AFM-Tip Functionalization for Single Molecule Recognition Imaging

F. Kienberger, A. Ebner, L. Chtcheglova, L. Wildling, T. Puntheeranurak, H. J. Gruber and P. Hinterdorfer

Institute for Biophysics, Johannes Kepler University of Linz, Altenbergerstr. 69, A-4040 Linz, Austria, peter.hinterdorfer@jku.at

ABSTRACT

The high force sensitivity of the atomic force microscope (AFM) has opened the possibility of measuring inter- and intramolecular forces of biomolecules on the single molecule level. Covalent binding of bioligands to AFM tips converts them into monomolecular biosensors by which cognate receptors can be localized on the sample surface and fine details of ligand-receptor interaction can be studied. Tethering of the bioligand to the AFM tip via a ~6 nm long, flexible poly(ethylene glycol) linker (PEG) allows the bioligand to freely reorient and to rapidly 'scan' a large surface area while the tip is at or near the sample surface. Using suchlike modified AFM tips, a recent technology development lead to molecular recognition imaging, where topography and recognition sites are simultaneously localized with nanometer accuracy. Thus, the AFM can identify specific components on complex biological sample and retain its high resolution in imaging.

Keywords: AFM, ligand-receptor, antibody-antigen, biosensor, molecular recognition

1 INTRODCUTION

At present, atomic force microscopy (AFM) is extensively used in a wide range of disciplines such as molecular biology, solid state physics and materials science. The major application is imaging of surfaces on scales from micro- to nanometers with the objective to visualize and properly characterize surface textures and shapes. AFM is unique in providing sub-nanometer resolution at a reasonable signal-to-noise ratio under physiological conditions, which is particularly important for biological investigations. In addition to high-resolution imaging of biological specimens like proteins, nucleotides, membranes and living cells, the measurement of mechanical forces at the molecular level has provided detailed insights into function and structure of biomolecular systems [1].

In molecular recognition experiments, defined forces are exerted on a receptor-ligand complex and the dissociation process is followed over time. Dynamic aspects of recognition are addressed in force spectroscopy experiments, where distinct force-time profiles are applied to monitor the changes of conformational and states during receptor-ligand dissociation. Consequently, dynamic force spectroscopy allows to detect energy barriers not detectable by conventional near equilibrium assays and to probe the free energy surface of proteins and molecular complexes [2].

The capability of AFM to resolve nm-sized details, together with its force detection sensitivity, has led to the development of molecular recognition imaging. By combining topographical imaging with force measurements, receptor sites are localized with nanometer accuracy. Topography and recognition of target molecules are thereby simultaneously mapped.

2 AFM-TIP CHEMISTRY

The AFM tip is converted into a specific biosensor by stable attachment of a bioligand which has the ability to specifically bind to a complementary molecule on the biological specimen which is scanned by this functionalized AFM tip. The bioligand can itself be a biomolecule (antibody, hormone, single- or double-strand nucleic acid, etc.) or a synthetic molecule or whatever structure that is being recognized by a complementary molecule on the surface of the biological sample to be studied. Linear poly(ethylene glycol) (PEG) chains have frequently been used to attach one or a few sensor molecules to an AFM tip because the PEG itself is chemically and physically inert and it allows for rapid and free reorientation of the sensor molecule when the AFM tip approaches the surface. Thus, even a single sensor molecule on the tip can recognize its cognate molecule on the target surface with up to 25 % probability in one force-distance cycle at standard conditions.

Furthermore, the 6-8 nm long PEG linker between the tip and the probe molecule facilitates data analysis in molecular recognition experiments because the nonlinear elasticity of PEG allows for easy discrimination of specific unbinding events (which are preceded by nonlinear PEG stretching) from nonspecific tip adhesion. Moreover, 6-8 nm long PEG linkers are indispensable for a new method in which surface topography and a map of recognition sites are simultaneously acquired in one fast surface scan (cf. Chapter 4). Finally, the PEG-linked sensor molecule appears to regularly escape the danger of being crushed between tip and sample surface in each force-distance cycle, as is deduced from preservation of single sensor molecules over many thousands of cycles.

