Chapter 7

Functionalization of AFM Tips and Supports for Molecular Recognition Force Spectroscopy and Recognition Imaging

A. Ebner, L. Wildling, and H. J. Gruber

Abstract

Linking of sensor molecules (e.g., antibodies) to an AFM tip converts it into a biosensor by which single target molecules (e.g., antigens) can be detected and localized on the sample surface. Moreover, the mechanism of interaction can be studied by force spectroscopy if purified target molecules are linked to an ultra-flat surface, such as mica or silicon (nitride). Rapid imaging of the binding sites and force spectroscopy studies are greatly facilitated if 6–10 nm long polyethylene glycol (PEG) chains are used as flexible tethers between the sensor molecule and the tip. Here, we describe a set of methods by which a variety of proteins, oligonucleotides, or small molecules can be tethered to silicon (nitride) tips or to mica. Methods are included which afford site-specific and oriented coupling of the sensor molecules.

Key words Atomic force microscopy, AFM tip functionalization, Force spectroscopy, Molecular recognition, Biospecific detection, Antibody–antigen interaction

1 Introduction

Attachment of a sensor molecule (e.g., an antibody) to the surface of an AFM tip converts it into a specific biosensor which can be used to localize complementary target molecules (e.g., antigens) on the sample surface (for reviews *see* refs. 1, 2). Insertion of a long, flexible polyethylene glycol (PEG) chain between tip surface and sensor molecule proved advantageous, for several reasons: (1) The sensor molecule can reorient rapidly and "palpitate" the sample surface, thus binding to cognate target molecules is greatly facilitated. (2) In force spectroscopy experiments, specific binding is much easier to discriminate from nonspecific tip-surface adhesion when using a 6–9 nm long PEG linker between tip and sensor molecule [3–5]. (3) Rapid simultaneous imaging of Topography and RECognition sites (TREC) is intrinsically dependent on the use of 6–9 nm long PEG linkers [6–8].

Gold-coated AFM tips were frequently used in molecular recognition force spectroscopy but (with few exceptions [9–11]) the

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linkers between tip and sensor molecules were usually very short [2].

Silicon and silicon nitride tips have the advantage that they are sharper and can be functionalized with virtually any probe molecule, based upon a versatile toolbox of PEG linkers and linking protocols [1, 2, 12]. The functionalization procedure typically involves three steps: (1) aminofunctionalization, i.e., introduction of NH_2 groups, (2) unilateral attachment of a PEG linker, and (3) attachment of the probe molecule to the free-tangling end of the PEG chain.

For studies of cells or organelles, these can be adsorbed to suitable supports (e.g., culture dishes). For studies on isolated target molecules, however, these need to be immobilized on ultra-flat supports. This can either be done by the same methods as used on the tip surface (Subheadings 3.3–3.7) or by use of much shorter crosslinkers if a more rigid attachment is desired (Subheading 3.2).

2 Materials

2.1 Aminofunctionalization of AFM Cantilevers or Silicon Nitride Chips or Mica Sheets

1. AFM cantilevers (silicon or silicon nitride).
2. Argon gas 5.0 in a cylinder with pressure reducer for adjustment of low pressure and a variable flow.
3. Chloroform (analytical grade or HPLC grade, *see* **Note 1**).
4. 30–60 μL Aminopropyltriethoxysilane (APTES, corrosive, irritant, mutagenic! *See* **Note 2**).
5. 10–20 μL Triethylamine (*see* **Notes 3, 4,** and **5**).
6. Glass desiccator (5 L, *see* **Note 6**) with a large silicone O-ring as seal between cover and jar. No grease is allowed.
7. Two lids cut from the top of two 1.5 mL Eppendorf™ reaction vials.
8. Small glass crystallization dishes (e.g., OD 40 mm) for cantilever washing.
9. Small polystyrene Petri dish (e.g., OD of lid 38 mm) for cantilever storage.
10. Optional alternative to cantilevers: Silicon nitride chips, precut to a size that fits within the AFM fluid cell (e.g., 6 × 6 mm, *see* **Note 7**).
11. Optional alternative to cantilevers: mica sheets (with dimensions that fit the liquid cell of the AFM, *see* **Note 7**), adhesive tape (e.g., Scotch tape™), clean tweezers, and a clean flat poly (tetrafluoroethylene) (PTFE) block (*see* **Note 8**) with an area somewhat larger than the mica sheets.

2.2 Immobilization via Short Crosslinker

1. Ethylene glycol bis(succinimidyl succinate) (EGS, 1 mg/mL, typically 10 mg in 10 mL) is dissolved in chloroform (*see* **Note 1**) and triethylamine is added (0.5% v/v, typically 50 µL in 10 mL *see* **Notes 3, 4,** and **5**).
2. Glass crystallization dishes (e.g., OD 40 mm) for the EGS reaction and for washing.
3. Small polystyrene Petri dishes (e.g., OD of lid 38 mm).
4. Phosphate-buffered saline (PBS) contains 150 mM NaCl, 5 mM NaH_2PO_4, adjusted to pH 7.4 with NaOH.
5. A buffer solution containing the protein of interest (1–2 µM, e.g., streptavidin, *see* **steps 6** and **7** below), preferably in PBS (*see* **Note 9**). Higher concentrations are recommended for DNA (10–100 µM, *see* **Notes 10** and **11**) or small molecules (1–2 mM, *see* **Notes 10** and **11**). Typically, 20–200 µL is required to form a drop on APTES-treated mica or silicon, which does not evaporate during the coupling reaction (Subheading 3.2).
6. Optional: Streptavidin stock solution (20 µM, *see* **Notes 12** and **13**). 1 mg recombinant streptavidin (60,000 g/mol, 15 nmol) is dissolved in 833 µL 150 mM NaCl. The solution is divided into 20 µL aliquots which are frozen in liquid nitrogen and stored at −25 °C.
7. Optional: Streptavidin working solution (2 µM, *see* **Note 12**): One 20 µL aliquot 20 µM streptavidin is thawed and mixed with 180 µL PBS.

2.3 Coupling of PEG-Biotin to Tip, Adsorption of Avidin on Mica

1. Reaction chamber with cover (*see* **Notes 14** and **15**).
2. Biotin-PEG-NHS (1 mg per batch, *see* **Note 16**), preferably with a molecular mass between 1000 and 2000 (approximately 15–30 ethylene glycol units, e.g., from www.nanocs.com or www.iris-biotech.de).
3. Chloroform (*see* **Note 1**) and triethylamine (*see* **Notes 3, 4,** and **5**).
4. Glass crystallization dishes (e.g., OD 40 mm) for washing of cantilever(s) in chloroform.
5. Avidin stock solution: Dissolve wild-type avidin in 150 mM NaCl at an avidin concentration of 1 mg/mL (typically 2.0 mg in 2.0 mL). Prepare 30 µL aliquots, freeze in liquid nitrogen, and store at −25 °C.
6. Avidin working solution No. 1: Thaw one 30 µL aliquot of the 1 mg/mL avidin stock solution and mix with 270 µL pure water. Use immediately.

7. Avidin working solution No. 2: Pipet 4 mL pure water into a 15 mL Falcon® tube. Thaw one 30 µL aliquot of the 1 mg/mL avidin stock solution and mix by vortexing. Transfer 10 µL of the thawed 1 mg/mL avidin stock solution into the Falcon® tube containing the 4 mL water portion and mix well. Use immediately.
8. Biotin stock solution (10 mM, *see* **Note 13**): 12.2 mg biotin is weighed into a 20 mL screw-cap glass vial or a 25–50 mL Erlenmeyer flask. 5 mL 50 mM Na_2HPO_4 solution (*see* **Note 17**) is added and biotin is dissolved by stirring. The solution is quickly divided into 20 µL aliquots, frozen in liquid nitrogen, and stored at −25 °C. For each blocking experiment, a fresh 20 µL aliquot is thawed and used immediately.
9. Optional: One 20 µL aliquot of 20 µM streptavidin (Subheading 2.2) is thawed and immediately used for one block experiment.

2.4 Immobilization via Acetal-PEG-NHS

1. Reaction chamber with cover (*see* **Notes 14** and **15**).
2. Acetal-PEG18-NHS or acetal-PEG27-NHS (1 mg per batch, *see* **Note 16**).
3. Chloroform (*see* **Note 1**) and triethylamine (*see* **Notes 3, 4,** and **5**).
4. Glass crystallization dishes (e.g., OD 40 mm) for washing of the cantilevers with chloroform.
5. Polystyrene Petri dishes (e.g., OD 38 mm) for citric acid treatment, for washing with water, for coupling of the sensor molecule, and for washing with buffer.
6. One strip of Parafilm™ (e.g., 16 mm × 16 mm).
7. Citric acid solution (1% w/v in water, aliquots can be stored at −25 °C).
8. Prepare a 100 mM solution of NaOH (4 mg/mL) in water, split into aliquots (100 µL), and store in the freezer at −25 °C. Thaw one vial for preparing the 1 M stock solution of $NaCNBH_3$ (next item).
9. Immediately before coupling of proteins to the tip, a 1 M solution of sodium cyanoborohydride ($NaCNBH_3$) is prepared: Weigh 13 mg $NaCNBH_3$ into a small screw-cap glass vial (caution, *see* **Note 18**). Add 20 µL 100 mM NaOH (*see* previous step). Add 180 µL pure water and mix with the pipet tip to dissolve all $NaCNBH_3$. Use within ~1 h and dispose of the unused rest (*see* **Note 18**).
10. 100 µL of a 1–2 µM solution of the protein of interest (e.g., an antibody with a concentration of 0.15–0.3 mg/mL) in PBS (*see* **Notes 9**, **10**, and **11**). Higher concentrations are required

for amine-modified DNA or for small molecules with amino groups (*see* **Notes 10** and **11**).

11. One aliquot (5–50 µL) 1 M ethanolamine hydrochloride solution (pH 8.0, *see* **Note 19**).

2.5 Immobilization via Maleimide-PEG-NHS

1. Reaction chamber with cover (*see* **Notes 14** and **15**).
2. Maleimide-PEG-NHS (1 mg per batch, *see* **Note 16**), preferably with a molecular mass between 1000 and 2000 (approximately 15–30 ethylene glycol units, e.g., from www.creativepegworks.com or www.polypure.com).
3. Chloroform (*see* **Note 1**) and triethylamine (*see* **Notes 3**, **4**, and **5**).
4. Glass crystallization dishes (e.g., OD 40 mm) for washing of the cantilevers with chloroform.
5. Polystyrene Petri dishes (e.g., OD of lid 38 mm) for coupling of the sensor molecule and for washing with buffer.
6. One strip of Parafilm™ (e.g., 16 mm × 16 mm).
7. HBS (HEPES-buffered saline) contains 150 mM NaCl, 10 mM HEPES (2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid), adjusted to pH 7.4 with NaOH.
8. HBS/EDTA: HBS is supplemented with 2 mM EDTA (ethylenediaminetetraacetic acid, disodium salt) and the pH is readjusted to 7.4 with NaOH. Alternatively, 0.02 volumes of the 100 mM EDTA stock solution listed below can be added.
9. 100 µL buffer containing the sensor molecule (1–2 µM thiol-protein, 10–100 µM thiol-DNA, 1–2 mM of a small molecule with an SH group, *see* **Note 10**), preferably in HBS/EDTA (*see* **Note 20**).
10. 2 µL EDTA (100 mM EDTA disodium salt, pH 7.5 adjusted with NaOH, stored frozen in many small aliquots, *see* **Note 21**).
11. 5 µL HEPES (1 M HEPES acid, pH 7.5 adjusted with 20% NaOH, stored frozen in many aliquots, *see* **Note 22**).
12. 2 µL 100 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in water, without pH adjustment (very acidic, use one freshly thawed aliquot, *see* **Note 22**).
13. 2 µL basic HEPES stock solution (nominally 1 M, prepared from an *exactly* 1 M stock solution of HEPES acid by addition of 20% NaOH until pH 9.6 is reached, *see* **Note 22**).

