General information on the functionalization of atomic force microscopy (AFM) tips via long, flexible polyethylene glycol (PEG) chains

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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. Insertion of a long, flexible polyethylene glycol (PEG) chain (**Figure 1**) between the tip surface and the sensor molecule proved highly advantageous, for several reasons: (i) The sensor molecule can reorient rapidly and "palpitate" the sample surface, thus specific binding to cognate target molecules is greatly facilitated. (ii) In force spectroscopy experiments, specific binding is much easier to discriminate from non-specific tip-surface adhesion when using a 6-9 nm long PEG linker between tip and sensor molecule [Riener et al., 2003a and 2003b; Kamruzzahan et al., 2006]. (iii) Rapid simultaneous scanning of Topography and RECognition sites (TREC) is intrinsically dependent on the use of 6-9 nm long PEG linkers [Ebner et al., 2005].

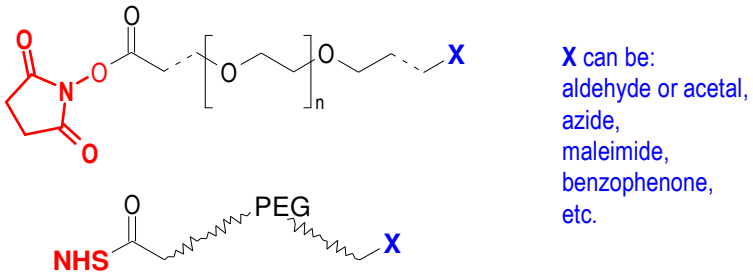

Figure 1: Typical structure of polyethylene glycol (PEG) linkers used for AFM tip functionalization. The linker contains a long PEG chain (n = 18 or 27) and one so-called NHS ester function ("NHS" stands for *N*-hydroxysuccinimide) which reacts with an amino group (NH_2) on the AFM tip to give a stable amide bond (see **Figure 2**). The other end of the PEG chain carries a different functional group (**X**) which serves to couple a sensor molecule to the AFM tip (see **Figure 2**). The dashed lines indicate minor variations in the linkages between the PEG chain and the terminal groups.

The most widely used procedure for AFM tip functionalization via flexible PEG linkers is shown in **Figure 2**. It employs three steps: (1) Generation of amino group (NH_2) on the tip surface, (2) reaction of these amino groups with one end of the PEG linker only, and (3) attachment of the sensor molecule to the free-tangling end of the PEG chain.

For step 2 in **Figure 2** it is essential that the reactive group **X** on the second end of the PEG linker does not react with the amino groups on the tip surface. This rule was only partially fulfilled with the outdated linker "Aldehyde-PEG-NHS" (see manual *AFM_tip_with_aldehyde*) [Ebner et al., 2007]. The latter problem was solved by replacing the aldehyde function with an acetal function which can be converted into an aldehyde function after PEG linker attachment to the tip surface (see **Figure 3** and the manual *AFM_tip_with_acetal*) [Wildling et al., 2011].