Our standard method of tip functionalization [3] involves three steps (Figure 1): (i) generation of NH_2

groups on the silicon nitride tip, (ii) attachment of a heterobifunctional PEG linker with one end by amide bond formation, and (iii) linking of a sulfhydryl-containing sensor molecule to the outer end of the PEG tentacle on the tip by disulfide bond formation. All three steps have been thoroughly analyzed and optimized in recent model studies [3]. With respect to the first step, gas-phase aminosilanization with APTES or silanol group esterification with ethanolamine hydrochloride proved to be equally suitable to create a sufficient number of grafting sites for attachment of up to 2 x 10^3 μm^{-2} PEG-biotin tentacles on silicon nitride chips [4, 5]. The heterobifunctional crosslinker most widely used for tip-PEG-protein linking is PDP-PEG-NHS. Its NHS ester function forms a stable amide bond with an amino group on the AFM tip and subsequently its pyridyldithiopropionyl (PDP) group reacts with a protein thiol, resulting in a disulfide linkage between PEG and protein. Use of this method is appropriate if the protein of interest contains a free thiol group (e.g. in case of half antibodies). Most extracellular proteins (e.g. antibodies), however, possess no free thiol group, requiring artificial thiol functions be introduced, e.g. with SATP (N-succinimidyl 3-(acetylthio)propionate). The procedure for SATP derivatization is quick and simple, however, unreacted SATP must rigorously be removed to ensure coupling of SAPT-derivatized antibody rather than of free SATP to the AFM tip. Consequently, gel filtration must be used for removal of free SATP which cannot be done at a small scale. Moreover, re-freezing of the modified protein is undesirable because of its low concentration after gel filtration.

Recently, a new heterobifunctional crosslinker has been introduced that has two different amino-reactive functions offering a convenient alternative with minimal protein consumption (e.g. 5 µg of protein in 50 µl buffer) and no pre-derivatization. One end of the crosslinker is an activated carboxyl (N-hydroxysuccinimide ester) which is must faster to react with the amino groups of the AFM tip than the benzaldehyde function on its other end. The reactivity of the latter is sufficient, however, to covalently bind lysine residues of proteins via Schiff base formation. The method has been critically examined using biotinylated IgG as bioligand on the tip and mica-bound avidin as complementary receptor. In summary, this new crosslinker offers a straightforward method for the tethering of proteins via one of their lysine residues, eliminating the need for endogenous or artificially introduced thiol groups. Minute amounts of protein are required to functionalize one AFM cantilever within 1-2 h incubation time.

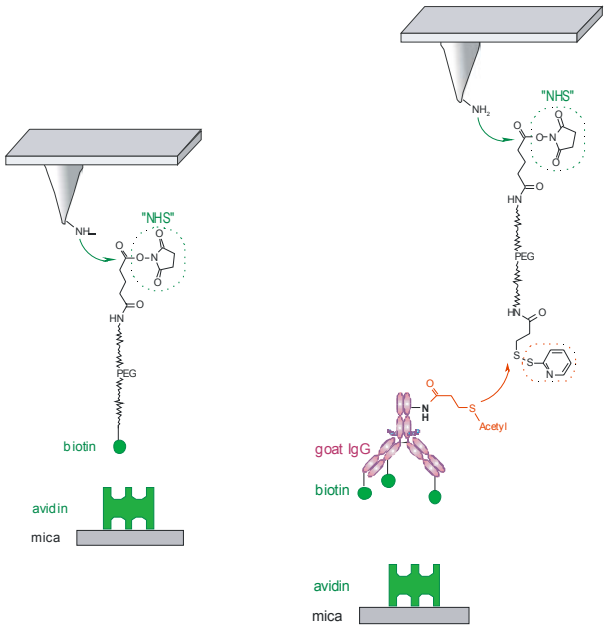

Figure 1: Left: A simple test system for avidin-biotin interaction. The succinimidyl ester of biotin-PEG-COOH was reacted with ethanolamine-derivatized tips. Right: Linkage of an antibody to the tip via disulfide bond formation. In the first step, the crosslinker PDP-PEG-NHS reacts with an amino group on the tip, with concomitant loss of the NHS leaving group. Hydroxylamine-induced de-acetylation of the SATP group on the antibody exposes a free SH group which reacts with the 2-pyridyldithio group on the free crosslinker end.

3 MOLECULAR RECOGNITION MEASUREMENTS

Interaction forces of single ligand-receptor pairs are measured in force-distance cycles using a ligand-carrying tip and a target surface with firmly attached receptor molecules (Figure 2). At a fixed lateral position, the tip vertically approaches the surface and is subsequently retracted. During this cycle, the cantilever deflection (i.e. force) is continuously measured and plotted versus tip-surface separation (i.e. distance). At the beginning of the tip-surface approach, the cantilever deflection remains zero. Upon tip-surface contact, the cantilever bends upward, consistent with a repulsive force that linearly increases with the distance. Subsequent tip-surface retraction first leads to relaxation of the cantilever bending until the repulsive force drops to zero. Upon further retraction, the cantilever progressively bends downwards, reflecting an attractive force that increases with increasing tip-surface separation.

