SPM for Functional Identification of Individual Biomolecules

Robert Ros[a*], Falk Schwesinger[b], Celestino Padeste[a], Andreas Plückthun[b], Dario Anselmetti[c],
Hans-Joachim Güntherodt[d], and Louis Tiefenauer[a]

[a]Molecular Nanotechnology, Paul Scherrer Institute, 5232 Villigen PSI, Switzerland
[b]Institute of Biochemistry, University of Zürich, Winterthurer Strasse 190, 8057 Zürich, Switzerland
[c]Novartis Services AG, 4002 Basel, Switzerland
[d]Physics Department, University of Basel, Klingenbergstrasse 82, 4056 Basel, Switzerland

ABSTRACT

The identification of specific binding molecules is of increasing interest in the context of drug development based on combinatorial libraries. Scanning Probe Microscopy (SPM) is the method of choice to image and probe individual biomolecules on a surface. Functional identification of biomolecules is a first step towards screening on a single molecule level.

As a model system we use recombinant single-chain Fv fragment (scFv) antibody molecules directed against the antigen fluorescein. The scFv's are covalently immobilized on a flat gold surface via the C-terminal cysteine, resulting in a high accessibility of the binding site. The antigen is immobilized covalently via a long hydrophilic spacer to the silicon nitride SPM-tip. This arrangement allows a direct measurement of binding forces. Thus, closely related antibody molecules differing in only one amino acid at their binding site could be distinguished. A novel SPM-software has been developed which combines imaging, force spectroscopic modes, and online analysis. This is a major prerequisite for future screening methods.

Keywords: Atomic Force Microscope, Binding Force, Force Spectroscopy, Antibody, scFv-Fragment

1. INTRODUCTION

Among the scanning probe microscopy (SPM) techniques, atomic force microscopy[1] (AFM) is commonly used for biological samples, because it allows measurements in liquid environments where electric conductivity is not required. AFM was first applied for imaging[2] of proteins, viruses and DNA under physiological conditions. Because biological samples are soft and fragile, gentle measurement modes were required[3]. Furthermore, the sample preparation turned out to be crucial, especially for biological samples. Recently, AFM has also been used to probe functions: Binding forces between receptor-ligand systems like biotin-streptavidin[4,5] or P-selectin complexes[6], between oligonucleotides[7], and between antigen-antibody pairs[8-11] were measured. In all these experiments one interacting partner is attached to the AFM-tip and the other to the surface. The AFM tip is approached to and then retracted from the surface while the cantilever deflection is recorded.

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* E-mail: Ros@psi.ch; http://www.psi.ch/lmn; Phone: +41-56-310 2580; Fax: +41-56-310 2646

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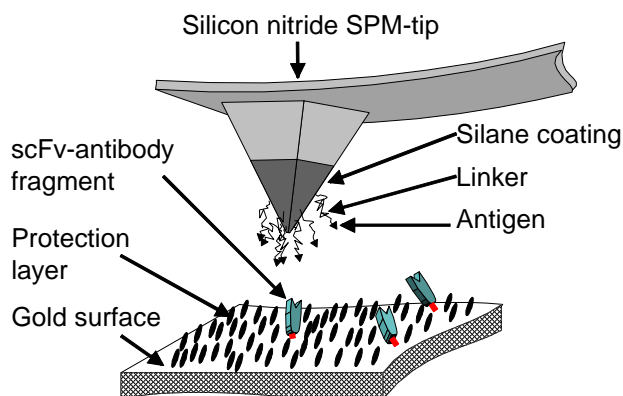
Fig. 1: Surface and tip functionalization. The antigen fluorescein is immobilized covalently via a 40 nm long hydrophilic linker to the AFM-tip, whereas the recombinant antibody molecules are attached to a gold surface via a thiol-group.

