COST-STSM-TD1002-12101: Dynamics of the receptor-ligand recognition process

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Abstract

Specific interactions occurring at receptor-ligand binding can be investigated in molecular recognition force spectroscopy (MRFS) experiments using functionalized tips. This report focuses on the activities concerning such experiments on avidin-biotin system during COST-STSM. There are also two other aspects of the STSM which will be briefly discussed: additional measurements concerning imaging of biological samples with AFM, particularly DNA; and also discussion of potential future collaboration.

I. INTRODUCTION

Atomic force microscope (AFM) [1] is a powerful tool for surface studies at molecular level, with ability to probe structure as well as the interactions involved. There are many variations and upgrades to the original technique which enable measurements of various surface properties [2–8]. There is a wide range of applications where AFM is used for exploration of biologically relevant processes on molecular level and under practically physiological conditions. One of those techniques is molecular recognition force microscopy (MRFM) and spectroscopy (MRFS) [9, 10], where specific ligands are covalently attached to AFM tips while their cognitive receptors are fixed at the surface under investigation. Upon tip approach to the surface receptor-ligand bond can be formed, which than gives additional information in an image or a force-distance curve.

This report will focus on the MRFS, as getting introduced to the technique was primary object of this COST-STSM. Activities

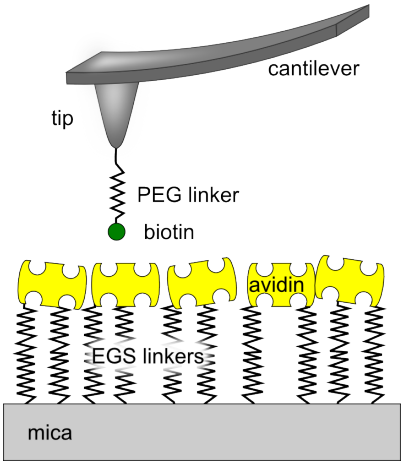

Figure 1: *Schematic representation of MRFS measured system. Avidin is attached to mica via EGS linker, while biotin is bound to tip via PEG linker. The interaction between avidin and biotin is measured in force-distance cycles and pulling speed is varied.*

that were covered are tip and sample preparation, FS measurements and data analysis. For this introductory study the avidin-biotin system was used, which is well studied and this provided the consistency check of obtained results. AFM tip was aminofunctionalized and a linker with ligand (biotin) was subsequently attached. Sample was also aminofunctional-

ized, and receptor (avidin) was bound to it via a different linker. Schematic view of the experiment is shown in Figure 1.

Along with learning the techniques and conducting experiments in MRFS, there were also two other activities during STSM that will be described in this report. First concerns possible future visits and further scientific collaboration while other one describes additional AFM measurements that focused on imaging of DNA on well defined flat surfaces, which is addressed in Appendix.

II. MATERIALS AND METHODS

In order to conduct the MRFS experiment, both tip and sample have to be treated prior to measurements. Additionaly, they have to be stored in adequate environments, in order to preserve characteristics of the specimens studied, i.e. their biological function.

Tip preparation

Functionalization of tips consists of two main parts: aminofunctionalization and subsequent attachment of linker with ligand. There are several developed methods for aminofunctionalization of which APTES coating proves to be most useful, due to lowest concentration of linkers [11]. Suitable concentration of linkers is neccessary in order to have only one ligand on the tip which has access to surface and therefore reduce the possibility of double and multiple bindings. APTES coating of previously cleaned tips is performed according to Ref. [11]. Prior to use, APTES is distilled under vacuum. A desiccator is flooded with argon in order to remove air and excess moisture. After obtaining suitable conditions 30 ml of APTES and 10 ml of triethylamine (TEA) are pipetted into two small separate plastic trays that are placed inside the desiccator. The AFM tips are then put nearby on a clean inert surface (usually Teflon), and the desiccator is closed for the 120 minutes of incubation. After incubation trays with APTES and TEA are removed and the desiccator is again flooded with argon for 5 min. The tips are left inside for 2 days in order to ''cure'' the APTES coating.

