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Measurement of Single Molecular Interactions by Dynamic Force Microscopy

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1. Introduction

Unbinding forces of weak, noncovalent bonds have been measured by scanning force microscopy ***(1)*** or biomembrane force probes ***(2)***. Initially, these scanning force microscopy measurements focused on feasibility studies to measure single biomolecular interactions ***(3–5)***. Recently, however, a few groups showed that these single molecule experiments give a direct link to bulk experiments where thermodynamic data are experimentally acquired ***(6–9)***. In contrast with bulk experiments where averaged properties are measured, a single molecular approach gives access to properties that are hidden in the ensemble. These experiments can give insight into the geometry of the energy landscape of a biomolecular bond ***(7,9–11)***. Some experiments even showed that intermediate states during unbinding (unfolding) exist which only can be detected by single molecule experiments ***(12–14)***.

To understand the relation between force and energy landscape, one can consider an atomic force microscope (AFM) experiment in which the spring used to measure the forces acting on the molecular complex is weak compared with the molecular bond stiffness. The ligand is immobilized on a sharp tip attached to a microfabricated cantilever and the receptor is immobilized on a surface. When approaching the surface to the tip, a specific bond may form between ligand and receptor, e.g., complementary DNA strands or antibody–antigen. The bond is then loaded with an increasing force when retracting the surface from the tip (dynamic force spectroscopy; **Fig. 1A**). At a certain force, ligand and receptor unbind, giving rise to an abrupt jump of the tip away from the surface (**Fig. 1B**). It has been demonstrated ***(6)*** that the unbinding is caused by thermal fluctuations rather than by a mechanical instability. If the thermal lifetime of the bond is short compared with the time it takes to build up an

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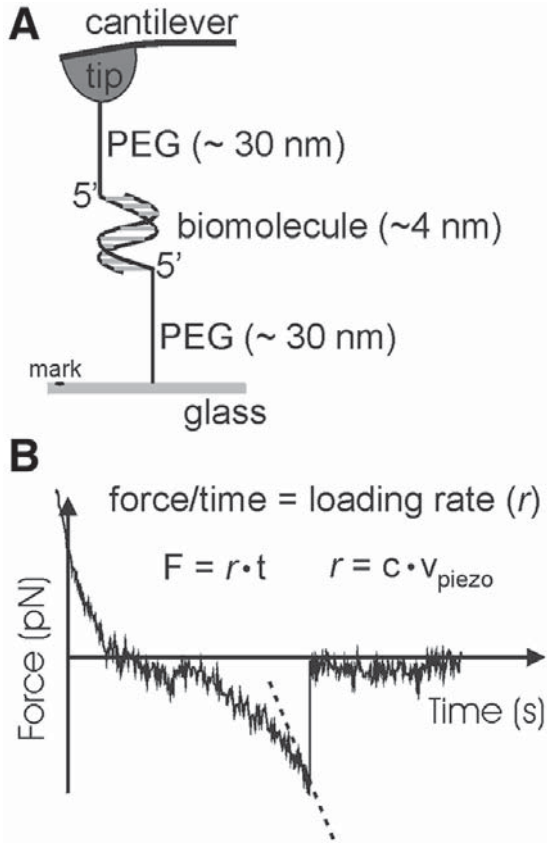

Fig. 1. **(A)** Single molecular interactions scheme measured by dynamic force microscopy. **(B)** Single biomolecule pulling experiment. The signal recorded is the force [pN] vs the time [*s*] (*c* = cantilever spring constant, v_{piezo} = pulling speed of the z piezo element). Keep in mind that the pulling of a biomolecule complex has to be performed at various pulling speeds in order to gather thermodynamic data. In the left part of the curve the cantilever is in its repulsive regime (above the time axis). As soon as the cantilever passes the equilibrium position and the biomolecular interaction has occurred, the cantilever is being deflected downwards. If a flexible cross-linker is fixing the biomolecule to the surface the linker is stretched as visible in the figure. At the point of rupture of the complex there is a sudden drop in force and the cantilever is ready for an additional pulling cycle. To extract the loading rate on the complex the derivative of the very last part of the force curve is fitted (dotted line).

observable force during a slow loading process, no unbinding event is observed. With faster loading, finite unbinding forces are observed. Therefore, unbinding forces depend on the rate of loading and on the details of the functional

relationship between bond lifetime and an applied force. The theory for these experiments has been described in great detail in the articles from Strunz et al. ***(15)***, Evans ***(10)***, and Merkel ***(11)***.