Recombinant scFv fragments[12] are the minimal size of antibodies which still comprise the complete antigen binding site. They can be generated against all conceivable antigenic targets, and designed mutant proteins can be produced. In our experiments, the antibody molecules are immobilized at a low density on a surface and the corresponding antigen on a tip (Fig. 1). AFM-images can be acquired with the antigen functionalized tip and individual, well separated molecules are selected before force spectroscopy measurements are started. The „strength" of antigen-antibody binding is commonly assessed by its affinity constant. It is still not clear whether the unbinding forces correlate with the affinity constant, the enthalpy or the entropy. Investigations on the streptavidin-biotin system[13] suggest that unbinding forces probe the enthalpic barrier to ligand-receptor dissociation, but also depend on the loading rate[6,14,15].

Using the mentioned method, scFv molecules which differ in only one amino acid have been discriminated[16]. At present we are investigating a series of further well characterized scFv-mutant proteins. The precise determination of unbinding forces and the extensive characterization of thermodynamic and kinetic parameters allow to further develop correlations. We report here on recent progress in piconewton force measurement techniques, in particular on an improved control software which combines imaging and force spectroscopy, and automated data processing.

2. EXPERIMENTAL

2.1 SPM-measurements

All SPM measurements were performed in buffer solution (50 mM phosphate buffer, pH 7.4) using a commercial SPM (Explorer, Topometrix, Santa Clara CA, USA). The images were acquired in contact mode, 1 µm scan range and a scan velocity of 5 µm/s. For force spectroscopy the vertical velocity was 1 µm/s. The software, written in Visual Basic (version 5.0, Microsoft), is based on the library "spmtools" (Topometrix) and runs on a PC under Windows NT 4.0 (Microsoft).

2.2 Tip modification

The procedure for tip modification has recently been described elsewhere[16]. Briefly, the SPM-tips (Si_3N_4-Microlever, Park Scientific Instruments, Sunnyvale, CA, USA; k $\approx$ 0.03 N/m) were first activated and silanized in a solution of 2% aminopropyl-triethoxysilane (Sigma) in dry toluene for 2 h. After washing with toluene, the cantilevers were incubated with 1 mg/ml fluorescein-poly(ethyleneglycol)-$OCH_2CH_2CO_2$-N-hydroxysuccinimide (Fluor-NHS5000, Shearwater Polymers Inc., Huntsville AL, USA) in 50 mM phosphate buffer pH 8.5 overnight at 4 °C. The cantilevers were washed with

phosphate buffer and used for AFM-imaging and force-distance experiments. Modified tips were stable for at least two weeks if stored in the refrigerator (4° C).

2.3 Chip surface preparation

Two scFvs of the antibody FITC-E2, specific for fluorescein[17], were used: The wild type protein and the mutant His(H59)Ala in which the histidine residue at position 59 in the heavy chain was exchanged by alanine. The C-terminal cysteine present in the peptitic linker allows a directed and stable immobilization on a gold surface.

PDMS-stamps with lines of 800 µm width were used for the microcontact printing (µCP)[18] of hydrophobic lines of octadecanethiol (ODT) on 1 cm^2 gold chips. The gold surfaces with the hydrophobic structures were then incubated for 2 h in 50 mM mercaptoethanesulfonate (dissolved in water/ethanol, 1:1, v/v), washed with ethanol and phosphate buffer. The resulting hydrophilic squares on the chip allow now to place different protein solutions (Fig. 3). 15 µl (for the four quadrant chip) or 3 µl (for the nine quadrant chip) of the scFv solutions (1 µg/ml) were pipetted on the hydrophilic squares, incubated for 20 min and carefully washed to avoid mixing of the protein solutions.

