Keysight Technologies
Single Molecule Force Spectroscopy (SMFS) Using the 7500AFM
Application Note

Introduction

The atomic force microscope (AFM) [1] is well known for its high imaging resolution even at ambient environment and room temperature. Besides that, it can be used to measure forces, accurately. Its force resolution at room temperature is high enough to measure forces as small as weak bindings between biological molecules [2, 3, 4, 5, 6]. Those abilities make the AFM, especially force distance based AFM methods a valuable tool to investigate biological functions [7, 8].

For biological processes weak interactions play a crucial role. Usually, ligand-receptor type complexes of proteins (e.g. antibodies, enzymes) and/or other organic biomolecules (e.g. vitamins, hormones) are formed. The lifetime and stability of such formed complexes is optimized for its biological function. Therefore measuring that weak interaction of proteins is important to understand its function.

Here, we show how the Keysight Technologies, Inc. 7500 AFM can be utilized to improve the molecular understanding of complex biological systems. In particular we show how interactions between proteins can be studied by Single Molecule Force Spectroscopy (SMFS), yielding the energy landscape.

Avidin-Biotin Interaction Study Using SMFS

Single Molecule Force Spectroscopy can be used to study the interaction of proteins. For this, an upgraded sensor design is necessary. One binding partner has to be bound to the apex of the AFM tip using a flexible linker [9, 10, 11], while the other binding partner is immobilized on the sample surface. Interaction force measurements are monitored by performing force distance cycles. Hereby, the tip is approached until it touches the surface. Eventually this results in a bond forming between the ligand (e.g. an organic molecule, peptide, toxin of full protein) bound to the tip and the receptor molecule (typically a protein) immobilized on the sample surface. Subsequent retraction results in loading of the bond with an increasing force ramp. At a certain force the bond ruptures and the bent cantilever jumps off the surface into its equilibrium position. The force at which the bond breaks is termed “rupture force”.

The breaking of the bond is driven by thermal fluctuations rather than by purely mechanical dissociation [12]. In fact, the loading of the bond results in lowering the energy barrier, which leads to a shorter lifetime of the interaction, i.e. faster loading yields larger rupture forces. Therefore, rupture forces depend on the applied force load-

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ing rate and the details of the functional relation of bond lifetime and applied force. A detailed theoretical consideration yields a direct link between such single molecule pulling experiments and bulk experiments. But the single molecule approach gives access to the full spectrum of individual properties of the ligand-receptor pair instead of an averaged value gained by bulk experiments [13].

In this paper we show how the energy landscape of the avidin-biotin interaction can be determined. Avidin is a protein made of four identical subunits (homotetramer), where each can bind to biotin with a high degree of affinity and specificity. It is produced in oviducts of birds, reptiles and amphibians and deposited in the whites of their eggs. In chicken white egg it makes up approximately 0.05% of the total protein content. The biological function of avidin is not exactly known, although it has been postulated that it is a bacterial growth inhibitor, by binding biotin which bacteria need. Biotin, also known as vitamin H, is a water soluble B-vitamin (B_7) and is necessary for cell growth, the production of fatty acids, and the metabolism of fats and amino acids.

Avidin and biotin bind with a dissociation constant of roughly 10^{-15}M, making it one of the strongest non-covalent bonds in nature. In fact, the affinity is so high that it is extremely resistant to any type of denaturing agent. This stability has prompted its use for ***in situ*** attachment of labels in a broad variety of applications, including immunoassays (like ELISA), DNA hybridization and localization of antigens in cells and tissues.

Here, the avidin-biotin interaction was investigated using single molecule force spectroscopy. For this, biotin was covalently bound to the AFM tip and avidin was immobilized to a freshly cleaved mica sheet. Single molecule interactions were monitored by acquiring force distance cycles using the above prepared tip and sample. Interaction between avidin and biotin was represented as a characteristic nonlinear force signal, arising from the elastic extension of the distensible PEG linker stretching, allowing for distinguishing specific unbinding events from unspecific adhesion (Figure 1). Evaluation of many of such unbinding events (>100) yielded a distribution of rupture forces with a mean value of ≈50 pN and a standard deviation of ≈19 pN at a force loading rate of 900 pN/s. The nature of this distribution are not measurement errors of consecutive measurements, in fact it is the stochastic nature of the unbinding process, itself. It can be understood by assuming that for unbinding, an energy barrier needs to be crossed in a thermally activated regime [12].