The technique has been applied to model systems such as biotin/(strept-)avidin ***(12,13)***, antibody–antigen ***(8)***, or ssDNA–ssDNA ***(9)*** where thermodynamic data existed. But now because the link from single molecule experiments to thermodynamic ensemble experiments is clearly made the technique is applicable to other systems. The applicability of single molecule experiments for gathering thermodynamic properties now permits measurement in biomolecules in which the quantity of expressed molecules is barely suitable to allow a thermodynamic approach. In these systems (for example, the binding of a drug to specific receptors) the off rate (measurable by the single molecule techniques) of the ligand–receptor plays a key role for further studies or development.

In these days when more and more precious genetically engineered drugs are being developed and used, the storage of theses compounds is crucial. If the compound is interacting with the surface of the storage container and no carrier substances like human serum albumin are allowed to be added, then the concentration of the substance in the containment is difficult to maintain during the shelf life of a drug. In a first study it was shown that force microscopy gives a suitable way to gather data from drug/storage container interactions and therefore to allow optimization of the storage container surface and buffer conditions in cases where storage problems occur ***(16)***.

2. Materials

2.1. Conversion of the Reactive Groups on the Surfaces

1. Ultrasonic bath.
2. Strong ultraviolet (UV) light source (UV-Clean, Boekel Scientific, Feasterville, PA).
3. Argon.
4. Glass slides, thickness approx 0.4 mm, cut into 0.5 × 0.5 mm pieces.
5. Microcantilever, spring constant < 0.03 N/m.
6. Dry toluene, crown cap, molecular sieve.
7. Amino-propyl-triethoxy-silane (APTES).
8. Mercapto-propyl-triethoxy-silane (MPTES).

2.2. Activation of the Reactive Groups by Heterobifunctional Cross-Linkers

1. Sulfosuccinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate (SMCC) (Pierce, Rockford, IL).
2. Poly (ethylene glycol)-α-maleimide-ω-*N*-hydroxy-succinimide-ester (MAL-PEG-NHS), molecular weight 3400, length approx 30 nm (Shearwater Co., Huntsville; AL).

3. Dimethyl sulfoxide (DMSO).
4. Buffer, e.g., *N*-hydroxyethylpiperazine-*N*'-2-ethanesulfonate (HEPES), 2-(*N*-morpholino)ethanesulfonic acid (MES; free of primary amines) adjusted to pH 7.0–7.5, phosphate-buffered saline (PBS; pH 7.3; Life Technologies, Rockville, MD).

2.3. Coupling of the Biomolecules to the Activated Surface

1. Biomolecules (i.e., thiol-modified ssDNA, water-soluble proteins exposing free cysteins).
2. Ethyl-acetate.
3. Peltier element (Melcor, Trenton, NJ).
4. Thermocouple (Thermocoax, Suresnes, France).
5. Glove box (can be used but not mandatory).

3. Methods

The methods described below outline (1) the conversion of the surface active groups to allow activation by heterobifunctional cross-linkers, (2) the activation of the newly generated groups by heterobifunctional cross-linkers, (3) the coupling of the selected biomolecules to the surface, (4) the set-up of the instrument to allow dynamic force spectroscopy, and (5) the extraction of the specific interaction parameters from the acquired data.

3.1. Conversion of the Reactive Groups on the Surfaces

The first steps of this procedure involve the cleaning of the individual surfaces (i.e., the flat glass surface and the microcantilever).

1. Mark two glass slides and clean the slides in ethanol for 10–20 min in an ultrasonic bath.
2. Dry under a stream of argon.
3. Replace ethanol in the beaker in the ultrasonic bath
4. Repeat this treatment twice.

From now on, the glass slide surfaces, and the AFM-tips (e.g., Si_3Ni_4-Microcantilever, Park Scientific, Sunnyvale, CA), are treated in parallel (*see* **Note 1**).