3. RESULTS AND DISCUSSION

3.1 Measurement software

Commercial SPM software programs provide a multitude of measurement modes, but they usually do not allow to combine different modes and to perform customized data analysis. We developed therefore a control software, which combines (I) imaging, (II) multiple force distance measurements at a selected point, and (III) online calculation of the adhesion forces (Fig 2). The imaging module comprises both, the topological and the sensor signal. Before force spectroscopy can be started,
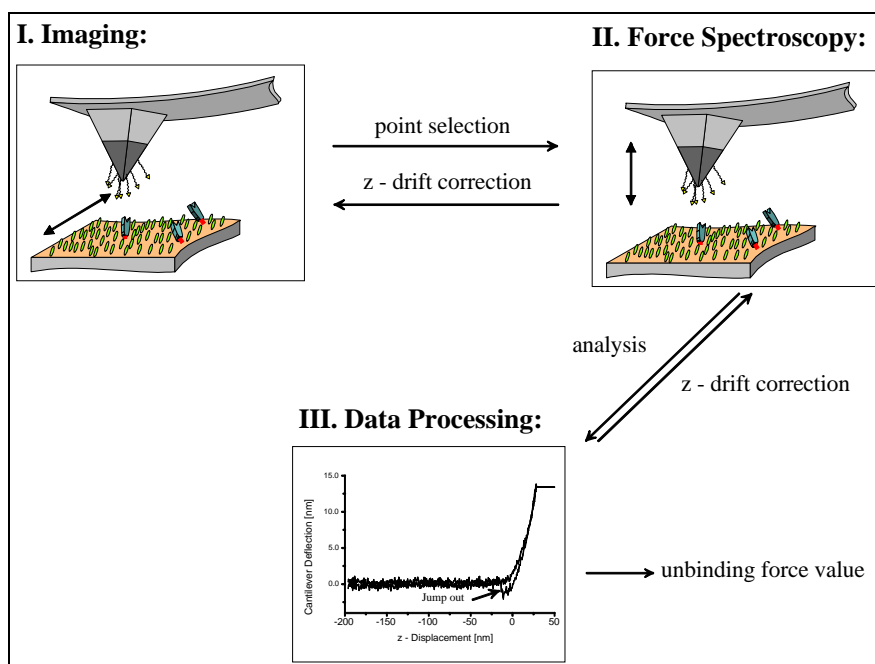

Fig. 2: Novel software concept. The software combines three modules: In the imaging mode (I) topography and sensor signal are acquired allowing the selection of x- and y-coordinates for force spectroscopy. The force spectroscopy module (II) acquires a preselected number of force distance curves and the data processing module (III) extracts by an algorithm the "jump out" values from which a mean value is calculated. Drifts of the instrument are automatically corrected during the measurement.

well-separated molecules are selected from an image. The program now allows to measure and save a high number of curves. In the force distance curves the jump out in the retracting part are considered as unbinding forces. These values are automatically extracted[8] and saved in a file for further statistical analysis. A severe problem, especially in long duration experiments, is the drift of the sensor signal. In order to prevent the application of destructive forces, the software detects the base signal after each force distance curve in order to adjust set point and maximal cantilever deflection proportional to the sensor signal drift.

This software allows fast and reproducible measurements of single antibody-antigen interactions. Since an entire experiment can now be carried out within one hour - compared to the four hours previously required - the risk of inactivating biological samples during the time of the measurement is much lower.
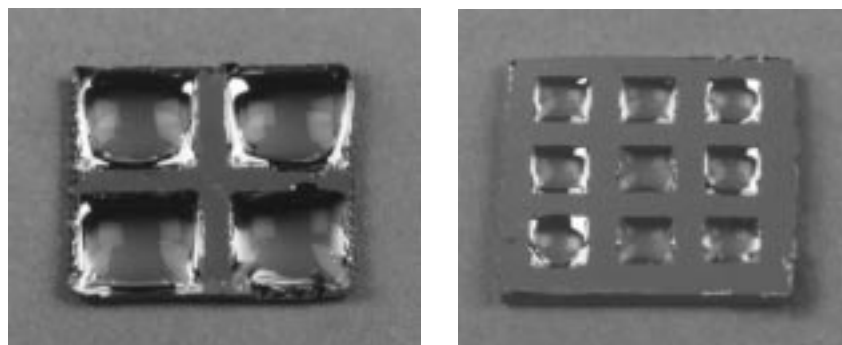

Fig. 3: Immobilization of different antibody types, well separated on the same chip. Different protein solution droplets are separated by stamped hydrophobic lines. Chip size 1×1 cm^2.