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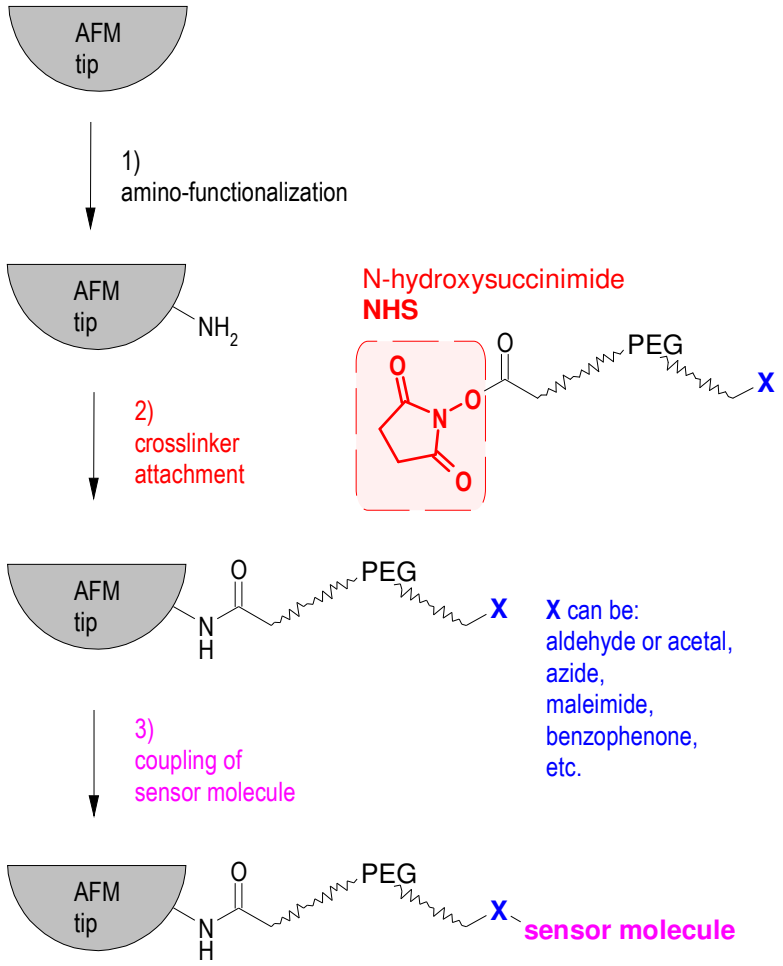

Figure 2: Three-step procedure of AFM tip functionalization with long, flexible PEG linkers: (1) amino-functionalization of the oxidized silicon nitride tip, (2) attachment of one end of the PEG linker by amide bond formation, (3) attachment of the sensor molecule to the free end of the PEG linker.

Figure 3 (see next page) shows an optimized procedure which is the new "work horse" for coupling of proteins but also of small molecules like ATP to AFM tips (both aspects are exemplified in Zhu et al. [2013]). It is applicable to all commercially available AFM tips (made from silicon or silicon nitride), also to the new generation of MAC levers. The citric acid treatment was not applicable to the old-style MAC levers [Wildling et al., 2011] but this statement in the original publication is outdated. At present, all commercially available silicon nitride (and silicon) cantilevers can be functionalized by the acetal linker procedure in **Figure 3**.