2.6 Coupling of Glycoproteins or Aldehydes to Hydrazide-Functionalized Tips (or Supports)

1. AFM tip (or support) functionalized with maleimide-PEG-NHS (Subheading 3.5).
2. 45 µL of aqueous buffer containing a glycoprotein (or aldehyde molecule) at a concentration of 0.4–2 µM (e.g., 0.06–0.3 mg/mL of antibody, *see* **Note 23**).
3. "Buffer 5.5" contains 100 mM sodium acetate and the pH is adjusted to 5.5 with HCl.
4. Slide-a-lyzer MINI cartridge with MWCO 10 kDa (Thermofisher product No. 69570) and foam pad (product No. 69588) for floating on the buffer surface.
5. One large glass beaker where the foam pad can be inserted into the beaker and one narrow 50 mL glass beaker where the foam pad covers the opening of the beaker.
6. 100 mM sodium periodate ($NaIO_4$) in water, *freshly* prepared by dissolving ~5 mg $NaIO_4$ in the volume of water, which gives the exact concentration of 21.4 mg/mL (*see* **Note 24**).
7. 3-(2-pyridyldithio)propionyl hydrazide (PDPH), dissolved in dimethylsulfoxide (DMSO) at a concentration of 25 mM (5.73 mg/mL), stored in 10 µL aliquots at −25 °C for up to several years.
8. 2 µL EDTA (50 mM EDTA disodium salt, pH 7.5 adjusted with NaOH, stored frozen in many small aliquots, prepared by diluting the 100 mM EDTA solution from Subheading 2.5, *see* **Note 21**).
9. 5 µL HEPES (500 mM HEPES acid, pH 7.5 adjusted with NaOH, stored frozen in many aliquots, prepared by diluting the 1 M stock solution from Subheading 2.5 with water by a factor of 2).
10. 2 µL TCEP (50 mM, without pH adjustment, very acidic, use one freshly thawed aliquot, prepared by diluting the 100 mM TCEP solution from Subheading 2.5 with water by a factor of 2).
11. 2 µL basic HEPES stock solution (nominally 500 mM, prepared by diluting the nominally 1 M HEPES solution with pH 9.6 from Subheading 2.5 with water by a factor of 2).
12. Polystyrene Petri dishes (e.g., OD of lid 38 mm) for coupling of the sensor molecule and for washing with buffer.
13. One strip of Parafilm™ (e.g., 16 mm × 16 mm).

2.7 Immobilization via Fmoc-PEG-NHS

1. Reaction chamber with cover (*see* **Notes 14** and **15**).
2. Fmoc-PEG-NHS (1 mg per batch, *see* **Note 25**), preferably with a molecular mass between 800 and 2000 (approximately 12–30 ethylene glycol units, e.g., from www.biomatrik.com or www.creativepegworks.com).

3. Glass crystallization dishes (e.g., OD 40 mm) for washing of the cantilevers with solvents.
4. Chloroform (*see* **Note 1**) and triethylamine (*see* **Notes 3**, **4**, and **5**).
5. *N,N*-Dimethylformamide (DMF) containing 20% (v/v) of piperidine: 4 mL DMF and 1 mL piperidine are mixed in a glass crystallization dish which has an outer diameter (OD) of 40 mm, resulting in a liquid level of 5 mm (*see* **Notes 4** and **5**).
6. *N,N,N′,N′*-Tetramethyl-*O*-(*N*-succinimidyl)uranium tetrafluoroborate (TSTU, 15 mg, 50 μmol).
7. *N,N*-Diisopropyl-*N*-ethylamine (DIEA, 100 μmol, 17.5 μL, *see* **Notes 3**, **4**, and **5**).
8. Ligand with free carboxylic group (30 μmol, corresponding to 10.8 mg in case of testosterone 3-(*O*-carboxymethyl)oxime, which has a molecular mass of 361.5 [13]).

3 Methods

All reactions must be performed in a well-ventilated hood (exceptions are reactions with aqueous buffers, and only if no toxic $NaCNBH_3$ is included in the buffer). Latex gloves will protect from chloroform or methanol for 1–2 s only and must be removed immediately after contact. Nitrile gloves are better but do not provide permanent protection either. Safety goggles are mandatory when using vacuum, e.g., for filtration of buffers through 0.2 μM filters. The 5 L desiccator used for aminofunctionalization and for storage of functionalized tips is never evacuated, only flushed with argon.

All solvents, reaction mixtures with organic solvents, and unused leftover of organic reagents (APTES, triethylamine, etc.) must be saved in a suitable bottle or tank in a well-ventilated area and handed over to a waste disposal company.

Coupling of PEG linkers to AFM tips is best performed in a special PTFE reaction chamber that is covered with a PTFE disk (*see* **Note 14**). Alternatively, an improvised glass chamber can be used (*see* **Note 15**). The chambers must be small enough to ensure complete immersion of the AFM cantilevers with a solution of 1 mg of PEG linker in 0.5 mL chloroform solution and to minimize evaporation of solvent.

3.1 Aminofunctionalization of Cantilever Tips and Supports

The traditional aminosilanization protocols with aqueous ethanol as solvent cannot be used because this generates long, sticky polymer networks which would result in dramatic adhesion between tip and support. No such problems are encountered when using ethanolamine hydrochloride in DMSO [4], aminophenyl

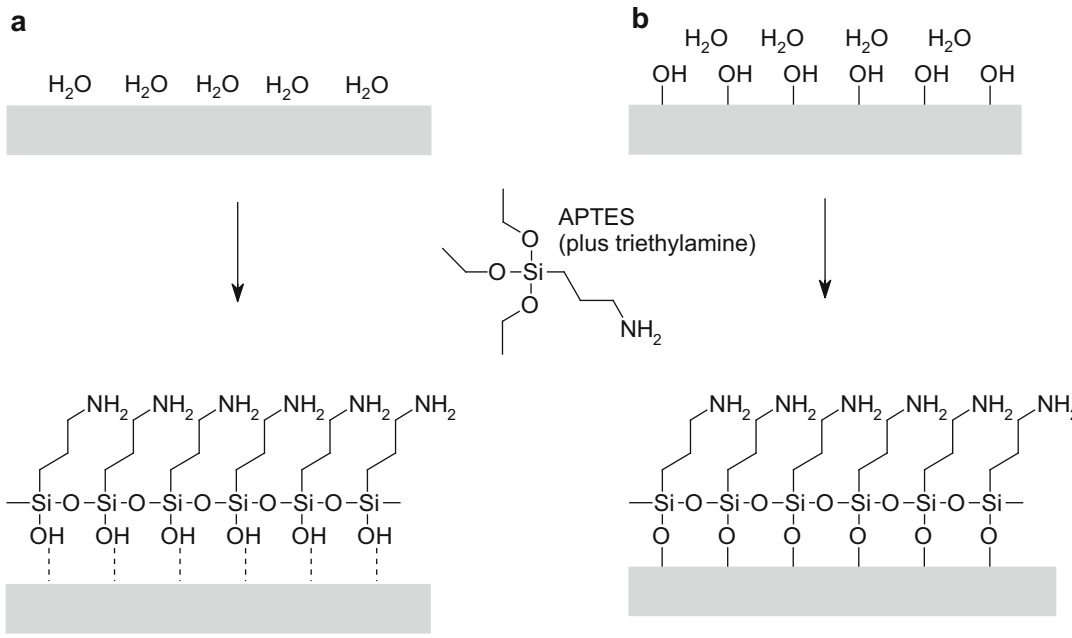

Fig. 1 Aminofunctionalization of mica (**a**) or of surfaces with silanol groups (**b**), such as oxidized silicon, oxidized silicon nitride, or glass. 3-(Aminopropyl)triethoxysilane (APTES) and triethylamine are applied in the gas phase [16]. The reaction is performed under argon atmosphere to reduce the amount of adsorbed water and to minimize oxidation of the amino groups

trimethoxysilane (APhS) in toluene [14], 3-aminopropyl triethoxysilatran in water [4, 15], APTES in toluene [4], or APTES/triethylamine in the gas phase [16]. As shown in Fig. 1, the APTES molecules are hydrolyzed by surface-bound water, resulting in crosslinking among the APTES molecules. In case of mica, the APTES network is attached to the mica by hydrogen bridges only (Fig. 1a), while on surfaces with silanol groups (e.g., on glass, oxidized silicon, or oxidized silicon nitride) the APTES network is also covalently bound via siloxane bridges (Fig. 1b).

1. Immerse silicon (nitride) cantilevers (or ultra-flat silicon nitride chips or freshly cleaved mica, *see* **Notes 26** and **27**) in chloroform in a glass crystallization dish for 5 min. Repeat 2× with new clean dishes and fresh chloroform (*see* **Note 28**).
2. Dry the cantilevers (or chips or mica) in a stream of argon or nitrogen gas and store dust-free in a polystyrene Petri dish.
3. Remove the top valve from the desiccator cover and flood the 5 L desiccator with argon gas through the top opening for 5 min to remove air and moisture.
4. Reinsert the valve into the cover of the desiccator.
5. Remove the cover of the desiccator and place the two lids of the reaction vials inside the desiccator, with a mutual distance

of ~10 cm distance. Argon is denser than air and stays inside (*see* **Note 6**).

6. Pipet 60 μL APTES into one lid and 20 μL triethylamine into the other lid (*see* **Note 29**).
7. Place the cantilevers (or chips or mica) on a glass tray (e.g., the lid of a glass Petri dish) inside the desiccator. The location is not critical but, if possible, the same distance from APTES and triethylamine is preferred.
8. Close the cover of the desiccator, flush the desiccator through the top opening for 30 s with argon gas (*see* **Note 29**), reinsert the top valve, and incubate for 120 min.
9. Remove the two Eppendorf reaction vial lids (with APTES and triethylamine) from the desiccator, close desiccator with cover, remove the top valve, flush for at least 5 min with argon gas through the top opening, and reinsert the top valve. It is essential to remove any traces of triethylamine by argon flushing.
10. Incubate the aminofunctionalized cantilevers (or mica sheets or silicon nitride chips) inside the desiccator for at least 2 days to maximize the crosslinking among the APTES molecules (Fig. 1a) and between APTES and surface ("curing," *see* Fig. 1b). The incubation/storage inside the argon-filled desiccator (at room temperature!) can be prolonged for at least two months without significant loss of the amino groups on the cantilever surface. Much longer storage times are also often possible but not recommended (*see* **Note 30**).

3.2 Immobilization of Biomolecules on Mica or Silicon Nitride Chips via a Short Crosslinker

Recognition force microscopy/spectroscopy requires sensor molecules on the AFM tip and target molecules on a sample surface. If the target molecules are located on the surface of a biological cell, then the cell can be anchored on a culture dish or adsorbed to a polylysine-coated surface. Alternatively, individual purified target molecules can be linked to an ultra-flat support in two different ways: (a) by the same long, flexible PEG linkers, as used on the AFM tip, or (b) with short crosslinkers.

Surface linking via PEG linkers does not lead to dense coverage of the surface with target molecules [4] but the lateral densities are high enough to allow for simultaneous interaction of a tip-linked sensor molecule with more than one surface-linked target molecule [17] (Traxler et al., manuscript in preparation).

Often it is desired to immobilize the target molecules at very high lateral density and without motional freedom [16, 18, 19]. This can be achieved by a three-step procedure: (a) generation of amino groups (as depicted in Fig. 1 and described in Subheading 3.1), (b) coupling of a short crosslinker with two NHS ester functions (Fig. 2), and (c) coupling of biomolecules

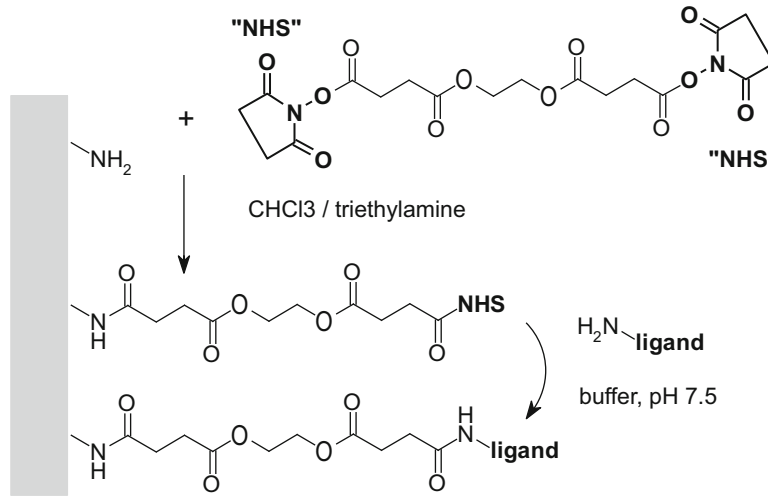

Fig. 2 Covalent coupling of proteins (or other molecules with NH_2 groups) to aminofunctionalized surfaces. The aminofunctionalized surface is first reacted with a short homobifunctional crosslinker (EGS = ethylene glycol bis(succinimidyl succinate)), which contains two NHS ester groups. NHS esters easily react with primary amino groups, resulting in stable amide bonds. In the first step, the EGS molecule couples to the amino group on the surface and in the second step to the amino group on the ligand molecule (reproduced from ref. 2 with permission from Springer-Verlag Berlin Heidelberg)

with NH_2 groups to the free-tangling ends of the surface-linked crosslinkers (Fig. 2) [18, 19]. The same procedure can be used to immobilize a dense layer of lectin molecules, which bind the glycoproteins on cell surfaces, affording tight immobilization of cells for subsequent studies by AFM [18, 20].