3.2 Localized immobilization

If different proteins are present on the same surface, the time consuming stabilization of the horizontal drift after a sample exchange can be avoided. Various techniques exist for a localized immobilization of different proteins on the same surface: photolithography, inkjet techniques and µCP. The latter method allows a direct pattering of surfaces with biomolecules down to the nanometer scale. We used the µCP[18] of alkane-thioles on gold in order to generate stable hydrophobic patterns in the millimeter range which serve as barriers for aqueous droplets (Fig. 3). A stable layer is formed within seconds when the stamp is slightly pressed onto the gold surface. In order to prevent denaturation of proteins, the gold surfaces are treated in a
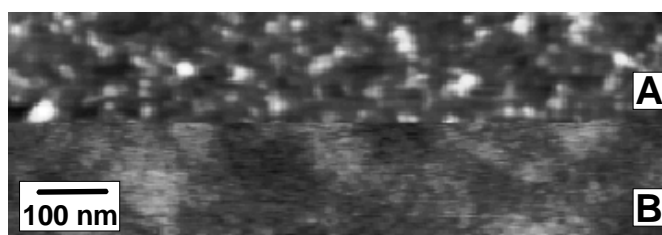

Fig. 4: Control AFM images confirming that no scFv-mixing occurs on a four quadrant chip using the described preparation technique. AFM-images of A) a surface with scFv proteins and B) adjacent quadrant without proteins.

next step with a hydrophilic charged thiol compound. The small droplets of protein solutions applied thereafter do not dry out during incubation time and functional scFv's can be immobilized at very low surface densities.

There is some risk of cross-contamination when the samples are washed at the end of the scFv-incubation. Therefore, the samples are placed on a conventional photoresist spinning device and spun at 2000 rpm for 10 seconds while directing a strong stream of washing buffer to the center of the chip. This washing method efficiently prevents mixing as confirmed by a sample on which one square was incubated only with buffer. In the corresponding AFM-image no proteins can been recognized (Fig. 4).

3.3 Unbinding force values

Using a tip of a spring constant k ≈ 0.03 N/m and the automated software presented, binding forces of (55 ± 3) pN for the FITC-E2 wild type and (47 ± 3) pN for the mutant His(H59)Ala were measured. The ratio of unbinding forces of the mutant to the wild type scFv is 0.85 ± 0.08. These results agree with the previous measurements[16] based on a standard software and scFv-preparation on unstructured substrates.

4. CONCLUSION

Considering future applications for functional screening, a control software will be required for the following processes: Identification of a few isolated molecules, automated multiple measurements thereon, extraction of force values from the measured curves and statistical analysis of these values. The most delicate step is the determination of force values from the curves. Beside the problems of calibration, the thermal noise limits the sensitivity and the precision of force values. Further improved data acquisition will allow a more precise definition of the rupture point and the base line of the curve. The novel software for AFM force spectroscopy acquisition, processing and preparation for statistical database analysis presented here is an important step toward application of AFM in drug screening for medical purposes.

However, sample preparation seems to be the bottleneck for a future application. Microcontact printing is a simple way to immobilize a few different types of functional proteins on a single surface. In order to generate a pattern of thousands of different proteins on a surface, automated dispensing will be required and the identity of a purified protein may be encoded by its position on the chip. Non specific binding is a serious problem and must be minimized in order to achieve an unambiguous identification of a binding protein. Furthermore, it is still not known in which way the measured unbinding forces correlate to the affinity constants or other kinetic and thermodynamic parameters. It has to be shown how binding forces can be used for the selection of binding proteins.

Considering all these problems, AFM will not be ready for functional screening in the near future. Nevertheless, AFM is a potent new tool for a direct identification and selection of specific binding proteins. The presented results are encouraging to reach further for this ambitious aim.

REFERENCES

1. G. Binnig, C. F. Quate, and C. Gerber, "Atomic Force Microscope", *Phys.Rev.Lett.* **56**, 930-933, 1986.

2. H. G. Hansma and J. H. Hoh, "Biomolecular imaging with the atomic force microscope", *Annu.Rev.Biophys.Sruct.* **23**, 115-139, 1994.