In the next step, PEG linker (heterobifunctional poly(ethylene)glycol derivative of 18 units corresponding to 8 nm extended length) with the ligand (biotin) is attached to aminofunctionalized tips. This biotin-PEG-NHS complex is previously synthesized in the chemistry lab from PEG_{800} diamine according to Ref. [12]. Tips are immersed for two hours in the solution of 1 mg of Biotin-PEG-NHS dissolved in 0.5 ml of chloroform in which 30 μl of TEA (to catalyze the reaction) is added. The glass reaction chamber containing the solution is covered, to ensure that the chloroform does not evaporate within a few minutes. After two hours the tips are washed by immersion in chloroform for 10 minutes 3 times and dried with nitrogen. Tips are stored in PBS buffer (pH 7.0) in fridge until use.

Sample preparation

For a sample, avidin covered flat mica surface was prepared. For first experiments freshly cleaved mica incubated with avidin and subsequently rinsed was used but this gave too low probability. This was due to a blocked tip, caused either by leftover free avidin, or avidin that wasn't firmly attached to surface

so that it got pulled free. In order to prevent this, avidin was bound to APTES functionalized mica via EGS linker (Ethylene glycol-bis(succinimidylsuccinate)) according to following protocol. Mica substrates are immersed in 1 mg/ml solution of EGS in chloroform. Additionally, 100-300 μl of TEA to serve as a catalyst is added and solution is covered in order to prevent chloroform evaporation. After two hours of incubation, avidin (c = 0.2 mg/ml) is added, followed by two additional hours of incubation. After the second incubation substrates are washed 50 times with PBS, and stored in PBS in refrigerator until measurement.

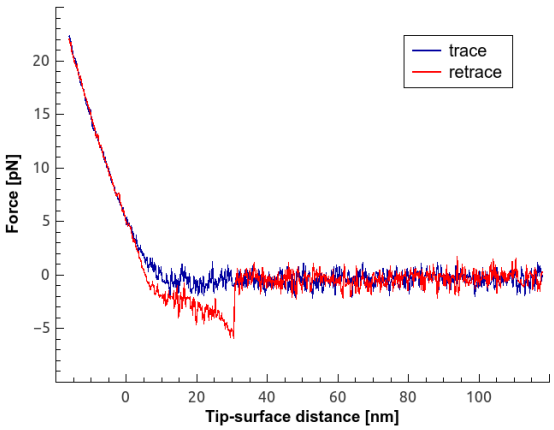

Figure 2: *Trace and retrace of a force-distance cycle with specific unbinding event clearly visible in a parabolic curve following retracting of the tip from the surface and ending upon unbinding. In a case of force-cycle when there is no binding, retrace looks just like a typical trace.*

Force spectroscopy

Force spectroscopy measurements were performed in a contact mode with a PicoSPM LE instrument (Molecular Imaging) in buffer solutions (PBS with pH 7.0). Tip on cantilever C of silicon nitride probes (MSCT probes, Brucker Corporation) was used (with nominal spring constant of 10 pN/nm and nominal resonant frequency of 7 kHz).

Measurements were performed in force-distance cycles during which cantilever deflection (which is proportional to the tip–probe force according to Hooke's law) is measured in dependence on the tip–surface distance Δz. First, the tip is approached to the surface while the lateral position of the cantilever is kept fixed. During the approach the cantilever deflection is zero until surface is reached. Upon reaching the surface a ligand-receptor complex can be formed, due to a flexible and extensive linker which allows the ligand to practically freely diffuse about the tip and search for the receptor. When the contact between the tip and the surface is established the cantilever starts to bend and the tip is pushed into the surface (if the surface is soft enough). Upon retraction cantilever straightens, and if receptor-ligand bond has been formed bends in the opposite direction. This bending increases until the ligand–receptor bond breaks and the cantilever straightens back. Shape of the typical force curves during these events is shown in Figure 2. In a case of specific binding retrace shows non-linear, parabolic-like shape governed by the elastic properties of the flexible crosslinker. Force at which bond breaks is certain critical force called unbinding force.