Alternative methods are analogous to Fig. 2, except that EGS is replaced by other short crosslinkers like glutaraldehyde [16] or by *N*-succinimidyl 4-(dimethoxymethyl)benzoate (SDMB, *see* **Note 31**).

1. The aminofunctionalized mica sheet (or silicon chip) is removed from the argon-filled desiccator (Subheading 3.1, *see* **Note 30**) and transferred into a glass crystallization dish with a chloroform solution containing EGS (1 mg/mL) and triethylamine (0.5%, v/v), covered with a glass lid, and incubated at room temperature for 1 h.
2. The EGS-functionalized mica sheet or silicone chip is washed (3×) in glass crystallization dishes by incubation in chloroform (5 min in each step), dried with a stream of nitrogen or argon gas, and placed in a polystyrene Petri dish.
3. The protein of interest (1–10 μM, *see* **Note 10**), or the amine-modified DNA (10–100 μM, *see* **Note 10**), or a small molecule with an amino group (*see* **Note 11**) is dissolved in PBS (*see*

Note 9). This solution is pipetted onto the EGS-functionalized support. For example, 200 µL of the 2 µM streptavidin working solution is applied to a mica sheet which fits the typical AFM fluid cell. The Petri dish is covered, and coupling is allowed to proceed for 2 h at room temperature.

4. The protein-functionalized support is washed (3×) in polystyrene Petri dishes by incubation in fresh PBS portions (5 min per incubation) and stored in PBS (or HBS, *see* Subheading 2.5) at 4 °C. It is recommended to use the functionalized support as soon as possible. If the immobilized protein is sufficiently stable, then prolonged storage is possible at 4 °C, provided that the storage buffer contains a preservative that prevents microbial growth (e.g., 0.2–0.3 mg/mL of sodium azide, *see* **Note 32**).

3.3 AFM Tip with PEG-Biotin and Immobilization of Avidin on Mica.

Functionalization of AFM tips with PEG-linked biotin is depicted in Fig. 3a. After aminofunctionalization (Subheading 3.1) the tip is reacted with a PEG linker that carries a biotin on one end and an NHS-ester on the other end (*see* Fig. 3a). In the coupling reaction, the NH_2 group on the tip replaces the NHS group, resulting in the formation of a stable amide bond (*see* Fig. 3a).

The linker biotin-PEG-NHS is unique because here the sensor molecule (i.e., biotin) is already part of the PEG linker. In all other cases described here, the aminofunctionalized tip is first reacted with one end of the PEG linker and in second step the sensor molecule is linked to the free-tangling end of the PEG chain (Figs. 4, 5, 6, and 7).

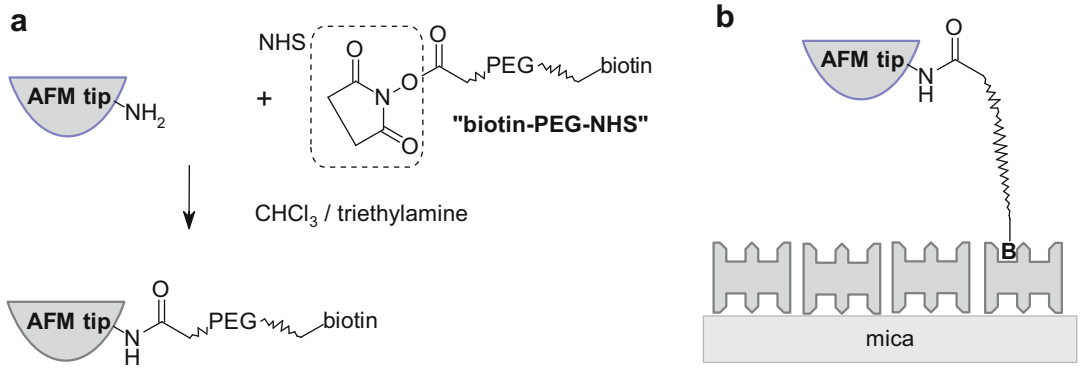

Fig. 3 Flexible tethering of biotin residues to AFM tips and molecular recognition of tip-bound biotin by mica-bound avidin molecules [4]. (**a**) After aminofunctionalization (compare Fig. 1), the amino group on the tip is coupled with "biotin-PEG-NHS," which is a 6–10 nm long polyethylene glycol (PEG) chain carrying an NHS ester function on one end and a biotin residue on the other end (reproduced from ref. 2 with permission from Springer-Verlag Berlin Heidelberg). (**b**) Freshly cleaved mica is coated with avidin molecules by electrostatic adsorption and the specific binding of biotin to avidin is characterized by molecular recognition force spectroscopy (MRFS) [4] or localized by *t*opography-and-*rec*ognition (TREC) imaging [8]

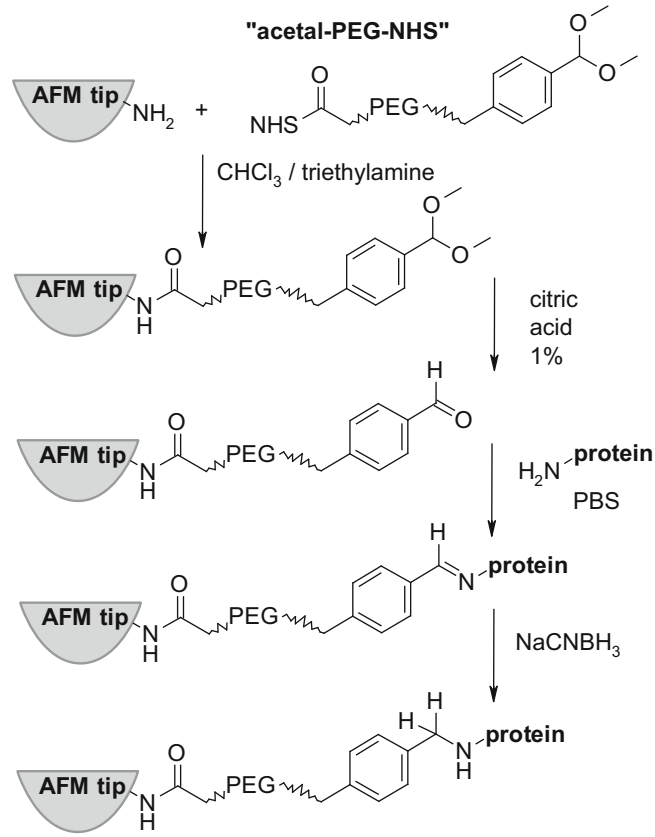

Fig. 4 Flexible tethering of NH_2-containing ligand molecules to NH_2-functionalized AFM tips [12] (or to NH_2-functionalized mica [27]). In the first step, an amide bond is formed between the NH_2 group on the AFM tip and the NHS ester function of the 6–10 nm long "acetal-PEG-NHS" linker. In the second step, the acetal group on the outer end of PEG is converted into an aldehyde by 15 min incubation in 1% citric acid. In the third step, the tip is incubated in phosphate-buffered saline (PBS) containing the ligand and $NaCNBH_3$ is added. Hereby, the NH_2 group of the ligand and the aldehyde group on the PEG linker first form an unstable –CH=N– bond, which is subsequently converted into a stable $–CH_2–NH–$ bond by $NaCNBH_3$. An interesting option with wide applicability is covalent coupling of streptavidin: it provides for subsequent binding of a biotinylated protein which then serves as the sensor molecule on the tip [48]

The flexible PEG chain provides for a high binding probability of biotin to the biotin-binding sites of avidin (or streptavidin) molecules which are immobilized on an ultra-flat support (*see* Fig. 3b) [4, 8, 19]. A particular advantage of avidin is that it can easily be immobilized on mica by electrostatic adsorption, either at high [4] or at low coverage [8].

The purpose for tip functionalization with biotin can be diverse: (1) It provides a simple test system for beginners. (2) It

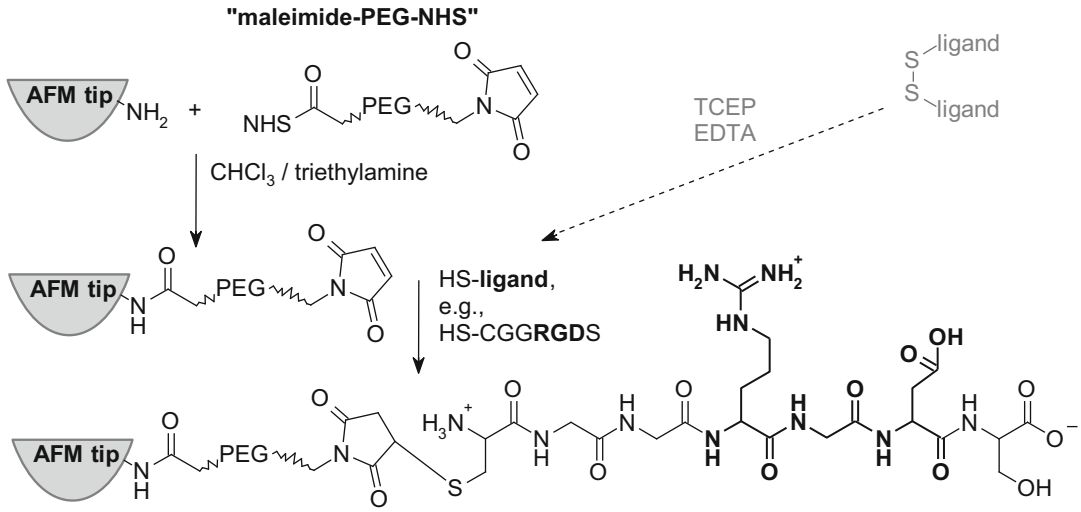

Fig. 5 Flexible tethering of HS-containing ligand molecules ("thiols") to NH_2-functionalized AFM tips. In the first step, the NH_2-group on the AFM tip forms an amide bond with the NHS ester function of the long linker ("maleimide-PEG-NHS"). In the second step, the maleimide group on the outer end of the PEG chain couples to the HS-group of the ligand molecule. In the example shown here, a peptide containing the fibronectin-binding **RGD** sequence (shown in boldface) was coupled via its cysteine residue [33]. Thiol groups (HS) have a high tendency to give disulfides (S-S) under ambient conditions. For this reason, TCEP and EDTA should be present during thiol coupling. Fortunately, TCEP causes quick cleavage of S-S into HS groups but does not harm maleimide groups at low concentration. EDTA prevents re-oxidation of HS groups into S-S groups, even in absence of an argon atmosphere [39]

affords a convenient model system with clear-cut controls when testing new AFM modes, such as TREC [8], or when characterizing new nanostructures on a flat support [21]. (3) Tips with PEG-biotin are ideal for characterization of new mutants/chimeras of (strept)avidin or other new biotin-binding proteins [19].