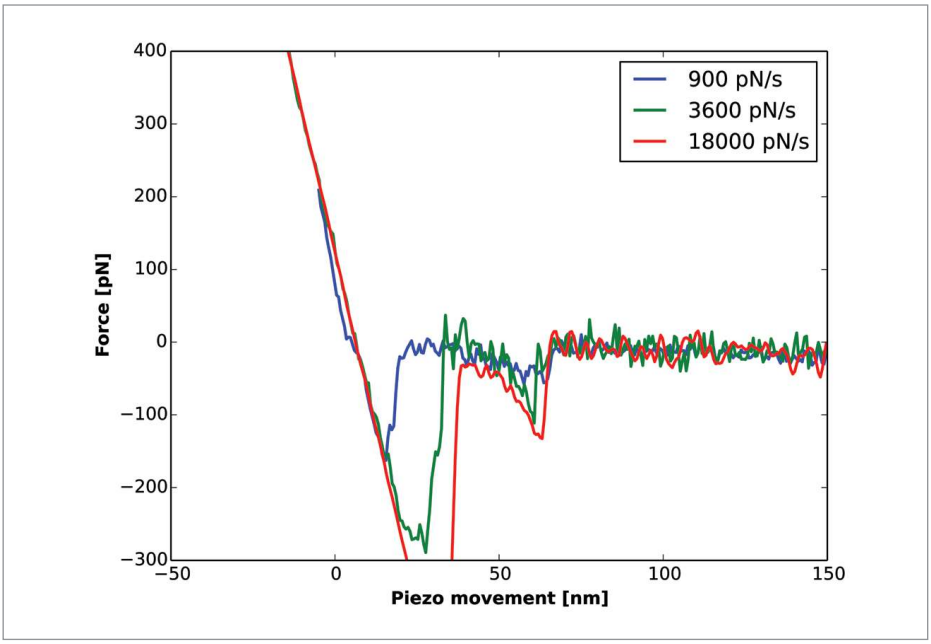

Figure 1. Force distance cycles showing avidin-biotin unbinding events. Avidin-biotin interaction is indicated by negative forces and a characteristic non-linear stretching of the tether (~30-60 nm). An unbinding event is characterized by a sudden increase of the force due to a rupture of the bond (~65 nm). The rupture force (force at which unbinding occurs) is depending on the force loading rate. The higher the loading rate (900, 3600 and 18000 pN/s) the higher the rupture forces.

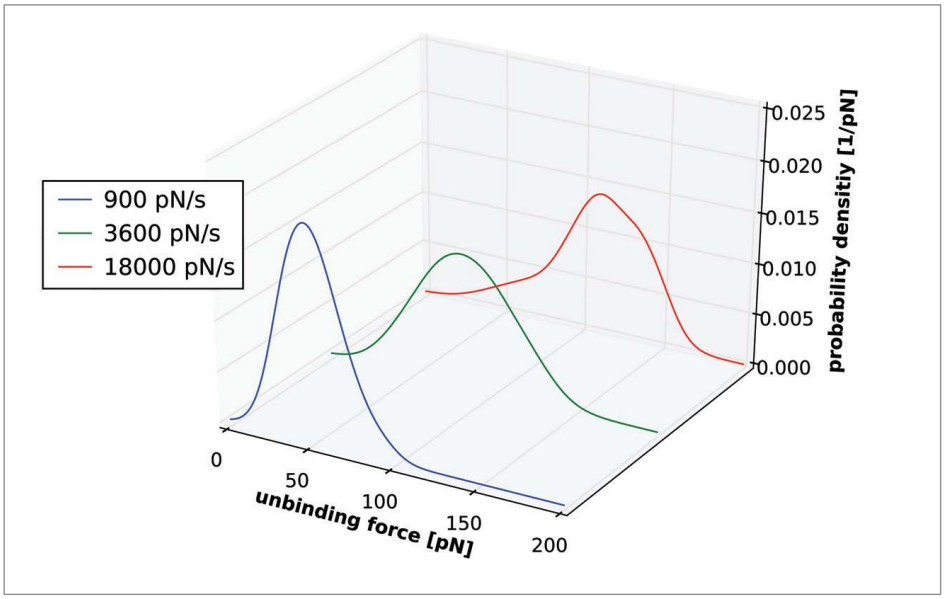

Figure 2. Distribution of rupture forces as a function of the applied loading rate. Applying higher force loading rates result in a shift of rupture forces to larger values.

Another consequence of the stochastic nature is that the unbinding forces depend on the time scale of the measurements. The most probable unbinding force is shifted to higher values for larger force

loading rates (Figure 1 and Figure 2). In fact there is a linear dependence of the most probable unbinding force and the logarithm of the loading rate (Figure 3). From this dependence the separation of the energy barrier from the equilibrium position x_β and the kinetic off-rate constant k_{off} can be determined [12]. Therefore the unbinding forces were measured at different pulling speeds, ranging from 300 nm s^{-1} to 24000 nm s^{-1}. The force loading rate was calculated from the retraction velocity times the effective spring constant, which is the slope of the force distance curve at the point of rupture. From the most probably unbinding force versus force loading rate dependence (Figure 3) x_β and k_{off} were determined to be 0.16±0.03 nm and 5±2.5 s^{-1}, respectively.