Dozen of functionalized tips were tested in shorter measurements, and those that gave highest binding probabilities were used later. Pulling speed was varied between 400 nm/s and 30000 nm/s and for each speed 1000 force-distance cycles were recorded.

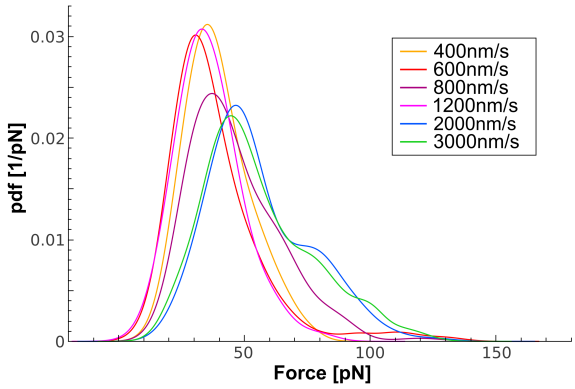

Figure 3: *Probability density functions for 6 measured pulling speeds. At higher speeds unbinding force shows increase, and width of distributions are broadening which means increase in uncertainty.*

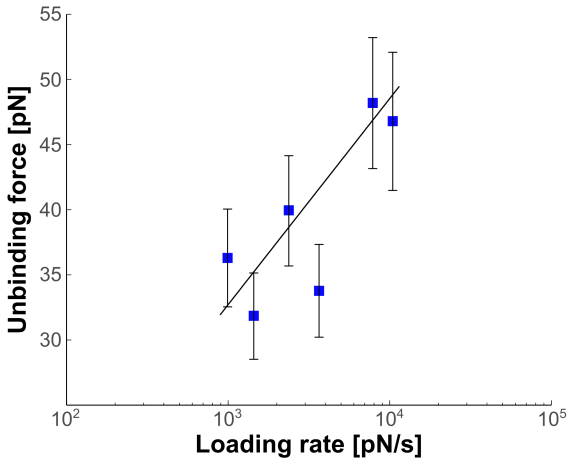

Figure 4: *Unbinding force dependence on the loading rate of the measured biotin-avidin system.*

III. RESULTS AND DISCUSSION

Recorded curves were analyzed with Matlab subroutine developed in host's group [13]. For events where multiple bindings occurred, only the last one was used in the analysis, because reliable height detection can be obtained only for full cantilever relaxation. For each set of data distribution of unbinding forces was calculated, and is shown in Figure 3. With increasing pulling speed both unbinding force and also it's variation are increasing, as expected from previous studies [9, 14].

Unbinding force is thus dependent on the kinetics of the experiment. Important quantity which is commonly used to best describe this dependence is loading rate r, which is effective force increase [9]:

$$r = \frac{\mathrm{d}f}{\mathrm{d}t} \tag{1}$$

and can also be expressed as product of pulling velocity and effective spring constant. In dependence of the loading rate unbinding force shows linear growth on the half-logarithmic scale (Figure 4), which characterizes single energy barrier in the thermally activated regime [14]. To put it simply, if we wait long enough bond will eventually break without the applied force due to thermal fluctuations. However, if the bond is pulled apart, higher force is needed with faster pulling.

IV. CONCLUSIONS

Activities during STSM covered all elements of scientific research: preparation of samples, introducing to experiment and data evaluation. Being part of a group that comes from solid surface state physics and is now moving toward soft matter and biophysics, this technique is a novelty and opens new possibilities that can broaden applicant's research. This STSM is a great opportunitiy to start a future collaboration beyond the topic of the STSM, which was discussed during several meetings. These were concentrated on graphene-DNA hybrid systems that are object of applicant's PhD thesis.