5. Treat the surfaces with a strong UV light source for 60 min. Place the mark on the flat surfaces towards the UV light source.
6. Mixing of silanization solution: Insert one short syringe needle with an attached balloon containing argon through the crown cap (never remove the crown cap!). Take a syringe with a syringe needle long enough to reach the silanization solution (APTES). Withdraw enough solution from stock to enable a 2% dilution in *dry* toluene. Remove dry toluene comparable with two syringes through the crown cap.
7. Put the glass slides with the mark on the top and the AFM tips in a glass container that can be sealed. Immediately silanize the surfaces in a 2% solution of APTES in dry toluene for 2 h or overnight.
8. Rinse extensively with toluene and dry under a stream of argon (*see* **Note 2**).

3.2. Activation of the Newly Generated Groups by Heterobifunctional Cross-Linkers

Immerse the surface in a 1-m*M* solution of MAL-PEG-NHS in DMSO for 2–3 h. The surfaces are again rinsed with DMSO and then with PBS buffer, pH 7.3. (*See* **Notes 3** and **4**.)

3.3. Coupling of the Selected Biomolecules to the Surface

1. The oligonucleotides with a 5π-SH modification were synthesized by Microsynth (Balgach, Switzerland) and were stored in a PBS buffer at pH 7.3 containing 10 m*M* dithiothreitol (DTT) at 4°C until use. Immediately before use, the oligonucleotides are diluted to a final concentration of 25 m*M* with PBS buffer.
2. Extract DTT from an aliquot of typically 200 µL by washing three times with 1 mL of ethyl-acetate!
3. A 50-µL drop of the oligonucleotide solution is then incubated on the poly (ethylene glycol)-ω-maleimide-modified surfaces. Put the solution of one ssDNA oligonucleotide on top of flat glass slide (mark visible on the topside) and the complementary ssDNA oligonucleotide on the AFM-tips, which are on top of a piece of Teflon.
4. To avoid drying create a humidity box. Put some water bubbles on the wall of the box and close the box with a parafilm. Incubate overnight at room temperature in a humid chamber.
5. Rinse with PBS buffer, and then the tips and surfaces are ready for use in the force experiments (*see* **Notes 5–9**).

3.4. Set-Up of the Instrument to Allow Dynamic Force Spectroscopy

Dynamic force spectroscopy measurements were performed using a commercial AFM instrument (Nanoscope IIIa, Digital Instruments, Santa Barbara, CA). The instrument has been expanded using the breakout box available from Digital Instruments. This allows an alternative control of the AFM by an external digital input–output board. We use the multifunctional DAQ board (PCI-MIO-16XE-10) from National Instruments (Austin, TX) to have additional functionality to control the approach-retract function of the force microscope. The only functions of the Nanoscope used are the initial approach using the integrated stepper motor and the feedback for the first approach towards the sample. Additional features, which are controlled through the LabVIEW software package (National Instruments, Austin, TX), are:

Changing of the retract speed after each individual interaction. The speed is increased or decreased in exponential steps starting with speeds <10 pN/s and then increased up to approx 10000 pN/s. Normally we choose six different speeds within the speed-range of four orders of magnitude.

The number of individual pulling cycles at a certain site can be defined before the location to another site on the sample is changed with nm precision.

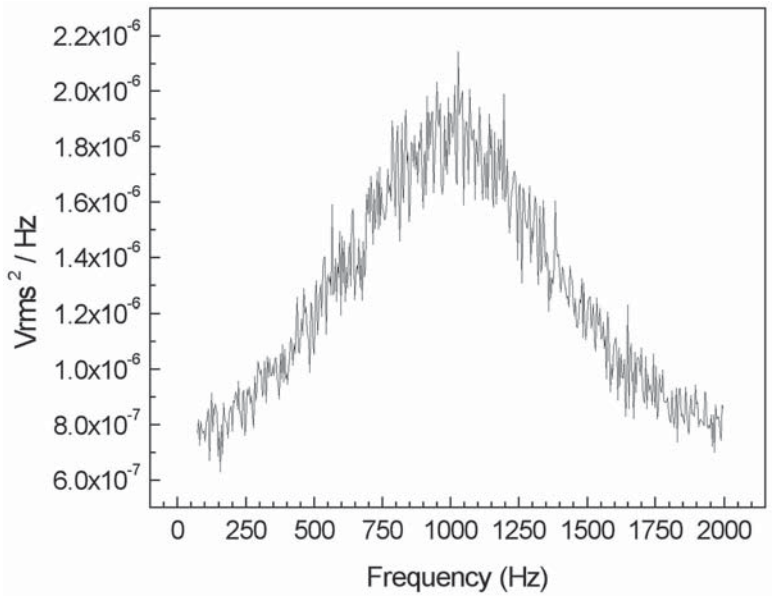

Fig. 2. The sampling rate is 4000 Hz, the frame size 1024 (step-size 3.90625 Hz), the number of points 512, spectrum shown is an average of 200 spectrums. The area under the curve is 0.00244 V_{rms}^2. With a sensitivity value of 0.0563V/nm, the spring constant is 5 pN/nm ± 10 % (compared with 10 pN/nm nominal value given by the company). The fitting and the integration of the volume can be done with commercial software (e.g., LABVIEW or Origin, OriginLab Corporation, Northampton, MA).