The shape of this non-linear force-distance profile is determined by the entropic properties of the flexible PEG-crosslinker and shows parabolic-like characteristics, which

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mirrors the increase of the spring constant of the polymer chain during extension. The physical connection between

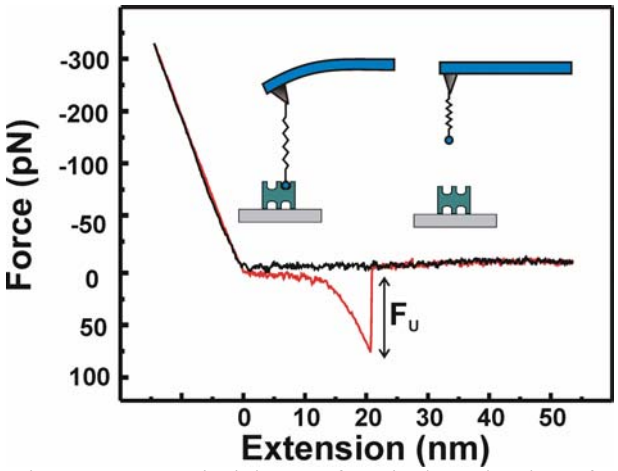

Figure 2: Principles of single-molecule force measurements. Avidin was adsorbed onto mica, and biotin was attached to an AFM tip via a PEG linker. A force-distance cycle was acquired under buffer conditions. The approach curve (black line) and the retracting curve (red line) are shown.

the tip and surface exerts the increasing force until the ligand-receptor complex finally dissociates at a certain critical force, termed unbinding force f_u, whereupon the cantilever jumps back to the resting position. If the ligand on the tip does not form a specific bond with the receptor on the cell surface, the recognition event (i.e., the parabolic shaped curve) is missing and the retrace looks like the trace. The specificity of ligand-receptor binding is usually demonstrated by blocking experiments with free ligands, which are injected into the solution in order to block the receptor sites on the sample surface. As a consequence, almost all specific recognition signals completely disappear and only occasional adhesion events are observed [2].

After acquiring thousands of force-distance cycles, empirical probability density functions (pdf) from the detected unbinding forces f_u can be constructed (Figure 3, upper panel). The maximum of the distribution reflects the most probable force upon which a single ligand-receptor bond dissociates under the force ramp used. In force spectroscopy experiments, the dynamics of the experiment (i.e. the pulling speed) is varied and the most probable unbinding force determined at different loading rates. The loading rate r can be deduced from r = df/dt, being equal to the product of pulling velocity and effective spring constant. The combination of the Boltzmann ansatz with the stochastic description of the unbinding process predicts the unbinding force distributions at different loading rates *r*. The maximum of each force distribution, $f^*(r)$, thereby reflects the most probable unbinding force at the respective loading rate *r*. f^* is related to *r* through $f^*(r) = k_BT/x\ ln(r.x/k_BT.k_{off})$, with T being the Temperature, k_B the Boltzmann constant, k_{off} the kinetic off-rate and x the length scale of the energy barrier. Apparently, the unbinding force f^* scales linearly with the logarithm of the loading rate. For a single barrier, this would give rise to a simple, linear dependence of the force on the logarithm of the loading rate. In cases where more barriers are involved along the escape path, the curve will follow a sequence of linear regimes, each of which marks a particular barrier. Force spectroscopy experiments lead to detailed structural and kinetic information of the molecular interaction. Length scales of energy barriers are obtained from the slope of the spectroscopy plot (i.e., force versus loading rate) and extrapolation to zero forces yields the kinetic off-rate for the dissociation of the complex in solution.

Figure 3 (lower panel) shows single force spectra for the binding of Ran, a molecule that regulates assembly and disassembly of the receptor-cargo complexes in the nuclear pore, to the nuclear import-receptor importin β. By use of recognition force spectroscopy it was found that the complex of RanGTP and importin β1 alternates between two distinct conformational states with different interaction strength. The force distributions shifted to higher forces by increasing the loading rate. For Ran-GTP, these distributions also had a unique bi-modal appearance (two Gaussian fits shown in black at each loading rate). The results indicate that the interaction between RanGTP and importin β can lead to two distinctly different bound states, each associated with an individual dissociation pathway [1].