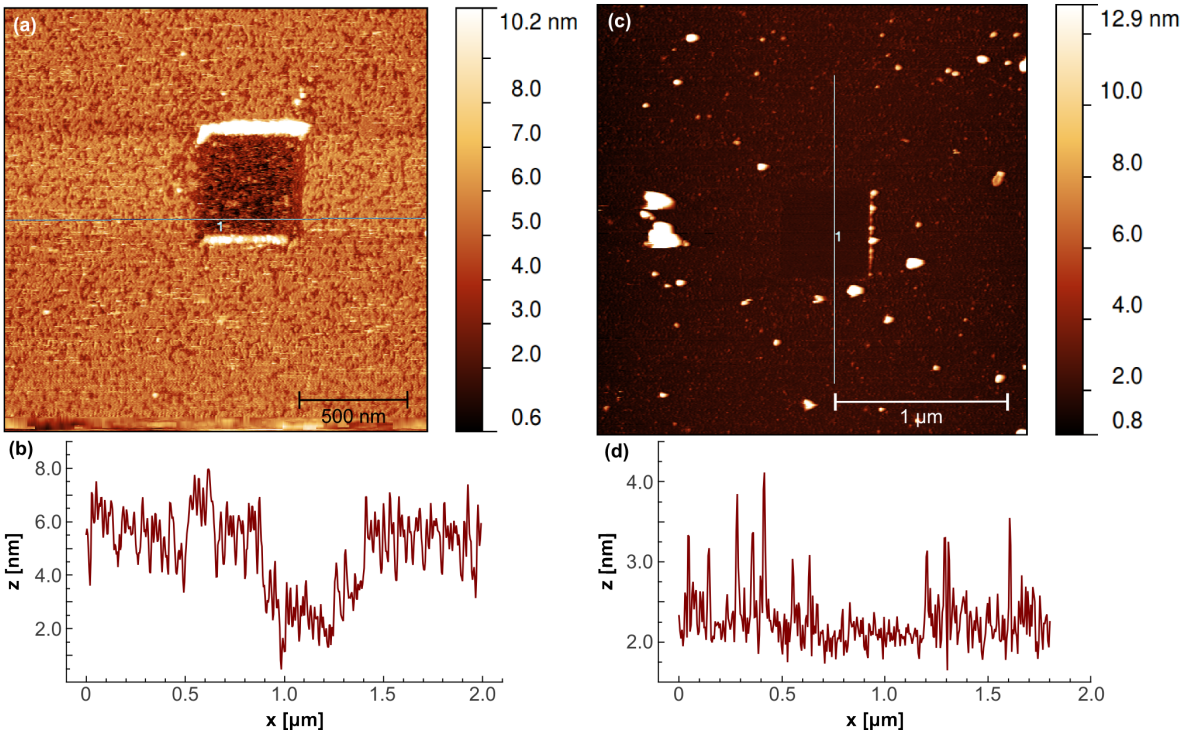

Figure 5: *(a) DNA layer adsorbed on avidin covered mica. After the scratching experiment, DNA layer is mostly moved away or "combed" leaving hole of a characteristic depth. (b) Profile of DNA layer on avidin covered mica after scrathing experiment. (c) DNA layer adsorbed on freshly cleaved mica. In scratching experiment strands are moved away. (d) Profile of DNA layer on mica after scratching experiment.*

Appendix

In spare time short-stranded DNA on flat mica surface was imaged. Aim of these simple experiments was to learn sample preparation and AFM imaging of soft samples. DNA used was 110* bp dsDNA (prepared from purchased 110 bp ssDNA from Mycrosynth A.G.), with biotin attached on one end and Cy5 fluorophore on other. DNA was adsorbed either on freshly cleaved mica previously incubated with $NiCl_2$ and Tris or mica with electrostatically bound avidin layer.

Imaging was performed on a Pico SPM Plus setup (Molecular Imaging) under aqueous conditions. Magnetic ac mode was used with magnetically coated cantilevers (MAC levers, Type VII, Agilent, 140 pN/nm nominal spring constant, 18 kHz resonant frequency). Representative image for both types of sample preparation are shown on Figure 5.

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