1. Aminofunctionalization of the cantilever (or mica, or silicon nitride chip) as described in Subheading 3.1.
2. Dissolve biotin-PEG-NHS (1 mg) in chloroform (0.5 mL, *see* **Notes 1, 4**, and **5**). Transfer this solution into the reaction chamber (*see* **Notes 14** and **15**) and add triethylamine (30 μL, *see* **Notes 3, 4**, and **5**). Quickly proceed to the next step.
3. Immerse one or several aminofunctionalized cantilevers in the reaction mixture. Cover the chamber with the lid and incubate for 2 h at room temperature.
4. Wash the cantilever(s) (3×) in glass crystallization dishes by incubation in chloroform (10 min per incubation).
5. Dry the cantilever(s) with a stream of nitrogen or argon gas, store for up to several weeks under argon gas. Use the tip within this time for force spectroscopy or recognition force

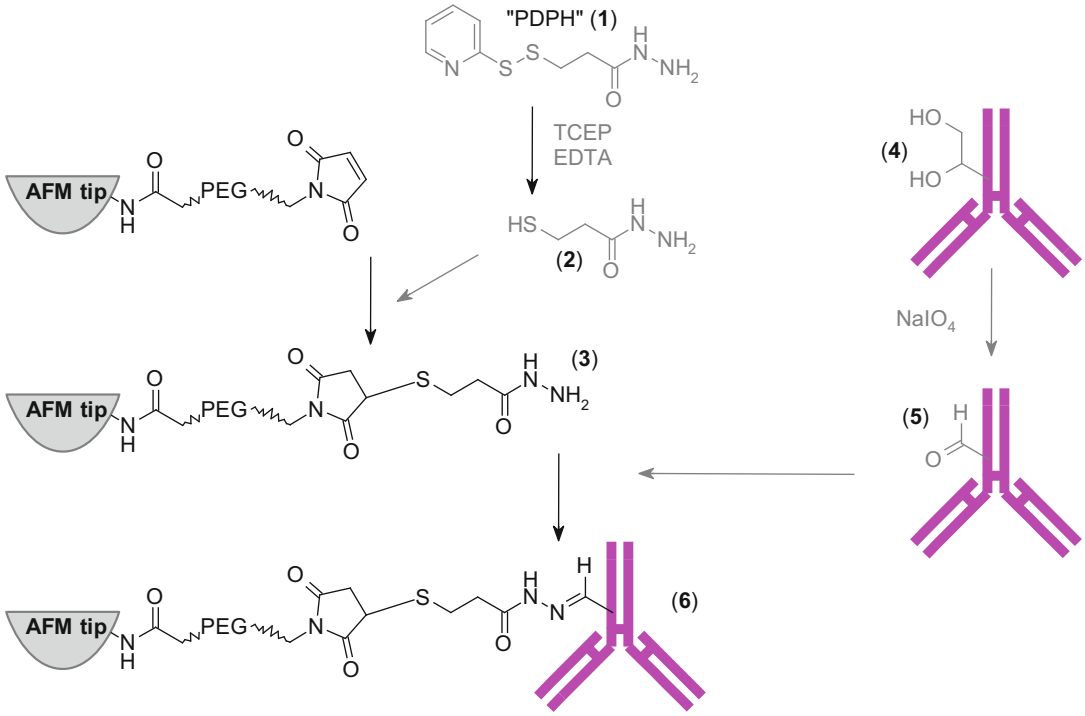

Fig. 6 Oriented coupling of periodate-treated antibodies (or other glycoproteins) to maleimide tips. PDPH (**1**) is mixed with TCEP in the presence of EDTA, resulting in generation of 3-mercaptopropionylhydrazide (**2**), which couples to the maleimide tip (**3**). In a separate reaction, an antibody (**4**, or another glycoprotein) is treated with sodium periodate ($NaIO_4$) which converts vicinal diols (e.g., in sialic acid residues) into aldehydes (**5**). The by-product (formaldehyde, not shown) must be removed by dialysis before the oxidized antibody can be coupled to the hydrazide tip (**3**), yielding a stable hydrazone linkage (**6**). Molecules with aldehyde groups can also be used in place of periodate-treated glycoproteins

microscopy on a sample with an immobilized biotin-binding protein (avidin, streptavidin, anti-biotin, etc.).

6. Cleave a mica sheet which is larger than the fluid cell (*see* **Note 27**) and mount in the fluid cell.
7. For force spectroscopy of the avidin–biotin interaction [4], avidin is adsorbed to mica at high lateral density, as described in the subsequent steps 8–10 (*see* **Note 33**).
8. Transfer 200 µL avidin working solution No. 1 into the fluid cell and incubate for 15 min.
9. Rinse with 1 mM NaCl (10×) and with PBS (10–50×). Add 300 µL PBS.
10. Keep wet and proceed to AFM measurements of the avidin–-biotin interaction, as described [4]. Use 15 µL from a freshly thawed 20 µL portion of 10 mM biotin (or of 20 µM streptavidin) for specific block of the avidin–biotin interaction (*see* **Note 13**).

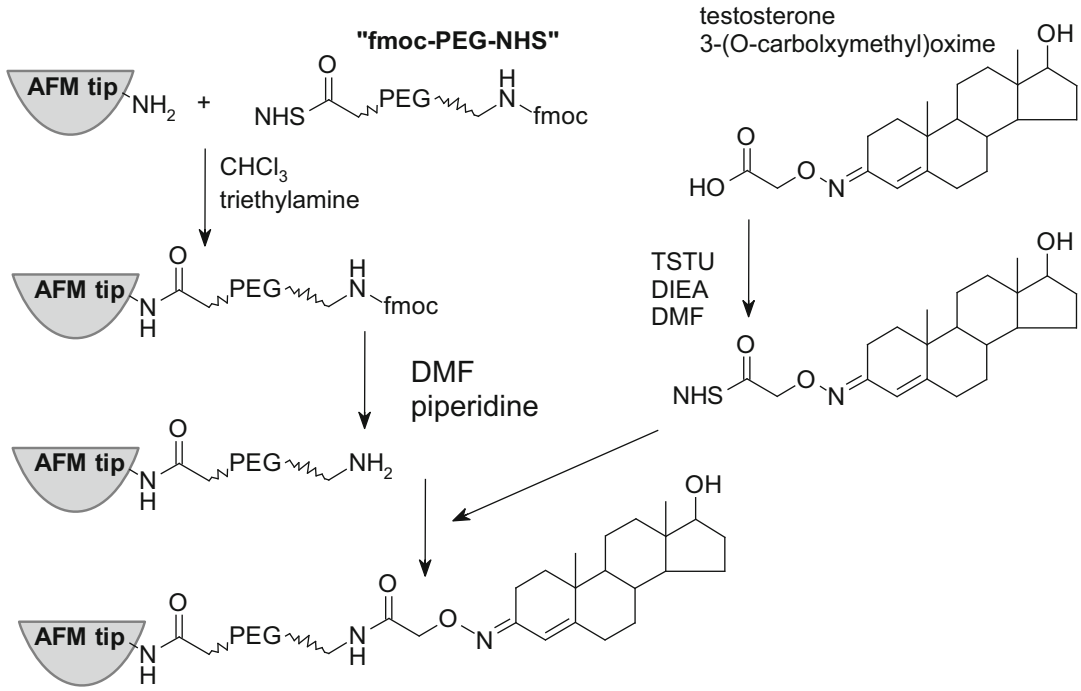

Fig. 7 Flexible tethering of ligands with carboxyl group (HOOC-ligand) to NH_2-functionalized AFM tips [13, 42]. In the first step, the NH_2 group on the tip forms an amide bond with the NHS ester function of the long linker "fmoc-PEG-NHS." In the second step, the protecting group (fmoc) on the outer end of PEG is removed by a mixture of *N,N*-dimethylformamide (DMF) and piperidine (4/1, v/v), resulting in a free amino group on the outer end of the PEG linker. In the third step, the ligand with the free COOH group is dissolved in DMF, mixed with TSTU and *N,N*-diisopropyl-*N*-ethylamine (DIEA), and added to the AFM tip. TSTU converts HOOC-ligand into the NHS ester of the ligand which then forms an amide bond with the NH_2 group on the PEG linker

11. For recognition force microscopy on single avidin molecules [8], avidin is adsorbed to mica at very low lateral density, as described in the subsequent **steps 12–14**.
12. Pipet 200 µL of the dilute avidin working solution No. 2 into the fluid cell and incubate for 20 min.
13. Rinse with 1 mM NaCl (10×) and with PBS (50×). Add 300 µL PBS.
14. Keep wet and proceed to TREC measurements as described [8]. Use free biotin or free streptavidin for specific block (*see* **Note 13**).

3.4 AFM Tip or Support with PEG-Acetal for Coupling of Proteins and Small Amines

Traditionally, the preferred method for chemical activation of the tip surface is aminofunctionalization, exemplified by gas phase silanization with APTES/triethylamine (Subheading 3.1). At the same time, the most abundant reactive groups on proteins are the amino groups of the lysine residues (e.g., 80–90 on one antibody

molecule [22]). Theoretically, an NH_2-group on the tip could be linked to an NH_2-group on the protein molecule by using a PEG with an NHS ester group on each of its two ends ("NHS-PEG-NHS"). In practice, this will mainly lead to crosslinking of adjacent NH_2-groups on the tip surface, unless special precautions are taken [23].

Several strategies were developed to minimize loop formation of the crosslinker on the tip surface (*see* introduction in ref. 12). One strategy used a PEG linker with two amino-reactive groups that possess very different reactivity (aldehyde-PEG-NHS [24]). When used at high concentration, the amino groups on the tip quickly react with the NHS ester of the PEG linker, while the "slow" benzaldehyde function on the other end of PEG are spared for subsequent coupling of protein [24] or other kinds of sensor molecules [12].

The "slow" coupling kinetics of the benzaldehyde group is compensated for by two aspects: (1) Coupling of proteins is accelerated 10^3 to 10^4-fold by pre-adsorption (*see* **Note 10**). (2) In contrast to an NHS ester, the benzaldehyde group does not hydrolyze or otherwise degrade in aqueous solution. For this reason, it is possible to apply overnight reactions if the concentration of the sensor molecule is much lower than usual.

Figure 4 shows an optimized variant of this strategy [12]. Here the benzaldehyde is protected by an acetal function (acetal-PEG-NHS). In the first step, the NHS ester of the PEG linker reacts with the amino group on the tip, yielding a stable amide bond. This reaction is allowed to proceed until all accessible amino groups on the tip have been consumed. In the second step, the inert acetal group on the outer end of the PEG linker is converted into a benzaldehyde by 15 min incubation in aqueous citric acid. In the third step, the benzaldehyde reacts with an NH_2-group of the sensor molecule, yielding a moderately stable –CH=N– bond (Schiff base). $NaCNBH_3$ is included in the reaction which converts the reversible –CH=N– bond into the stable $–CH_2$-NH– bond (*see* **Note 34**).

Aldehyde-PEG-NHS and the newer acetal-PEG-NHS can be regarded as the "work horses" of tip functionalization because they enable tip functionalization with tiny amounts of virtually any protein (100 µL 1 µM protein, 0.1 nmol, e.g., 15 µg antibody, or 5× less if necessary). For AFM studies on cells, only the tip is functionalized with protein [25, 26]. For interaction studies between purified biomolecules, one component is tethered to the tip and the other component to mica [27]. The below procedure can easily be adapted for functionalization of mica when replacing the small reaction chamber by a glass crystallization dish that can hold the mica sheet. A larger amount of linker in a larger volume of chloroform will be required to cover the mica surface with the solution.

1. Aminofunctionalization of the cantilever (or mica, or silicon nitride chip) as described in Subheading 3.1.
2. Dissolve acetal-PEG-NHS (1 mg) in chloroform (0.5 mL, *see* **Notes 1**, **4**, and **5**). Transfer this solution into the reaction chamber (*see* **Notes 14** and **15**) and add triethylamine (30 μL, *see* **Notes 3**, **4**, and **5**). Quickly proceed to the next step.
3. Immerse the cantilever(s) in the reaction mixture. Cover the chamber with the lid and incubate for 2 h at room temperature.
4. Wash the cantilever(s) (3×) in glass crystallization dishes by incubation in chloroform (10 min per incubation). Dry with a stream of nitrogen or argon gas, store for up to several weeks under argon gas.
5. Immerse the cantilevers in a polystyrene Petri dish in 1% citric acid for 15 min.
6. Wash the cantilevers in pure water (3 × 5 min) in polystyrene Petri dishes, dry with a stream of nitrogen or argon, place in a dry polystyrene Petri dish, cover with the lid, and proceed to the next step.
7. Freshly prepare a 1 M solution of $NaCNBH_3$ (Subheading 2.4).
8. Place Parafilm™ with the clean side up in a polystyrene Petri dish. Press it on with the thumb of a freshly gloved hand (powder-free).
9. Place the cantilevers on the Parafilm™, with the tips pointing to the center.
10. Pipet 100 μL of the protein solution (1–2 μM, e.g., 0.15–0.3 mg/mL of an antibody, in PBS, *see* **Note 9**) on the cantilever(s) and proceed to the next step. Alternatively, amine-modified DNA (10–100 μM) or small molecules with amino groups (1–2 mM) can be coupled in a buffer containing 100 mM NaCl and 50 mM phosphate (*see* **Note 11**).
11. Add 2 μL of the 1 M $NaCNBH_3$ stock solution to the drop on the cantilever(s), mix cautiously, cover Petri dish with its lid, and incubate for 1–2 h. In the meantime, the unused remainder of the $NaCNBH_3$ stock solution is properly disposed of (*see* **Note 18**).
12. Add 5 μL of an aqueous solution of ethanolamine hydrochloride (1 M, pH 8.0, *see* **Note 19**) to the drop on the cantilever (s), mix cautiously, cover, and incubate for 10 min. In this step, unused aldehyde groups are quenched by reaction with ethanolamine.
13. Wash in PBS or any other buffer of choice (3 × 5 min) in polystyrene Petri dishes.