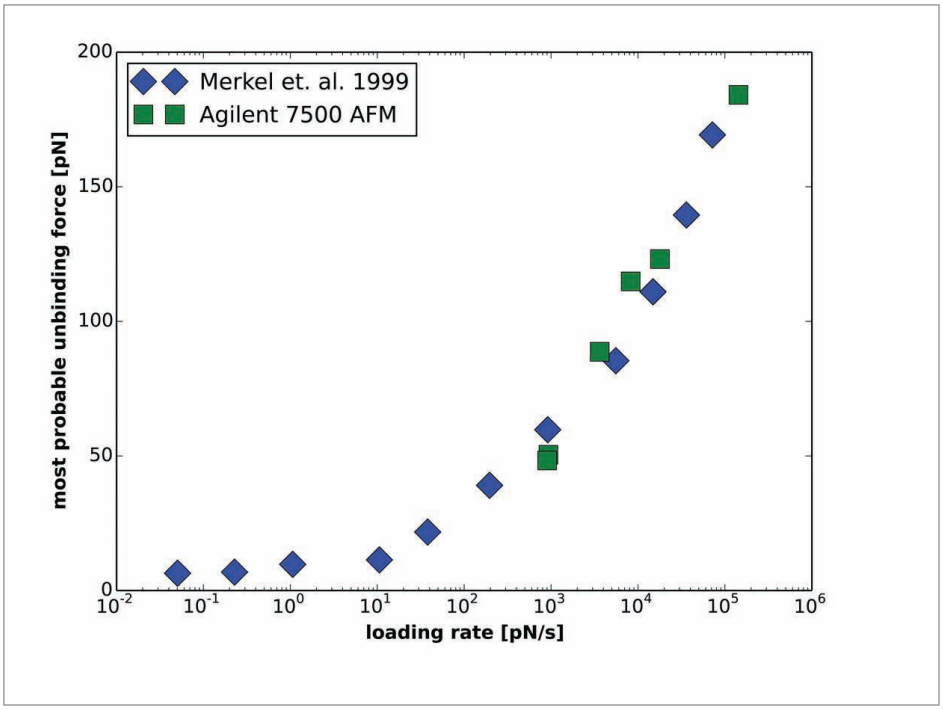

Figure 3. Dynamic force spectroscopy plot of avidin – biotin interaction. The most probable unbinding force is plotted versus the applied loading rate (green squares). Just as expected a semi-logarithmic dependence is found, allowing the extraction of parameters of the energy landscape (x_β = 0.16±0.03 nm and k_{off} = 5±2.5 s^{-1}). The force measured with the Keysight 7500 AFM agrees well with previously published values using a biomembrane force probe [14].

Conclusion

The AFM is revolutionizing Nano technological research. Since its invention, this unconventional microscopy technique has evolved into a multifunction toolbox. For the first time, single cellular machineries can be observed at work, and their functional state explored. Here we have shown how Keysight's 7500AFM can be used to investigate protein–protein interaction.

We have demonstrated the ability to study the interaction between a protein and its natural ligand (avidin and biotin) on the single molecule level. For this, avidin and biotin were immobilized to a mica sheet and an AFM tip, respectively. Unbinding events were monitored by force distance cycles (Figure 1). This allowed determining the unbinding force distribution (Figure 2) and the most probably unbinding force (Figure 3) as a function of the loading rate. Using the well-known single barrier model [12], the latter relation allowed calculating the separation of the energy barrier from the equilibrium position x_β and the kinetic off-rate constant k_{off} to be 0.16±0.03 nm and 5±2.5 s^{-1}, respectively. In addition we compared our measurements with previously published results [14]. Even though the published results were measured with a different technique (biomembrane force probe), both results agree well, indicating the superior force accuracy of the 7500 AFM using single molecule spectroscopy.

Materials and Methods

Commercially available AFM cantilevers (MLCT, Bruker) were amino-functionalized as described using the gas-phase silanization method [15], and subsequently biotinylated using biotin-PEG-NHS [16], a short activated poly (ethylene glycol) polymer chain. Avidin was electrostatically immobilized on a freshly cleaved mica sheet [16] applying a standard saline buffer solution (PBS – phosphate buffered saline). All force measurements were carried out in 10 times diluted PBS. Force distance cycles were performed at room temperature by using biotin coated tips with 0.02Nm^{-1} nominal spring constant, and mica immobilized avidin. Empirical force distributions of avidin-biotin unbinding events were calculated as previously described [17, 18]. For characterizing the energy landscape the applied loading rate was varied by performing measurements with different retraction speeds. For this, the sweeping range, and frequency was varied between 300 and 1200 nm, and between 0.5 and 10 Hz. The force loading rates were determined by multiplying the retraction speed with the effective spring constant, i.e. the mean slope just before rupture. The most probably unbinding force F is dependent on the force loading rate r through [12]:

$$F = \frac{k_B T}{x_\beta} \ln\left(\frac{x_\beta r}{k_B T k_{off}}\right),$$

where $k_\beta T$ is the thermal energy, x_β is the width of the prominent energy barrier from the energy minimum to the energy maximum of the ligand-protein complex, and k_{off} is the dissociation rate. The parameters x_β and k_{off} were extracted by fitting the equation above to the experimental data using a generalized least square fitting procedure, which accounts for errors in determining F and r [19].

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