AFM cantilevers used for this experiment had spring constants <30 pN/nm. Each cantilever was *in situ* calibrated according to the method of Hutter ***(17)***. In short a power spectrum of the thermal vibrations of the cantilever is recorded using the LabVIEW software package (*see* **Fig. 2**). The spectrum shows a resonance peak at the natural resonance frequency. The integrated volume under the peak is directly correlated to the spring constant of the cantilever. It is important to retract the cantilever far enough from the surface (>1 μm) to avoid hydrodynamic damping. The temperature was controlled using a home-built fluid cell in which the buffer solution that immersed both the probe surface and the AFM cantilever was in contact with a Peltier element (Melcor, Trenton, NJ), driven with a constant current source (*see* **Fig. 3**). The temperature of the buffer was monitored with a thermocouple (Thermocoax, Suresnes, France). The thermocouple is calibrated with a digital thermometer and temperature measurements at different points of the cell showed deviations <2°C.

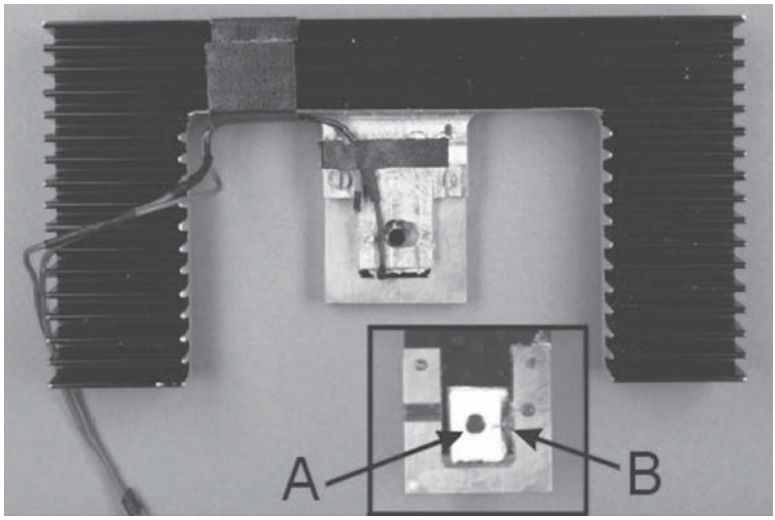

Fig. 3. Home-built fluid-cell platform allowing a precise temperature control. Big-picture complete device showing the top view of the cell including the big black heat sink block with lamellas for temperature stabilization. (*Inset*) Bottom view of the center of the fluid cell, which fits into the multimode head of the Nanoscope IIIa head. *Arrow A,* hole in the Peltier element for laser transmission. The hole is covered by a cover glass no. 1 to avoid fluid leaking and maintain angles of incidence of the laser. *Arrow B,* spring-to-clamp cantilever to the bottom of the Peltier element.

3.5. Extraction of the Specific Interaction Parameters from the Acquired Data

Write a small program to toggle through all the automatically collected force curves. The LABVIEW platform provides easy solutions, which can be expanded according to the operator's needs (*see* **Note 10**).

1. Select the force curves that show clear rupture force. Discard force curves that show no interaction or show unexpectedly long rupture distances.
2. Gather enough sample curves to obtain a reasonable number of data points to analyze your experiment. A way to analyze the data statistically is described by Izenman ***(18)***.
3. Expand your force-curve analysis software to allow automatic recognition of the last jump back of the cantilever to the equilibrium position, determination of the slope at this point, the force of unbinding, and the speed of the pulling cycle (*see* **Fig. 1B**).
4. It is important to determine the specificity of the interaction measured. Use the second sample coated with the unspecific biomolecules. Measure the interaction