14. Mount cantilever in the AFM setup or store in a 24 well plate or in a polystyrene Petri dish at 4 °C under buffer that contains sodium azide (0.2–0.3 mg/mL, *see* **Note 32**) for 1–2 weeks.

3.5 AFM Tip or Support with PEG-Maleimide for Thiol Coupling

Figure 5 depicts AFM tip functionalization with maleimide-terminated PEG chains and subsequent coupling of an SH group (thiol) to the maleimide. If desired, then the same coupling scheme can also be applied on mica or silicon (nitride) chips [28–31].

Thiol-maleimide coupling has the unique advantage that it allows for site-specific (oriented) coupling of a protein or a peptide to the maleimide group. This is made possible (a) because the maleimide does not react with amino groups (under typical reaction conditions) and (b) because genetically engineered proteins or peptides can be prepared with a single cysteine residue in the desired position [32, 33]. The latter is exemplified by the fibronectin-binding peptide CGGRGDS in Fig. 5. Single-stranded DNA can be custom-synthesized with a thiohexyl group on the 5′ or 3′ end and coupled to maleimide-functionalized tips [29, 34, 35]. Glucose, as well as the glucose transport inhibitor phlorizin, have been derivatized with a thiol linker for coupling to tips with maleimide groups or similar thiol-reactive coupling functions [36]. Moreover, maleimide tips can be reacted with thiol-tris-NTA [37] to equip the tip with a His_6-tagged protein [31, 33, 38].

In the absence of EDTA, thiols are easily oxidized to disulfides by air. Custom-synthesized thiol-DNA or cysteine peptides may already contain a significant fraction of disulfide. These problems are solved by the inclusion of 1–2 mM TCEP—but only if the protein of interest does not contain an intrinsic disulfide essential for its native structure and function (*see* **Note 22**).

1. Aminofunctionalization of the cantilever (or mica, or silicon nitride chip) as described in Subheading 3.1.
2. Dissolve maleimide-PEG-NHS (1 mg) in chloroform (0.5 mL, *see* **Notes 1**, **4**, and **5**). Transfer this solution into the reaction chamber (*see* **Notes 14** and **15**) and add triethylamine (30 μL, *see* **Notes 3**, **4**, and **5**). Quickly proceed to the next step.
3. Immerse one or several aminofunctionalized cantilevers in the reaction mixture. Cover the chamber with the lid and incubate for 2 h at room temperature.
4. Wash the cantilever(s) (3×) in glass crystallization dishes by incubation in chloroform (10 min per incubation). Dry with a stream of nitrogen or argon gas, store for up to several weeks under argon gas.
5. Place Parafilm™ with the clean side up in a polystyrene Petri dish. Press it on with the thumb of a freshly gloved hand (powder-free).

6. Place the cantilever(s) on the Parafilm™, with the tips pointing to the center.
7. Prepare (thaw) 100 µL of the sample with the sensor molecule (1–2 µM thiol-protein, or 10–100 µM thiol-DNA, or 1–2 mM of a small molecule with an SH group, *see* **Note 10**), preferably in HBS containing 1–2 mM EDTA (*see* **Note 20**).
8. If the sample with the sensor molecule does not contain EDTA, then 2 µL EDTA (100 mM, pH 7.5) should be added.
9. If the sensor molecule does not contain a disulfide essential for its native structure or function, then the following additions should be made (compare Fig. 5): (a) 5 µL HEPES (nominally 1 M, pH 7.5, Subheading 2.5), (b) 2 µL TCEP hydrochloride (100 mM in water, unbuffered, freshly thawed), 2 µL HEPES (nominally 1 M, pH 9.6, Subheading 2.5).
10. Pipet the mixture with the sensor molecule on the cantilever (s), cover the Petri dish, incubate for 2–4 h.
11. Wash in PBS or any other buffer of choice (3 × 5 min) in polystyrene Petri dishes.
12. Mount cantilever in the AFM setup or store in a 24 well plate or in a polystyrene Petri dish at 4 °C under buffer that contains sodium azide (0.2–0.3 mg/mL, *see* **Note 32**) for 1–2 weeks.

3.6 AFM Tip (or Support) with Hydrazide for Coupling of Oxidized Glycoproteins or Other Aldehydes

Due to the quantitative turnover of thiol coupling to maleimides, the maleimide-functionalized tip shown in Fig. 5 lends itself to the attachment of other coupling functions. A typical example is the attachment of thiol-tris-NTA to maleimide tips which provides for stable binding of His_6-tagged proteins on a time scale of hours [31, 33, 38].

Alternatively, the "maleimide tip" can be converted into a "hydrazide tip" when using the scheme depicted in Fig. 6 (Strasser et al., manuscript in preparation). Hereby the disulfide in PDPH (**1**) is cleaved by TCEP, resulting in 3-mercaptopropionylhydrazide (**2**) which then couples to the maleimide tip on the AFM tip (**3**), or some other surface with maleimide functions. The mixture of PDPH and TCEP can directly be applied to the maleimide tip, for two reasons: (a) The low concentration of TCEP used here does not react with maleimide. (b) The released fragment 2-mercaptopyridine (not shown) tautomerizes to 2-thiopyridone [39], which does not possess a reactive thiol group.

The next step shown in Fig. 6 is the spontaneous coupling of an aldehyde group, exemplified here by the aldehyde found on a periodate-treated antibody. Aldehyde-hydrazide coupling results in a stable hydrazone linkage [40]. No fixation by $NaCNBH_3$ is necessary here, in contrast to the –CH=N– bond formed between an aldehyde and a primary amine (compare Fig. 4).

The pretreatment of the antibody (or of another glycoprotein) with periodate (*see* Fig. 6) must be performed in a separate reaction because the oxidation of the vicinal diol groups of the sialic acid residues of the antibodies gives formaldehyde as by-product. The small formaldehyde molecule could outrun the oxidized glycoprotein in the reaction with the hydrazide groups on the AFM tip; therefore, the protein must be dialyzed extensively after periodate oxidation to remove the formaldehyde.

The main advantage of hydrazide coupling of periodate-treated antibody is the site-specificity of PEG linker attachment on the antibody molecule. The location and the approximate size of the two oligosaccharides on an antibody are indicated in Fig. 8. The two oligosaccharides are located on the two F_c domains, in close proximity to the central hinge of the antibody. Attachment of the PEG linker on the oxidized oligosaccharide gives an almost symmetric arrangement where both antigen-binding sites of the two F_{ab} arms have the same distance from the site of PEG attachment (Fig. 6), in contrast to random coupling of one of the many amino groups to an aldehyde-functionalized tip (Fig. 4).

It should be noted that RNA carries a vicinal diol group on the ribose which constitutes the 3′ end. It can also be oxidized with periodate for subsequent hydrazone coupling [41]. Of course it is

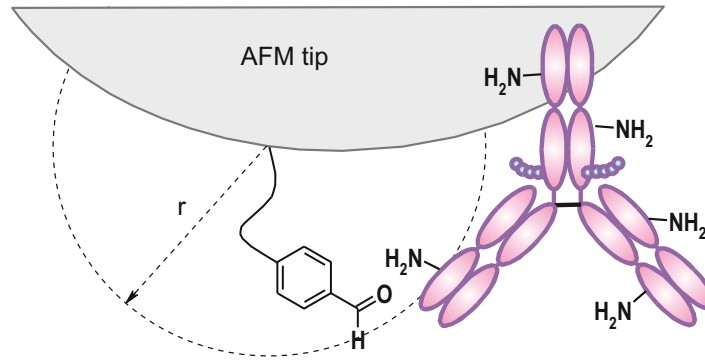

Fig. 8 Model for the acceleration of protein coupling by pre-adsorption to the tip surface. Kinetic experiments showed that efficient tip functionalization at micromolar protein concentrations can only be explained by pre-adsorption of protein before covalent coupling [5]. The outer end of the typical PEG linker can reach all points within a hemisphere with $r = 6$ nm. One adsorbed protein per hemisphere (450 nm^3) corresponds to a formal concentration of 4 mM [5]. The number of NH_2-groups per antibody is 80–90 [22]. If 10 NH_2-groups of the antibody are within reach of the PEG linker, then the formal concentration of NH_2-groups per hemisphere is 40 mM. This implies that pre-adsorption causes a more than 10^3 fold enrichment of protein concentration (from μM to mM values) and thereby greatly accelerates coupling from a time scale of weeks to minutes [5] (reproduced in with slight modification from ref. 5 with permission from the American Chemical Society)

also possible to couple any other ligand which contains a free aldehyde group.

1. Aminofunctionalization of cantilever or support (Subheading 3.1).
2. Coupling of maleimide-PEG-NHS (Subheading 3.4).
3. Dilute the glycoprotein (10–100 pmol, e.g., 2–10 μg antibody) with "buffer 5.5" to a final volume of 45 μL. Preferably, this step is already performed in the slide-a-lyzer MINI unit.
4. Mount the slide-a-lyzer Mini in the foam pad, place the foam pad on the surface of "buffer 5.5," and dialyze for 2 h at room temperature. Much longer dialysis times and several buffer changes are required if buffer components are present which can react with periodate (*see* **Note 23**).
5. Fill a 50 mL beaker with water (~25 °C, *see* **Note 35**) and place foam pad on top. The slide-a-lyzer bottom must not touch the buffer surface but should be rather close to it.
6. Add 5 μL 100 mM sodium periodate to the protein solution in the slide-a-lyzer and mix with digital pipette.
7. Cover the slide-a-lyzer tube with its lid and incubate for 1 h at room temperature in the dark (*see* **Note 36**).
8. Place the foam pad on fresh "buffer 5.5" and dialyze for 2 h. Exchange the buffer and dialyze overnight.
9. Place Parafilm™ with the clean side up in a polystyrene Petri dish. Press it on with the thumb of a freshly gloved hand (powder-free).
10. Place the cantilever(s) on the Parafilm™, with the tips pointing to the center.
11. Premix the following components in a reaction vial:
    (a) 50 μL pure water.
    (b) 2 μL EDTA (50 mM, pH 7.5).
    (c) 5 μL HEPES (500 mM, pH 7.5).
    (d) 4 μL PDPH (25 mM in DMSO) under stirring with pipet tip (*see* **Note 37**).
    (e) 4 μL TCEP hydrochloride (50 mM), mix shortly.
    (f) 4 μL HEPES (500 mM, pH 9.6) under stirring with pipet tip (*see* **Note 37**).
12. Pipet the mixture with the PDPH component onto the cantilever(s). Cover with lid and incubate for 2–4 h at room temperature.
13. Wash with water (3 × 5 min in fresh polystyrene Petri dishes) and dry with a gentle stream of nitrogen or argon gas. Proceed to the next step (*see* **Note 38**).

14. Place Parafilm™ with the clean side up in a polystyrene Petri dish. Press it on with the thumb of a freshly gloved hand (powder-free).
15. Place the cantilever(s) on the Parafilm™, with the tips pointing to the center.
16. Pipet the periodate-treated and dialyzed antibody (or other glycoprotein) onto the cantilever(s), cover with lid, and incubate for 2 h at room temperature.
17. Wash (3 × 5 min in fresh polystyrene Petri dishes) with any kind of buffer that is to be used later on, or preferred for storage.
18. Mount cantilever in the AFM setup or store in a 24 well plate or in a polystyrene Petri dish at 4 °C under buffer that contains sodium azide (0.2–0.3 mg/mL, *see* **Note 32**) for 1–2 weeks.

3.7 AFM Tip (or Support) with PEG-NH-Fmoc for Coupling of Ligands with Carboxyls

Some sensor molecules offer only a COOH group for coupling to the PEG linker. The best known example for this situation is biotin. In the special case of biotin, however, many companies offer PEG linkers where biotin is already pre-attached to the PEG linker (Fig. 3).

No comparable PEG conjugates are available for other typical ligand molecules. When we wanted to tether the carboxylic acid derivatives of aldosterone [42] or testosterone [13] to the AFM tip via a flexible PEG linker, then we had two choices: (1) synthesis of "hormone-PEG-NHS" with a structure that is analogous to "biotin-PEG-NHS" shown in Fig. 3, or (2) coupling of a PEG linker to the tip, followed by coupling of the hormone. For simplicity, the second option was chosen, as outlined in Fig. 7.

