access individual binding sites and to reduce possible unspecific background, and 4) single molecule manipulation techniques to allow preparation of "single molecule" sensor tips.

6. Acknowledgements

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[14] Streptavidin from Sigma, S-4762.
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[18] In SFM forces are measured via cantilever deflections. Since an uncertainty in cantilever spring constant affects directly the force measurement, it is very important to accurately determine the cantilever properties. Length and width of the cantilever can be determined by means of optical or electron microscopy methods. By measuring the cantilever resonance frequency the thickness can be deduced which allows calculation of the spring constant.
[19] Since parallel and serial binding mechanisms (specific and unspecific) can hardly be discriminated and distinguished from measuring the cantilever deflection, the experimental system should be designed as simple and straightforward as possible. From that reason we have chosen direct and covalent attachment of biotin to the probing surfaces.
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[22] Assuming a cantilever spring constant of 0.01 N/m and a resolution of the cantilever readout system of 0.01 nm, a force resolution of 0.1 pN can be achieved.