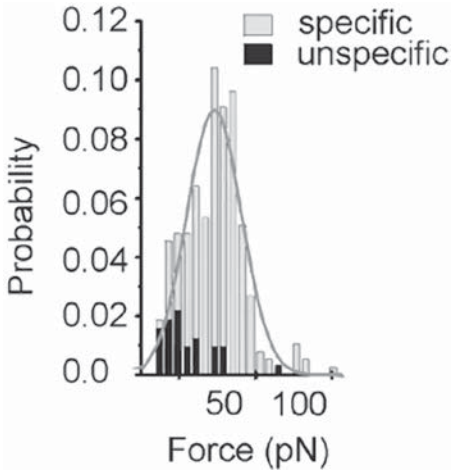

Fig. 4. Specificity check of the unbinding measurements. The force histogram can be fit with a gaussian distribution function to reveal the most-probable unbinding force (F*). Adjust the bin width according to Izenman ***(18)***. Gray bars, specific interaction; black bars, nonspecific interaction.

forces in the same way as with the 'specific' sample. You end up with a histogram comparable to **Fig. 4**.

5. Transfer your data into a diagram having the axes of F* (most probable unbinding force) and the rate of pulling. You should end up with a figure comparable to **Fig. 5**. Once you have collected and analyzed your data following our procedure you should be able to extrapolate the off rate of your system and all the other relevant parameters.

4. Notes

1. Plan to prepare the specific sample and the background sample in the same experiment. For instance we use two glass slides as substrates, one coated with the specifically interacting biomolecule (for example, ssDNA oligonucleotide) and the other with the unspecific molecule (for example, the nonhybridizing ssDNA oligonucleotide).
2. Silanization procedures provide an easy possibility to convert the surface reactive groups of silicon or glass or even silicon nitride tips of the cantilever. Remember that most groups have their own ideal preparation procedure. The activation of surfaces with trifunctional (for example, tri-ethoxy) silans easily

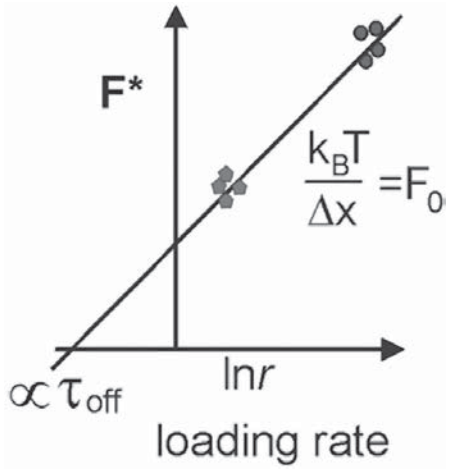

Fig. 5. Loading rate dependence of the unbinding measurements. The slope fit through the data sets provides insight into the energy landscape of the unbinding of the biomolecular interaction. If the precision of the data and the number of various pulling speeds is high enough to fit two slopes within this diagram then a probable intermediate state during unbinding can be detected.

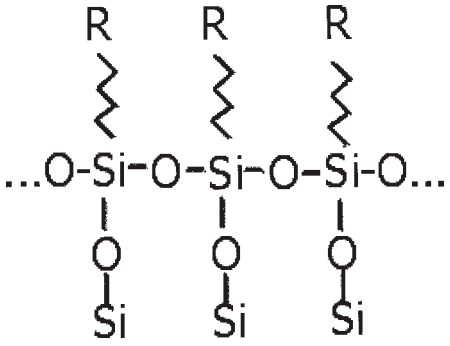

Fig. 6. Ideal surface activation (silanization).

can lead to monolayers on the surface of the sample (*see* **Fig. 6**), but there is a great chance that multilayer and aggregates of polymerized silans are formed (*see* **Fig. 7**). To reduce this possibility monofunctional silans can be used. The

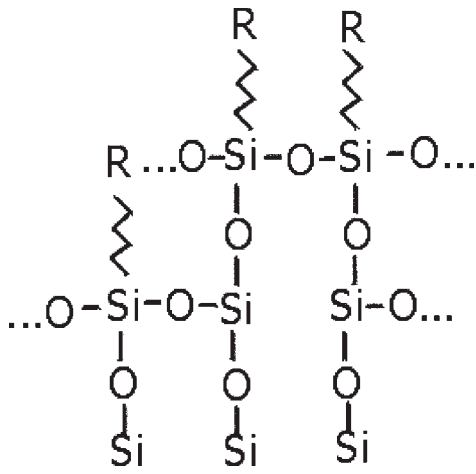

Fig. 7. Reality: partial hydrolysis during deposition, multiplayer formation, and formation of aggregates.

reactivity of these substances is comparable but one should work in an argon environment during the whole silanization procedure (glove box). Curing the surface by heating the sample (150°C) after silanization is possible but not necessary in the case of trifunctional silans.