In the first step, the tip was reacted with "fmoc-PEG-NHS" (*see* **Note 25**), resulting in the usual amide bond between tip and linker. In the second step, the masked amino group on the outer end of the PEG linker was unmasked by removal of the fmoc group. Subsequently, a mixture the carboxylic acid derivative of testosterone, the activator (TSTU), and a base (DIEA) were mixed in DMF at a concentration which resulted in formation of the NHS-ester of the carboxylic group. When the tip was immersed in this solution then an amide bond was formed between the PEG and the hormone derivative.

1. Aminofunctionalization of the cantilever (or mica, or silicon nitride chip) as described in Subheading 3.1.
2. Dissolve fmoc-PEG-NHS (1 mg) in chloroform (0.5 mL, *see* **Notes 1**, **4**, and **5**). Transfer this solution into the reaction chamber (*see* **Notes 14** and **15**) and add triethylamine (30 μL, *see* **Notes 3**, **4**, and **5**). Quickly proceed to the next step.

3. Immerse one or several aminofunctionalized cantilevers in the reaction mixture. Cover the chamber with the lid and incubate for 2 h at room temperature.
4. Wash the cantilever(s) (3×) in glass crystallization dishes by incubation in chloroform (10 min per incubation). Dry with a stream of nitrogen or argon gas, store for up to several weeks under argon gas.
5. In the meantime the reaction chamber is extensively washed with chloroform and dried with a stream of nitrogen or argon gas.
6. Prepare a 4/1 mixture of DMF and piperidine in a glass crystallization dish, with a liquid level of ~5 mm (*see* **Note 39**).
7. Immerse the cantilever(s) in the DMF/piperidine mixture for 20 min at room temperature to remove the fmoc group (*see* **Note 39**).
8. Wash the tip in glass crystallization dishes by incubation in DMF (1 × 1 min) and isopropanol (4 × 1 min) and dry with a stream of nitrogen or argon gas (*see* **Note 39**). Quickly proceed to the next step.
9. The following additions are made to the reaction chamber: 30 μmol of the ligand containing a COOH group (e.g., 10.8 mg of the testosterone derivative in Fig. 7), 50 μmol TSTU (15 mg), 100 μL DMF, and 100 μmol DIEA (17.5 μL, *see* **Note 3**). The solution is mixed whereupon all components will usually dissolve (*see* **Notes 39** and **40**).
10. Immerse the cantilever(s) in this reaction mixture and incubate for 2 h at room temperature.
11. Wash the cantilever(s) by incubation in DMF (3 × 3 min) and isopropanol (3 × 1 min) and dry with a stream of nitrogen or argon gas.
12. Mount cantilever in the AFM setup or store under argon gas for 1–2 weeks.

4 Notes

1. Traditionally, many of the reactions are performed in chloroform solvent. If chloroform is not available or forbidden in some lab environment, then DMSO is a good alternative for the reaction steps (but not for the washing steps). Smaller volumes can be used because DMSO does not evaporate. Smaller volumes lead to much faster coupling kinetics if the amounts of the reagents are not scaled down with the volume. The low volatility of DMSO implies that a volatile solvent (e.g., isopropanol) must be used for the subsequent washing steps.

For the initial washing of fresh tips, chloroform can be replaced by 5 min incubation steps in a series of mixtures of ethyl acetate/ethanol (first 9/1, then 5/5, and finally 0/10).

2. Freshly purchased APTES must immediately be divided into aliquots (~0.2–0.5 mL, uncritical, can be done with a Pasteur pipette), crimp-sealed under argon, and stored at −25 °C for up to 1 year. The 11 mm crimp caps should have a silicone septum with PTFE lining, not a rubber septum which becomes hard and leaky when frozen. Once opened, the portion must be consumed or disposed of in the organic solvent waste.
3. Triethylamine is mandatory for APTES functionalization in the gas phase (Subheading 3.1). In all other reactions, it would be possible to replace triethylamine with DIEA or vice versa (e.g., in Subheading 3.6). Please note: 1 μL DIEA can be replaced by 0.80 μL triethylamine, while 1 μL triethylamine can be replaced by 1.25 μL DIEA. Triethylamine must be stored under argon in order to slow the oxidation by ambient oxygen. For this purpose, a gentle stream of argon (not nitrogen) is blown into the bottle for 2 min from a Pasteur pipette while the outlet is covered as much as possible with the screw cap. The cap is quickly mounted when the pipette is retracted. The cap is sealed with Parafilm™ or similar and the glass bottle is stored in a sealed plastic jar which is also filled with argon and sealed with Parafilm™. DIEA usually comes with a septum seal which provides easier protection against oxidation than the screw cap on triethylamine. Withdrawal of DIEA portions must be made with a gastight Hamilton syringe *while* perfusing the gas phase with a gentle stream of argon or nitrogen, using one fine hypodermic needle (mounted on the barrel of a 1 mL plastic syringe where the "ears" have been cut off to attach silicon tubing) for the inlet and another fine hypodermic needle for the outlet. The argon cushion will minimize oxidation of the amine (*N*-oxide formation). The maximal storage time for the amines is 1 year. If the amines turn yellow, they must be disposed of. Do not store in a refrigerator because this will cause condensation and absorption of moisture.
4. Gastight Hamilton™ syringes (point style 2, with cemented needle) should preferably be used for all pipetting steps of organic solvents (e.g., chloroform, DMF) and organic reagents (e.g., APTES, triethylamine, DIEA). Of course, volumes of ≥1 mL can also be pipetted with graduated glass pipets and a pipet bulb. After use, the Hamilton™ syringes must immediately be washed with isopropanol or ethanol. Initially, alcohol is pulled into the syringe and discarded (3×). Then, the piston must repeatedly be removed and reinserted, whereby the whole barrel and piston are repeatedly rinsed with alcohol from the squeeze bottle. Subsequently, the alcohol must be blown off

with a stream of nitrogen or argon gas, also from the interior of the needle by inserting the needle of the syringe barrel into the Pasteur pipette which is used as nozzle for blowing with nitrogen or argon gas. Soft plastic Pasteur pipettes (where the pipetting bulb has been cut off) are preferred because of much less danger. When using a glass Pasteur pipette, it is mandatory to always hold the pipette and not the silicone tubing with the hand, otherwise the glass pipette can become a dangerous missile when the gas pressure is high. Moreover, a glass pipette might break and glass parts can clog the syringe.

5. As an alternative to a Hamilton™ syringe, the yellow tip of a digital pipette can be connected via a 5–10 cm piece of *thin* silicone tubing to a glass capillary (disposable glass micropipette). You can use the volume adjustment of the digital pipette for relatively accurate volume delivery if you observe the following rules: (a) Adjust the desired volume on the digital pipette. (b) Pull the solution into the capillary with this adjustment. You will see that the level of liquid in the capillary is too low. (c) Slowly deliver the liquid into an empty glass vial (for subsequent disposal). (d) Go back to the reagent bottle and once again pull the desired volume (according to setting on the digital pipette) of the same reagent into the identical capillary. You will see that the level of liquid is too high (the reason is that some liquid has remained inside the capillary during the first delivery; however, the same liquid will also stay inside in the second pipetting cycle, ensuring delivery of the desired volume). (e) Slowly deliver the liquid from the capillary into the reaction mixture where it should be added. Do not rinse the capillary by repeatedly pulling reaction mixture into the capillary because then too much liquid would be delivered from the capillary.
6. It is essential to use a 5 L desiccator and argon gas for amino-functionalization in the gas phase (Subheading 3.1). The relation between the desiccator volume and the amounts of APTES plus triethylamine is critical. Argon is denser than air and stays inside the desiccator while the cover is transiently removed. Nitrogen cannot be used because is less dense than air and would immediately be replaced by air when the cover is opened.
7. The silicon chip or silicon nitride chip should be smaller than the inner dimensions of the circular AFM fluid cell. The mica sheet should be larger than the outer dimensions of the circular AFM fluid cell which is then pressed onto the mica to give a circular bath.
8. After machining, all PTFE objects are sonicated in chloroform several times, rinsed with isopropanol, dried with a stream of

nitrogen or argon gas, and stored in a dust-free place. Rinsing with isopropanol and drying is sufficient after each use. Isopropanol is preferred over ethanol because the latter can more easily react with NHS ester functions than isopropanol.

9. PBS is the buffer which affords the best coupling yields of amines with NHS esters on EGS-treated supports (Subheading 3.2) or with PEG-aldehyde groups (Subheading 3.4). HEPES buffer may cause premature hydrolysis of the NHS esters on the EGS surface before the target molecule has been coupled. HEPES should be compatible with coupling to tip-PEG-aldehyde but we never tested it. Tris buffer must be avoided because Tris base will react with NHS ester or aldehyde groups. If a protein is obtained in Tris buffer from an external source, then it must be dialyzed against PBS. The same applies to protein solutions containing imidazole, glycine, ammonium salts, or reducing agents like 2-mercaptoethanol or dithiothreitol. In all such cases, the protein must extensively be dialyzed against PBS. Some proteins (especially antibodies) are supplied with bovine serum albumin (BSA) or other protein additives. Here it is mandatory to remove BSA before coupling. One option is gel filtration on a Superdex 200 increase column (10/300 mm); the other is Melon Gel, which removes albumin and gelatin additives.
10. Proteins should be coupled to tips or supports at 1–2 μM protein concentration, when using the procedures depicted in Figs. 2, 4, 5, and 6 [5, 24]. Even at 0.2 μM protein concentration, we achieved successful coupling to the tip. In contrast, millimolar concentrations of small molecules are required to ensure efficient tip functionalization [12, 36]. The reason is that proteins weakly pre-adsorb to the tip surface next to the site of linking, causing a local concentration in the millimolar range and an acceleration of the coupling kinetics by at least three orders of magnitude, as compared to the kinetics seen in homogeneous solution (Fig. 8) [5]. Millimolar concentrations at the site of coupling are required because of the slow intrinsic coupling kinetics of the typical coupling functions [5, 43]. Possibly, a similar favorable effect of pre-adsorption may also occur with nucleic acids because ~10 to ~100 μM DNA concentrations were found to be sufficient in several studies [34, 35, 44]. Please note that addition of a detergent will abolish the pre-adsorption mechanism, and thus prevent coupling of protein on a time scale of hours. By the same rationale, the coupling of a thiol protein to a maleimide tip (Fig. 5) will be impeded if an excess of a non-thiol protein is present, which dominates the pre-adsorption to the tip. The analogous problem applies to a mixture of a glycoprotein with a large excess of a carbohydrate-free protein (Fig. 6).

11. If a small molecule with an amino group should be coupled to EGS-functionalized surface (Subheading 3.2) or to an aldehyde-functionalized surface (Subheading 3.4), then its concentration should be 1–2 mM (*see* **Note 10**). The concentration of amine-modified DNA should be 10–100 μM under the same circumstances (*see* **Note 10**). In both cases, the 5 mM phosphate content may be too low to prevent an uncontrolled pH shift (DNA has many phosphates in each molecule); therefore, it is recommended to use a buffer with 100 mM NaCl and 50 mM NaH_2PO_4, adjusted to pH 7.4 with NaOH.
12. In Subheading 3.2, the streptavidin stock solution (20 μM) and working solution (2 μM) listed in Subheading 2.2 are only required if the intention is to couple streptavidin to a flat support.
13. For specific block of the avidin–biotin interaction, the buffer in the fluid cell is either mixed with 0.05 volumes of the 10 mM biotin stock solution (*see* Subheading 2.3) or with 0.05 volumes of the 20 μM streptavidin stock solution (*see* Subheading 2.2), resulting in final concentrations of 500 μM biotin or 1 μM streptavidin. In our fluid cell the bath volume is 300 μL; therefore, we add 15 μL of the 10 mM biotin stock solution or 15 μL of the 20 μM streptavidin stock solution, depending on whether we want to block the avidin on the support or the biotin on the tip, respectively. By experience, it is easier to block the biotin on the tip with free streptavidin than the avidin on the surface with free biotin. The probable reason is the difficulty to mix the solution in the fluid cell with a digital pipette. Hereby it seems easier to bring the free streptavidin to the cantilever tip than the free biotin to the surface of the fluid cell.
14. The reaction chamber for tip functionalization is prepared by milling a cylindrical hole with a perfectly flat bottom into a PTFE block, with a diameter and a height of 12 mm. A flat PTFE disk (or square platter) with the same base area as the PTFE block is used as cover to prevent evaporation of chloroform. The reaction chamber and the cover are rigorously cleaned after machining (*see* **Note 8**) but after each use rinsing with isopropanol and drying with a stream of argon or nitrogen gas is sufficient.
15. An improvised reaction chamber can be created by cutting a 4 mL screw-cap vial at the height of 12 mm and smoothening the sharp edges with a gas flame. However, it is less convenient to use than the PTFE chamber because the bottom is not perfectly flat and the AFM cantilevers may tip over. During the reaction, this glass chamber is covered by an inverted glass

beaker (5 mL or 10 mL), which must not touch the glass chamber to avoid tipping over of the cantilevers.