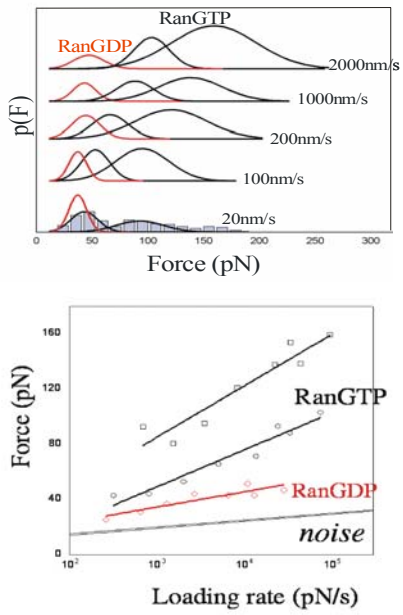

Figure 3: Dynamic force spectroscopy. Ran and importin β were immobilized onto the AFM tip and mica, respectively, and the interaction force was measured at different loading rates. Upper panel: Unbinding force distributions obtained at different loading rates. Lower panel: Force spectra obtained for complexes of importin β with RanGDP and RanGTP, respectively.

4 MOLECULAR RECOGNITION IMAGING

We have developed a method for the localization specific binding sites and epitopes with nm positional accuracy by combining dynamic force microscopy with single molecule recognition force spectroscopy [5]. A magnetically driven AFM tip containing a ligand covalently bound via a tether molecule was oscillated at 5 nm amplitude while scanning along the surface. Since the tether had a length of 6-8 nm, the ligand on the tip was always kept in close proximity to the surface and showed a high probability of binding when a receptor site was passed. The recognition signals were well separated from the topographic signals arising from the surface, both in space (z ~ 5 nm) and time (half oscillation period ~ 0.1 ms). Topography and recognition images were obtained simultaneously using a specially designed electronic circuit (Figure 4a). Maxima (U_{up}) and minima (U_{down}) of each sinusoidal cantilever deflection period were depicted, with U_{down} driving the feedback loop to record a height (topography) image and U_{up} providing the data for the recognition image. In this way, topography and recognition image can be gained simultaneously and independently with nm lateral resolution.

Figure 4b shows the topographic image of avidin molecules immobilized onto mica and the simultaneously acquired recognition image using a biotin-modified tip [6]. Almost all avidin molecules visible in the topographical image (Figure 4b, left panel) were also recognized as dark spots by the biotin-PEG functionalized tip (Figure 4b, right panel), yielding an overall success rate of ~90%. The good correlation between topography and recognition images is highlighted on some single molecules (marked with solid white circles). Some avidin molecules were not recognized by the biotinylated tip (dashed white circle), which could be caused by a partial loss in the functionality of avidin. The specificity of the recognition signals was proven by a block of the tip with free avidin and repeating of recognition imaging (not shown). Before blocking, almost all avidin molecules were recognized, while after the block only five spots remained in the recognition image, probably due to adhesive locations on the surface. This specific block demonstrates that the recognition events arise from the interaction of biotin on the tip with avidin on the surface, proving the overall specificity of the detected molecular recognition signals.

5 SUMMARY

Due to the high lateral resolution and sensitive force detection capability of the AFM, the exciting option of measuring intra- and intermolecular forces on the single-molecule level has also become possible. The proof-of-principle stage of the pioneering experiments has already evolved into established methods for exploring kinetic and structural details of interactions and molecular recognition

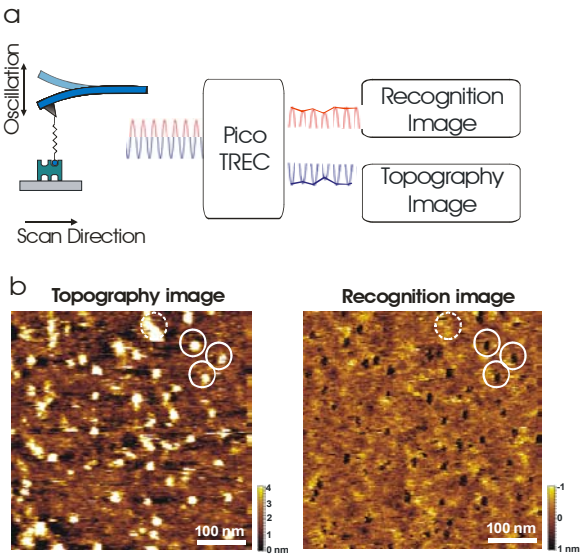

Figure 4: Simultaneous topography and recognition imaging. (a) Signal processing for obtaining topography and recognition images. (b) Topographical (left) and recognition image (right) of avidin molecules adsorbed to mica acquired with a biotin-tethered tip.

processes. Data obtained from force spectroscopy include physical parameters not measurable by other methods and opens new perspectives in exploring the regulation of the dynamics of biological processes. New instrumental developments like the recently developed recognition imaging mode allow the biochemical composition of the sample to be investigated. Together with improvements of the sensitivity and acquisition speed this has paved the way to exciting fields in nano-bioscience and nano-biotechnology.

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