3. Long flexible linkers are needed when single biomolecule interactions are being probed. When manipulating single globular proteins, tertiary folded RNAs or ssDNA–ssDNA pairs, additional problems must be overcome ***(19,20)***. As a result of their small dimensions (typically 1.5–6 nm) direct tethering to the surfaces is undesired. Furthermore, the nonspecific interactions of the surrounding surfaces would mask the signal of the small individual molecule. These interactions, and possible solvent exclusion within the gap, could lead to denaturation of the molecule or perturbation in the force profiles. A covalent attachment of these biomolecules to surfaces via long linkers minimizes the nonspecific interaction of the surfaces with the biomolecule, and allows probing the single protein molecules. Long linkers of some tens of nanometers from the surface allow space for free exchange of additional ligands or solution in the environment of the molecular interaction being probed. Additionally the use of long linkers allows easy discrimination of single events from multi-events. In cases were two molecular interactions happen a saw-tooth-like pattern in the retract curve is visible. The probability that two bonds break simultaneously is minimized because the length of the flexible cross-linker is comparable to the curving of the cantilever tip. Because the anchoring points are located on a highly curved surface, simultaneous ruptures are very unlikely.
4. In experiments were short linkers are applied the surfaces are immersed in a 1 m*M* solution of sulfo-SMCC in 50 m*M* HEPES buffer (pH 7.0) for 1 h at room temperature. After incubation rinse the surfaces with HEPES buffer only to remove

excess of cross-linkers. Short linkage of the ligand/receptor system on the surface is resulting in considerably more unspecific adhesion events. It is still possible to extract the "real" value of the unbinding force if the very last jump back to equilibrium is being considered ***(3–5)***. Important is the change in pulling speed during the experiment to obtain off rates and not just one interaction force. Owing to the fact that multiple binding events can rupture at the point of rupture a bimodal or higher modal distribution in the force histogram is to be expected

5. It is important to note that the activated cantilever surface has a limited lifetime ***(19,20)*** and therefore the speed of the following steps is crucial. The cantilever is thoroughly rinsed with the buffer used to store the biomolecule. Because the specificity of the cross-linker is changing with the pH and is sensitive towards free amino groups. Buffers like Tris-HCl should be avoided. Buffers of choice are PBS, HEPES, and MES, adjusted to the right pH. The thiol groups of the biomolecules are coupled at a pH ranging from 7.0–7.5 at room temperature.
6. Biomolecules have to be cleaned if stored under protective conditions like those mentioned for thiol-modified oligonucleotide (DTT has been added to prevent oxidation of the thiol to the disulfide). Extraction with organic solvent is an easy way to remove these compounds. By repeating the extraction the amount of DTT is reduced to levels that do not interfere with the covalent coupling to the maleimide group. Oligonucleotides remain in the water phase and DTT is transferred in the organic phase (top phase of the two).
7. In addition, be careful with the buffers in which the biomolecules are being delivered. Unsuitable buffers or protective agents quench the coupling reaction completely. These contaminants are removed using centrifugation membranes available from various companies (e.g., Amicon, Eppendorf, Sartorius). Calculate the dilution factor to be applied during this cleaning to bring the 'contaminant' to a level of approx 1/100 relative to the molecule to be coupled.
8. If instead of thiol groups of the biomolecule (thiol-modification on ssDNA end or free cysteine of protein) amino groups (amino-modification on ssDNA end or free lysine or terminal amino group on protein) have to be coupled to the surface, then instead of APTES, the silanization reagent MPTES can be applied and the protocol follows exactly the same description.
9. The cross-linking group maleimide has its optimal pH range from 6.5–7.5 and succinimide from 7.0–8.5. Some adjustments of pH during the individual coupling steps of the individual groups can improve the overall performance of the coupling reaction ***(19,20)***.
10. To calculate the spring constant of the cantilever and to express the cantilever deflection as a force, the cantilever deflection signal vs the voltage applied to the piezo tube (sensitivity) has to be measured. It is equal to the slope of the force curve while the cantilever is in contact with the sample surface (a cantilever deflection signal of 1–2 V on the photodiode is sufficient for obtaining a good value). Typically, with a spring constant of approximately 10 pN/nm, the sensitivity values are around 0.07–0.09 V/nm. So 1 V deflection signal on the photodiode corresponds to 110–150 pN.