16. Additional information on crosslinkers and protocols for special applications (e.g., linking of His_6-tagged proteins or click chemistry) is available from the internet at http://www.jku.at/biophysics/content.
17. The disodium salt of phosphoric acid (Na_2HPO_4, "dibasic") is dissolved in water at a concentration of 50 mM. The pH is not adjusted by addition of acid but left at its very basic value. This is necessary to deprotonate biotin, making it soluble in aqueous buffer. After mixing with biotin (10 mM), the effective pH of the solution is between 7 and 7.5. In contrast to biotin derivatives, free biotin is very susceptible to degradation under ambient conditions, with a half time of few hours [45]. No degradation is seen in frozen biotin samples. Thawed aliquots of biotin must be consumed or discarded.
18. Sodium cyanoborohydride ($NaCNBH_3$) is toxic. In acidic solution, it will release hydrogen gas and toxic HCN. Do not mix with acids! For disposal, the tiny amount of $NaCNBH_3$ (13 mg) used for one batch can be diluted with aqueous NaOH (three pellets with a total weight of ~1 g in 500 mL water) and flushed down the drain with copious amounts of water. This is safer than storing the remainders of many reactions in the lab. Avoid skin contact with $NaCNBH_3$ and inhalation of the dust! Store in a desiccator over desiccant to ensure long-term stability over many years. Exchange the desiccant ("blue gel" or "orange gel") when its color indicates that it is no longer dry (pink color or loss of orange color, respectively). Please note that "blue gel" is carcinogenic when its dust is inhaled, due to the cobalt content. "Orange gel" is less dangerous than "blue gel." Do not weigh $NaCNBH_3$ into a plastic vial because the static charge of the plastic vial may cause spreading of dust all around the vial.
19. 2.44 g ethanolamine hydrochloride is dissolved in water at a final volume of 25 mL and the pH is adjusted to 8.0 by cautious addition of 20% NaOH. Aliquots (50 µL) are frozen and stored at −25 °C. Only 5 µL is required for one batch of tip functionalization but pH adjustment may be difficult in volumes smaller than 25 mL.
20. In thiol-maleimide coupling (Fig. 5), the guidelines for the buffer composition of the sample are somewhat similar to those of amine coupling to NHS esters (Fig. 2) or to aldehydes (Fig. 4, *see* **Note 9**). Maleimides do not react with amines at 1–2 mM concentration but higher concentrations of amines (Tris buffer, glycine, ammonium salt, etc.) must be removed by dialysis against HBS containing 2 mM EDTA. Thiol

components like 2-mercaptoethanol or DTT are strictly forbidden and must rigorously be removed by extensive and repeated dialysis against HBS/EDTA buffer. In a slide-a-lyzer™ cartridge with a low filling level, 1 h of dialysis possibly lowers the concentration of the undesired component by one order of magnitude. This guess is made from the dialysis time course of chloride ions seen with a flat dialysis sack with ~1 mm layer thickness [46].

21. EDTA is essential to suppress premature oxidation of thiol groups into disulfides before the thiol has coupled to a maleimide group. EDTA binds $Fe^{2+/3+}$ ions, which are ubiquitous on the surfaces of glassware, causing complete suppression of thiol oxidation on a time scale of many hours [39].
22. **Steps 11–13** in Subheading 2.5 and **Step 9** in Subheading 3.5 are optional and sometimes even forbidden (*see* below). The sample is first mixed with a concentrated HEPES stock solution (pH 7.5) to increase the buffering capacity. Subsequently the identical volumes of a 100 mM stock solution of TCEP hydrochloride (pH ~2) and of a nominally 1 M stock solution of HEPES (pH 9.6). These additions automatically result in the desired pH range (7.5–7.7), without having to measure the pH in the small volume. TCEP causes conversion of disulfides into thiols on a time scale of seconds [47]. Fortunately, TCEP does not react with maleimide groups on a time scale of hours if the TCEP concentration is $\leq$2 mM. TCEP is forbidden to use with proteins where the native structure and function depend on intra- or inter-molecular disulfide bridges (e.g., in serum albumins or in antibodies). TCEP is mainly used in combination with custom-synthesized peptides, nucleic acids, or small molecules carrying thiol groups. Please note: The maleimide-functionalized tip must not be exposed to pH > 8, otherwise the maleimide will hydrolyze rapidly. This is one of the reasons why the sensor molecule, EDTA, TCEP, and HEPES are premixed before the mixture is placed on the cantilever(s).
23. If the glycoprotein concentration is higher than 2 μM (e.g., 0.3 mg/mL antibody), then the sample should be diluted with "buffer 5.5" to obtain a 2 μM concentration. If the original sample buffer contains trehalose, galactose, glycerol, ethylene glycol, ethanolamine, free serine, free threonine, 2-mercaptoethanol, 2-mercaptoethylamine, cysteine, or DTT (or some other reducing agent, or some other vicinal diol or vicinal amino alcohol), then the initial dialysis of the sample must be particularly extensive, with many buffer changes, in order to completely remove these components, which would seriously interfere with periodate oxidation and/or subsequent hydrazone formation.

24. The solid sodium periodate ($NaIO_4$) must be stored in a desiccator over desiccant and protected from light. For each glycoprotein oxidation reaction, a fresh stock solution of 100 mM $NaIO_4$ must be prepared.
25. In case that only fmoc-PEG-COOH (but not fmoc-PEG-NHS) is commercially available with the desired length of the PEG linker, then it can be mixed with TSTU and DIEA in DMF and reacted with aminofunctionalized tips (or supports). The procedure for this step is analogous to that described in this article for coupling of the carboxylic acid derivative of the hormone. Theoretically, fmoc-PEG-NHS could be replaced with "boc-PEG-NHS." In practice, this is not recommended because the strongly acidic conditions of boc removal may etch the tip surface or destroy a magnetic coating, especially if the solvent contains moisture.
26. Silicon nitride chips can be cut in the same way as glass chips. First, a line is scratched along a clean ruler with a clean glass cutter that has a tungsten carbide tip. Then the chip is broken along this line over a sharp edge [4].
27. A mica sheet is cut to the desired dimension with a pair of scissors and subsequently cleaved on both sides. For this purpose, a flat PTFE block (with an area somewhat larger than the mica sheet) is cleaned by extensive rinsing with isopropanol and dried with soft tissue (e.g., Kimwipe™, *see* **Note 8**). The cut mica sheet is placed on top and a strip of adhesive tape (e.g., Scotch tape™, longer than the mica sheet) is firmly pressed over the whole mica sheet. Then, one thumb is pressed onto the adhesive tape where the mica is underneath. The other hand pulls one end of the adhesive tape away from the surface while the thumb is rolled to the side to allow for lifting of the tape. A top layer of the mica sticks to the tape and a fresh mica surface is exposed. Then the remaining mica sheet on the PTFE block is turned upside down with a clean pair of tweezers and the procedure is repeated to expose a fresh, clean mica surface on the other side also. The fresh mica surface generated by the second cleavage should be used for chemical functionalization.
28. The silicon nitride tips can be pretreated with piranha or ozone to ensure oxidation of silicon nitride [4, 12]. Meanwhile, we found that the spontaneous oxidation of silicon nitride is sufficient and no additional oxidation step is necessary.
29. In the standard procedure, 30 µL APTES and 10 µL triethylamine were used for gas phase aminosilanization [4, 16,]. Meanwhile, we use 60 µL and 20 µL, respectively, because now we flush the desiccator with argon gas for 30 s through the top opening of the desiccator cover after the tips have been placed

inside. The stream of argon must not be so strong as to move the cantilevers on their tray.

30. After removal of some of the aminofunctionalized objects (cantilever, mica sheet, or silicon nitride chip) from the argon-filled desiccator, the desiccator must again be flushed with argon for 5–10 min, in order to replace all air by argon gas.
31. For functionalization of an ultra-flat support, acetal-PEG-NHS can be replaced by "SDMB" [12] which is analogous to acetal-PEG-NHS but lacks the long PEG chain (*see* **Note 16**). When following the same procedure as described for acetal-PEG-NHS by using SDMB, then proteins (or other molecules with amino groups) are firmly bound to NH_2-surfaces at high lateral density. Such immobilization via SDMB is an alternative to the EGS procedure in Subheading 3.2. EGS can crosslink adjacent amino groups on the solid surface and the NHS ester functions can hydrolyze before the coupling of a biomolecule. Both problems are absent when using SDMB; therefore, the coupling step can be extended to much longer times in order to enhance the coupling yield even at low concentrations of the target molecule.
32. Sodium azide is toxic and mutagenic. It must not be used with $pH < 7$ because this will cause protonation of the azide ion and cause release of HN_3 gas into the atmosphere which is also toxic and mutagenic.
33. Avidin and lysozyme are positively charged at neutral pH; therefore, they can be electrostatically adsorbed to mica. Please note that the NaCl concentration should be $\leq$15 mM during the adsorption process (*see* published correction to ref. 4) while the subsequent AFM experiments can be done in normal PBS or similar buffers. Other biotin-binding proteins (e.g., streptavidin or anti-biotin) do not stick to mica and must be covalently bound to mica (or silicon nitride chips), e.g., by performing the aminosilanization (Subheading 3.1) and EGS coupling (Subheading 3.2).
34. Like $NaCNBH_3$, $NaBH_4$ would also be able to convert the unstable –CH=N– bonds into stable $–CH_2–NH–$ bonds. However, $NaBH_4$ is far more reactive and (in contrast to $NaCNBH_3$) it would also convert the reactive benzaldehyde groups on the tip into unreactive benzyl alcohol groups, thereby preventing further coupling of amino groups. This is why $NaCNBH_3$ can be added to the reaction mixture at the very beginning, different from the procedure in the original publications [12, 24]. $NaCNBH_3$ will only reduce the –CH=N– bonds (with the coupled protein) into stable $–CH_2$-NH– bonds but not the important benzaldehyde

functions into benzyl alcohol. Another big advantage of $NaCNBH_3$ over $NaBH_4$ is that it does not cleave disulfide bonds into thiols [41].

35. The temperature of water in the beaker should be at least equal or slightly higher than the room temperature. This will prevent loss of water vapors from the bottom side of the dialysis membrane.
36. Much longer times than 1 h (or higher periodate concentrations) will be required if the glycoprotein does not contain a sialic acid. The terminal vicinal diol of a sialic acid residue is far more flexible and far more reactive toward periodate than a vicinal diol in a sugar ring (e.g., in the 3′-terminal ribose of RNA [41]).
37. Stirring with the pipet tip during the addition of PDPH is important to prevent precipitation of PDPH when the DMSO solvent is diluted with buffer. Stirring during addition of 1 M HEPES (pH 9.6) is important to prevent prolonged exposure of PDPH to high pH in a certain location of the sample.
38. In our workflow, it was convenient to use the hydrazide tips immediately for coupling of periodate-treated and dialyzed antibody. For this circumstantial reason, we never tested whether the hydrazide tip (**3**) can be stored under argon. Based on general chemical knowledge it is expected that the hydrazide tips (**3**) should be at least as stable as maleimide tips when stored for several weeks under argon.
39. After removal of the fmoc group, the free amino group on the PEG linker may be more susceptible to oxidation than the amino group on the APTES surface. For this reason, it is advisable (but not mandatory!) to bubble all solvents with argon gas, which come into contact with the tip while it is in this state. Due to its small volume, the reaction mixture with TSTU cannot be bubbled but, during mixing, argon gas can be blown into the reaction chamber (before immersion of the cantilevers) while slightly swirling the solution, in order to reduce the oxygen content.
40. The small volume of solvent and the high concentrations of the solid components were chosen in order to ensure tip functionalization within 2 h. The conditions are analogous to those for coupling of a small NHS ester molecule to PEG-amines in homogeneous solution. If the reaction is performed at lower concentrations of the components (lower amounts of solid and/or higher volume of solvent), then overnight incubation should be used for coupling of the NHS ester. If little of the ligand is available, then at least the concentrations of TSTU and DIEA should be similar as described here (in that case, free

N-hydroxysuccinimide should be added, e.g., 20–30 μmol, corresponding to 2.3–3.5 mg). Some ligands with COOH groups may be poorly soluble in DMF, even after addition of DIEA. A typical example is biotin. In this case, it helps to stir the covered solution with a clean magnetic stir bar. Hereby the COOH group will be converted into the NHS ester and thereby become much more soluble in DMF. Another alternative is the replacement of DMF by DMSO.