3. D. Anselmetti, M. Dreier, T. Richmond, E. Meyer, J. Frommer, and H.-J. Güntherodt, "Biological materials studied with dynamic force microscopy", *J.Vac.Sci.Technol.B* **12** , 1500-1503, 1994.

4. G. U. Lee, D. A. Kidwell, and R. J. Colton, "Sensing discrete streptavidin-biotin interactions with atomic force microscopy", *Langmuir* **10**, 354-357, 1994.

5. E.-L. Florin, V. T. Moy, and H. E. Gaub, "Adhesion forces between individual ligand-receptor pairs", *Science* **264**, 415-417, 1994.

6. J. Fritz, A. G. Katopodis, F. Kolbinger, and D. Anselmetti, "Force-mediated kinetics of single P-selectin/ligand complexes observed by atomic force microscopy", *Proc.Natl.Acad.Sci.USA* **95**, 12283-12288, 1998.

7. G. U. Lee, L. A. Chrisey, and R. J. Colton, "Direct measurement of the forces between complementary strands of DNA", *Science* **266**, 771-773, 1994.

8. U. Dammer, M. Hegner, D. Anselmetti, P. Wagner, M. Dreier, W. Huber, and H.-J. Güntherodt, "Specific antigen/antibody interactions measured by force microscopy", *Biophys.J.* **70**, 2437-2441, 1996.

9. P. Hinterdorfer, W. Baumgartner, H. Gruber, K. Schilcher, and H. Schindler, "Detection and localization of individual antibody-antigen recognition events by atomic force microscopy", *Proc.Natl.Acad.Sci.USA* **93**, 3477-3481, 1996.

10. S. Allen, X. Chen, J. Davies, M. C. Davies, A. C. Dawkes, J. C. Edwards, C. J. Roberts, J. Sefton, S. J. B. Tendler, and P. M. Williams, "Detection of antigen-antibody binding events with atomic force microscope", *Biochemistry* **36**, 7457-7463, 1997.

11. O. H. Willemsen, M. M. E. Snel, K. O. van der Werf, B. G. de Grooth, J. Greve, P. Hinterdorfer, H. J. Gruber, H. Schindler, Y. Kooyk, and C. G. Figdor, "Simultaneous height and adhesion imaging of antibody-antigen interactions by atomic force microscopy", *Biophys.J.* **75**, 2220-2228, 1998.

12. A. Plückthun, "Recombinant antibodies", *Immunol.Rev.* **130**, 151-188, 1994.

13. A. Chilkoti, T. Boland, B. D. Ratner, and P. S. Stayton, "The relationship between ligand binding thermodynamics and protein ligand interaction forces measured by atomic force microscopy", *Biophys.J.* **69**, 2125-2130, 1995.

14. A. G. Evans and K. Ritchie, "Dynamic strength of molecular adhesion bonds", *Biophys.J.* **72** , 1541-1555, 1997.

15. M. Rief, M. Gautel, F. Oesterhelt, J. M. Fernandez, and H. E. Gaub, "Reversible unfolding of individual titin immunoglobulin domains by AFM", *Science* **276**, 1109-1112, 1997.

16. R. Ros, F. Schwesinger, D. Anselmetti, M. Kubon, R. Schäfer, A. Plückthun, and L. Tiefenauer, "Antigen binding forces of individually addressed single-chain Fv antibody molecules", *Proc.Natl.Acad.Sci.USA* **95**, 7402-7405, 1998.

17. G. Pedrazzi, F. Schwesinger, A. Honegger, C. Krebber, and A. Plückthun, "Affinity and folding properties both influence the selection of antibodies with the selectivity infective phage (SIP) methodology", *FEBS Letters* **415**, 289-293, 1997.

18. A. Kumar and G. M. Whitesides, "Features of gold having micrometer to centimeter dimensions can be formed through a combination of stamping with an elastomeric stamp and an alkanethiol "ink" followed by chemical etching", *Appl.Phys.Lett.* **63**, 2002-2004, 1993.