Acknowledgments

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References

1. Dammer, U., Popescu, O., Wagner, P., Anselmetti, D., Güntherodt, H. J., and Misevic G. N. (1995) Binding strength between cell-adhesion proteoglycans measured by atomic-force microscopy. *Science* **267,** 1173–1175.
2. Evans, E., and Ritchie, K. (1997) Dynamic strength of molecular adhesion bonds. *Biophys. J.* **72,** 1541–1555.
3. Moy, V. T., Florin, E. L., and Gaub, H. E. (1994) Intermolecular forces and energies between ligands and receptors. *Science* **266,** 257–259.
4. Dammer, U., Hegner, M., Anselmetti, D., et al. (1996) Specific antigen/antibody interactions measured by force microscopy. *Biophys. J.* **70,** 2437–2441.
5. Hinterdorfer, P., Baumgartner, W., Gruber, H. J., Schilcher, K., and Schindler, H. (1996) Detection and localization of individual antibody-antigen recognition events by atomic force microscopy. *Proc. Natl. Acad. Sci. USA* **93,** 3477–3481.
6. Evans, E. (1998) Energy landscapes of biomolecular adhesion and receptor anchoring at interfaces explored with dynamic force spectroscopy. *Faraday Dis.* **111,** 1–16.
7. Strunz, T., Oroszlan, K., Schäfer, R., and Güntherodt, H. J. (1999) Dynamic force spectroscopy of single DNA molecules. *Proc. Natl. Acad. Sci. USA* **96,** 11277–11282.
8. Schwesinger, F., Ros, R., Strunz, T., et al. (2000) Unbinding forces of single antibody-antigen complexes correlate with their thermal dissociation rates. *Proc. Natl. Acad. Sci. USA* **97,** 9972–9977.
9. Schumakovitch, I., Grange, W., Strunz, T., Bertoncini, P., Güntherodt, H.-J., and Hegner, M. (2002) Temperature dependence of unbinding forces between complementary DNA strands. *Biophys. J.* **82,** 517–521.
10. Evans, E. (2001) Probing the relation between force-lifetime-and chemistry in single molecular bonds. *Annu. Rev. Biophys. Biomol. Struct.* **30,** 105–128.
11. Merkel, R. (2001) Force spectroscopy on single passive biomolecules and single biomolecular bonds. *Phys. Rep.* **346,** 343–385.
12. Merkel, R., Nassoy, P., Leung, A., Ritchie, K., and Evans, E. (1999) Energy landscape of receptor-ligand bonds explored with dynamic force spectroscopy. *Nature* **397,** 50–53.
13. Di Paris, R., Strunz, T., Oroszlan, K., Güntherodt, H.-J., and Hegner, M. (2000) Dynamics of molecular complexes under an applied force. *Single Mol.* **1,** 285–290.
14. Marszalek, P. E., Lu. H., Li, H. B., al. (1999) Mechanical unfolding intermediates in titin modules. *Nature* **402,** 100–103.
15. Strunz, T., Oroszlan, K., Schumakovitch, I., Güntherodt, H.-J., and Hegner, M. (2000) Model energy landscapes and the force-induced dissociation of ligand-receptor bonds. *Biophys. J.* **79,** 1206–1212.

16. Schwarzenbach, M. S., Reimann, P., Thommen, V., et al. (2002) Interferon α-2a interactions on glass vial surfaces measured by atomic force microscopy. *PDA J. Pharm. Sci. Technol.* **56,** 78-89.
17. Hutter J. L. and Bechhoefer, J. (1993) Calibration of atomic-force microscopetips. *Rev. Sci. Instrum.* **64,** 3342–3342.
18. Izenman, A. J. (1991) Recent developments in nonparametric density estimation. *J. Am. Stat. Assoc.* **86,** 205–224.
19. Hermanson, G. T. (1996) *Bioconjugate Techniques*, Academic Press, San Diego.
20. Hegner M. (2000) DNA handles for single molecule experiments. *Single Mol.* **1,** 139–144.