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References

1. Barattin R, Voyer N (2008) Chemical modifications of AFM tips for the study of molecular recognition events. Chem Commun 2008:1513–1532
2. Ebner A, Wildling L, Zhu R, Rankl C, Haselgrübler T, Hinterdorfer P, Gruber HJ (2008) Functionalization of probe tips and supports for single-molecule recognition force microscopy. In: Samori B (ed) STM and AFM studies on (bio)molecular systems: unraveling the Nanoworld. Springer, Berlin, pp 29–76
3. Hinterdorfer P, Baumgartner W, Gruber HJ, Schilcher K, Schindler H (1996) Detection and localization of individual antibody-antigen recognition events by atomic force microscopy. Proc Natl Acad Sci U S A 93:3477–3481
4. Riener CK, Stroh CM, Ebner A, Klampfl C, Gall AA, Romanin C, Lyubchenko YL, Hinterdorfer P, Gruber HJ (2003) Simple test system for single molecule recognition force microscopy. Anal Chim Acta 479:59–75
5. Kamruzzahan ASM, Ebner A, Wildling L, Kienberger F, Riener CK, Hahn CD, Pollheimer PD, Winklehner P, Hölzl M, Lackner B, Schörkl DM, Hinterdorfer P, Gruber HJ (2006) Antibody linking to atomic force microscope tips via disulfide bond formation. Bioconjug Chem 17:1473–1481
6. Stroh CM, Ebner A, Geretschläger M, Freudenthaler G, Kienberger F, Kamruzzahan ASM, Smith-Gill SJ, Gruber HJ, Hinterdorfer P (2004) Simultaneous topography and recognition imaging using force microscopy. Biophys J 87:1981–1990
7. Stroh C, Wang H, Bash R, Ashcroft B, Nelson J, Gruber H, Lohr D, Lindsay SM, Hinterdorfer P (2004) Single-molecule recognition imaging-microscopy. Proc Natl Acad Sci U S A 101:12503–12507
8. Ebner A, Kienberger F, Kada G, Stroh CM, Geretschläger M, Kamruzzahan ASM, Wildling L, Johnson WT, Ashcroft B, Nelson J, Lindsay SM, Gruber HJ, Hinterdorfer P (2005) Localization of single avidin-biotin interactions using simultaneous topography and molecular recognition imaging. ChemPhysChem 6:897–900
9. Chen G, Ning X, Park B, Boons G-J, Xu B (2009) Simple, clickable protocol for atomic force microscopy tip modification and its application for trace ricin detection by recognition imaging. Langmuir 25:2860–2864
10. Hukkanen E, Wieland J, Gewirth A, Leckband D, Braatz RD (2005) Multiple-bond kinetics from single-molecule pulling experiments: evidence for multiple NCAM bonds. Biophys J 89:3434–3445
11. Drew ME, Chworos A, Oroudjev E, Hansma H, Yamakoshi Y (2010) A tripod molecular tip for single molecule ligand-receptor force spectroscopy by AFM. Langmuir 26:7117–7125
12. Wildling L, Unterauer B, Zhu R, Rupprecht A, Haselgrübler T, Rankl C, Ebner A, Vater D, Pollheimer P, Pohl EE, Hinterdorfer P, Gruber HJ (2011) Linking of sensor molecules with amino groups to amino-functionalized AFM tips. Bioconjug Chem 22:1239–1248

13. Rangl M, Leitner M, Riihimäki T, Lehtonen S, Hytönen VP, Gruber HJ, Kulomaa M, Hinterdorfer P, Ebner A (2014) Investigating the binding behaviour of two avidin-based testosterone binders using molecular recognition force spectroscopy. J Mol Recognit 27:92–97
14. Ebner A, Hinterdorfer P, Gruber HJ (2007) Comparison of different aminofunctionalization strategies for attachment of single antibodies to AFM cantilevers. Ultramicroscopy 107:922–927
15. Shlyakhtenko LS, Potaman VN, Sinden RR, Gall AA, Lyubchenko YL (2000) Structure and dynamics of three-way DNA junctions: atomic force microscopy studies. Nucleic Acids Res 28:3472–3477
16. Wang H, Bash R, Yodh JG, Hager GL, Lohr D, Lindsay SM (2002) Glutaraldehyde modified mica: a new surface for atomic force microscopy of chromatin. Biophys J 83:3619–3625
17. Baumgartner W, Hinterdorfer P, Ness W, Raab A, Vestweber D, Schindler H, Drenckhahn D (2000) Cadherin interaction probed by atomic force microscopy. Proc Natl Acad Sci U S A 97:4005–4010
18. Klein DG, Stroh CM, Jensenius H, van Es M, Kamruzzahan ASM, Stamouli A, Gruber HJ, Oosterkamp TH, Hinterdorfer P (2003) Covalent immobilization of single proteins on mica for molecular recognition force microscopy. ChemPhysChem 4:1367–1371
19. Köhler M, Karner A, Leitner M, Hytönen VP, Kulomaa M, Hinterdorfer P, Ebner A (2014) pH-dependent deformations of the energy landscape of avidin-like proteins investigated by single molecule force spectroscopy. Molecules 19:12531–12546
20. Kamruzzahan ASM, Kienberger F, Stroh CM, Berg J, Huss R, Ebner A, Zhu R, Rankl C, Gruber HJ, Hinterdorfer P (2004) Imaging morphological details and pathological differences of red blood cells using tapping mode AFM. Biol Chem 385:955–960
21. Preiner J, Losilla NS, Ebner A, Annibale P, Biscarini F, Garcia R, Hinterdorfer P (2008) Imaging and detection of single molecule recognition events on organic semiconductor surfaces. Nano Lett 9:571–575
22. Dorner MM, Bassett EW, Beiser SM, Kabat EA, Tanenbaum SW (1967) Studies on human antibodies: V. Amino acid composition of antidextrans of the same and of different specificities from several individuals. J Exp Med 125:823–831
23. Ratto TV, Langry KC, Rudd RE, Balhorn RL, Allen MJ, McElfresh MW (2004) Force spectroscopy of the double-tethered concanavalin-A mannose bond. Biophys J 86:2430–2437
24. Ebner A, Wildling L, Kamruzzahan ASM, Rankl C, Wruss J, Hahn CD, Hölzl M, Kienberger F, Blaas D, Hinterdorfer P, Gruber HJ (2007) A new, simple method for linking of antibodies to atomic force microscopy tips. Bioconjug Chem 18:1176–1184
25. Francius G, Alsteens D, Dupres V, Lebeer S, De Keersmaecker S, Vanderleyden J, Gruber HJ, Dufrene YF (2009) Stretching polysaccharides on live cells using single molecule force spectroscopy. Nat Protoc 4:939–946
26. Lower BH, Yongsunthon R, Shi L, Wildling L, Gruber HJ, Wigginton NS, Reardon CL, Pinchuk GE, Droubay TC, Boily J-F, Lower SK (2009) Antibody-recognition force microscopy shows that outer membrane cytochromes OmcA and MtrC are expressed on the exterior surface of Shewanella oneidensis MR-1. Appl Environ Microbiol 75:2931–2935
27. Rankl C, Kienberger F, Wildling L, Wruss J, Gruber HJ, Blaas D, Hinterdorfer P (2008) Multiple receptors involved in human rhinovirus attachment to live cells. Proc Natl Acad Sci U S A 105:17778–17783
28. Fuhrmann A, Ros R (2010) Single-molecule force spectroscopy: a method for quantitative analysis of ligand–receptor interactions. Nanomedicine 5:657–666
29. Hegner M, Grange W, Bertoncini P (2004) Measurement of single molecular interactions by dynamic force microscopy. Methods Mol Biol 242:369–381
30. Lv Z, Roychaudhuri R, Condron MM, Teplow DB, Lyubchenko YL (2013) Mechanism of amyloid beta-protein dimerization determined using single-molecule AFM force spectroscopy. Sci Rep 3:2880
31. Rangl M, Ebner A, Yamada J, Rankl C, Tampé R, Gruber HJ, Rexach M, Hinterdorfer P (2013) Single-molecule analysis of the recognition forces underlying nucleo-cytoplasmic transport. Angew Chem Int Ed Engl 52:10356–10359
32. Nimmervoll B, Chtcheglova LA, Juhasz K, Cremades N, Aprile FA, Sonnleitner A, Hinterdorfer P, Vigh L, Preiner J, Balogi Z (2015) Cell surface localised Hsp70 is a cancer specific regulator of clathrin-independent endocytosis. FEBS Lett 589:2747–2753
33. Oh YJ, Hubauer-Brenner M, Gruber HJ, Cui Y, Traxler L, Siligan C, Park S, Hinterdorfer P (2016) Curli mediate bacterial adhesion to fibronectin via tensile multiple bonds. Sci Rep 6:33909

34. Lin L, Wang H, Liu Y, Yan H, Lindsay S (2006) Recognition imaging with a DNA aptamer. Biophys J 90:4236–4238
35. Strunz T, Oroszlan K, Schafer R, Güntherodt HJ (1999) Dynamic force spectroscopy of single DNA molecules. Proc Natl Acad Sci U S A 96:11277–11282
36. Neundlinger I, Puntheeranurak T, Wildling L, Rankl C, Wang L-X, Gruber HJ, Kinne RKH, Hinterdorfer P (2014) Forces and dynamics of glucose and inhibitor binding to sodium glucose co-transporter SGLT1 studied by single molecule force spectroscopy. J Biol Chem 289:21673–21683
37. Wruss J, Pollheimer PD, Meindl I, Reichel A, Schulze K, Schöfberger W, Piehler J, Tampé R, Blaas D, Gruber HJ (2009) Conformation of receptor adopted upon interaction with virus revealed by site-specific fluorescence quenchers and FRET analysis. J Am Chem Soc 131:5478–5482
38. Posch S, Aponte-Santamaría C, Schwarzl R, Karner A, Radtke M, Gräter F, Obser T, König G, Brehm MA, Gruber HJ, Netz RR, Baldauf C, Schneppenheim R, Tampé R, Hinterdorfer P (2017) Mutual a domain interactions in the force sensing protein von Willebrand factor. J Struct Biol 197:57
39. Riener CK, Kada G, Gruber HJ (2002) Quick measurement of protein sulfhydryls with Ellman's reagent and with 4,4′-dithiodipyridine. Anal Bioanal Chem 373:266–276
40. Sander EG, Jencks WP (1968) Equilibria for additions to carbonyl group. J Am Chem Soc 90:6154–6162
41. Proudnikov D, Mirzabekov A (1996) Chemical methods of DNA and RNA fluorescent labeling. Nucleic Acids Res 24:4535–4542
42. Wildling L, Hinterdorfer P, Kusche-Vihrog K, Treffner Y, Oberleithner H (2009) Aldosterone receptor sites on plasma membrane of human vascular endothelium detected by a mechanical nanosensor. Pflugers Arch 458:223–230
43. Hölzl M, Tinazli A, Leitner C, Hahn CD, Lackner B, Tampé R, Gruber HJ (2007) Protein-resistant self-assembled monolayers on gold with latent aldehyde functions. Langmuir 23:5571–5577
44. Krasnoslobodtsev AV, Shlyakhtenko LS, Lyubchenko YL (2006) Probing interactions within the synaptic DNA-SfiI complex by AFM force spectroscopy. J Mol Biol 365:1407–1416
45. Ebner A, Marek M, Kaiser K, Kada G, Hahn CD, Lackner B, Gruber HJ (2008) Application of biotin-4-fluorescein in homogeneous fluorescence assays for avidin, streptavidin, and biotin or biotin derivatives. Methods Mol Biol 418:73–88
46. Gruber H, Wilmsen H, Schurga A, Pilger A, Schindler H (1995) Measurement of intravesicular volumes by salt entrapment. Biochim Biophys Acta 1240:266–276
47. Burns JA, Butler JC, Moran J, Whitesides GM (1991) Selective reduction of disulfides by tris (2-carboxyethyl)phosphine. J Org Chem 56:2648–2650
48. Bozna BL, Polzella P, Rankl C, Zhu R, Salio M, Shepherd D, Duman M, Cerundolo V, Hinterdorfer P (2011) Binding strength and dynamics of invariant natural killer cell T cell receptor/CD1d-glycosphingolipid interaction on living cells by single molecule force spectroscopy. J Biol Chem 268:15973–15979