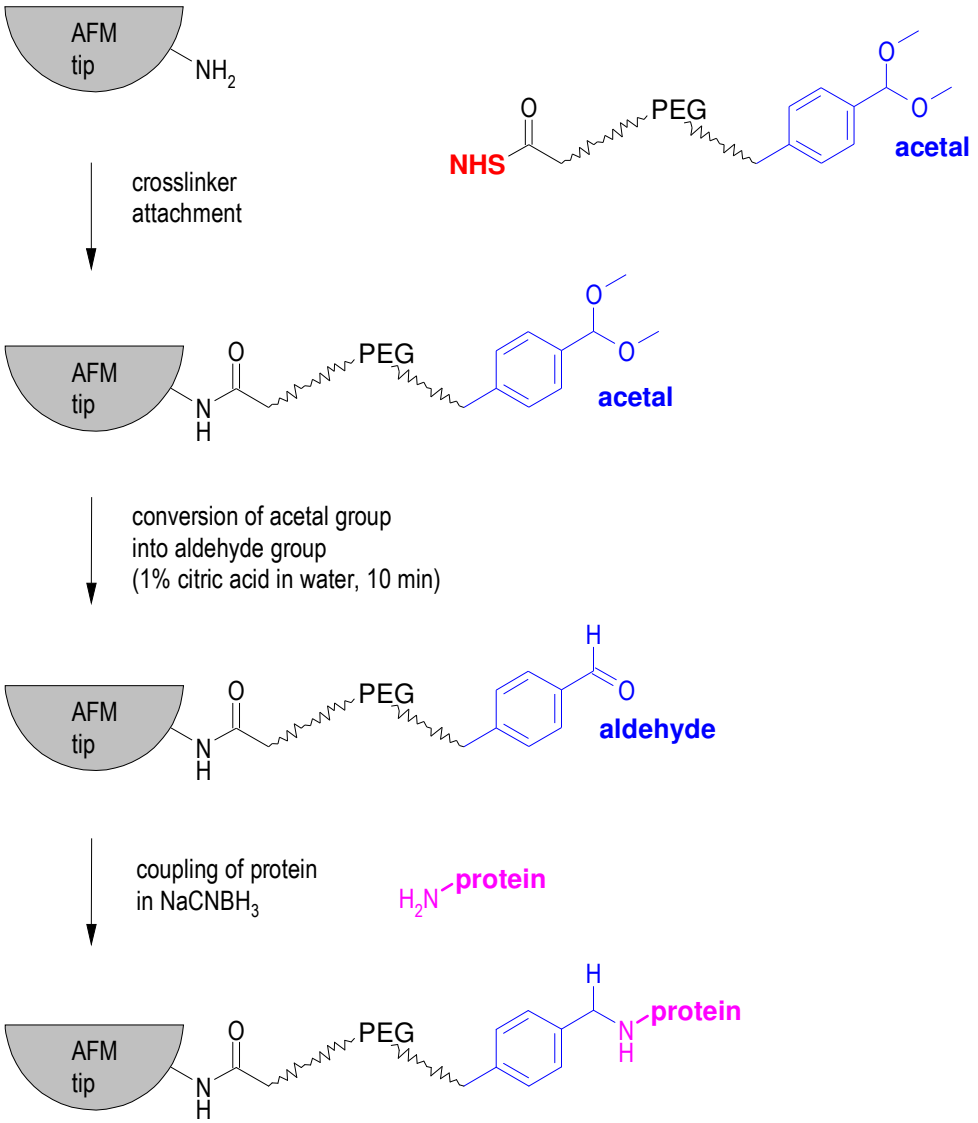

Figure 3: New standard method for protein coupling to AFM tips. The crosslinker molecule has one NHS ester function which is highly reactive towards the amino group (NH_2) on the tip surface, while the **acetal** group on the other end is unreactive. Subsequently, the acetal is converted into an **aldehyde** which couples NH_2 groups, such as the lysine residues of proteins.

Aldehyde functions are particularly useful for coupling of proteins because aldehydes react with amino groups (-NH_2) and most proteins have a high number of lysine residues (NH_2) on their surface (e.g., 80-90 per antibody [Dorner et al., 1967]). The disadvantage of aldehyde coupling is that one of the many amino groups is randomly chosen for coupling. In contrast, site-specific coupling is achieved by linking of cysteine mutants to AFM tips with maleimide groups (see **Figure 4**), or binding of His_6-tagged proteins to tips carrying tris-NTA functions (see **Figure 5**), or coupling of oxidized glycoproteins to hydrazide tips (see **Figure 6**).

In contrast to amino groups (NH_2), thiol groups (SH) possess a unique reactivity for maleimide groups (see **Figure 4**). This is fortunate because free SH groups (unpaired cysteines) are absent in most extracellular proteins and not very abundant in intracellular proteins. Often it is also possible to prepare cysteine-less mutants and then to introduce a new cysteine at a specific site of the protein of interest. Consequently, the protein can be coupled to the AFM tip with uniform geometry, using the addition of the SH group to the maleimide on the AFM tip.

Maleimide coupling is also ideal for AFM tip functionalization with DNA [Lin et al., 2006; Zhu et al., 2010] or with thiol derivatives of small molecules like glucose [Neundlinger et al., 2014; Puntheeranurak et al., 2006 and 2007] or serotonine [Wildling et al., 2012]. Details are found in the manual "*AFM_tip_with_maleimide*"

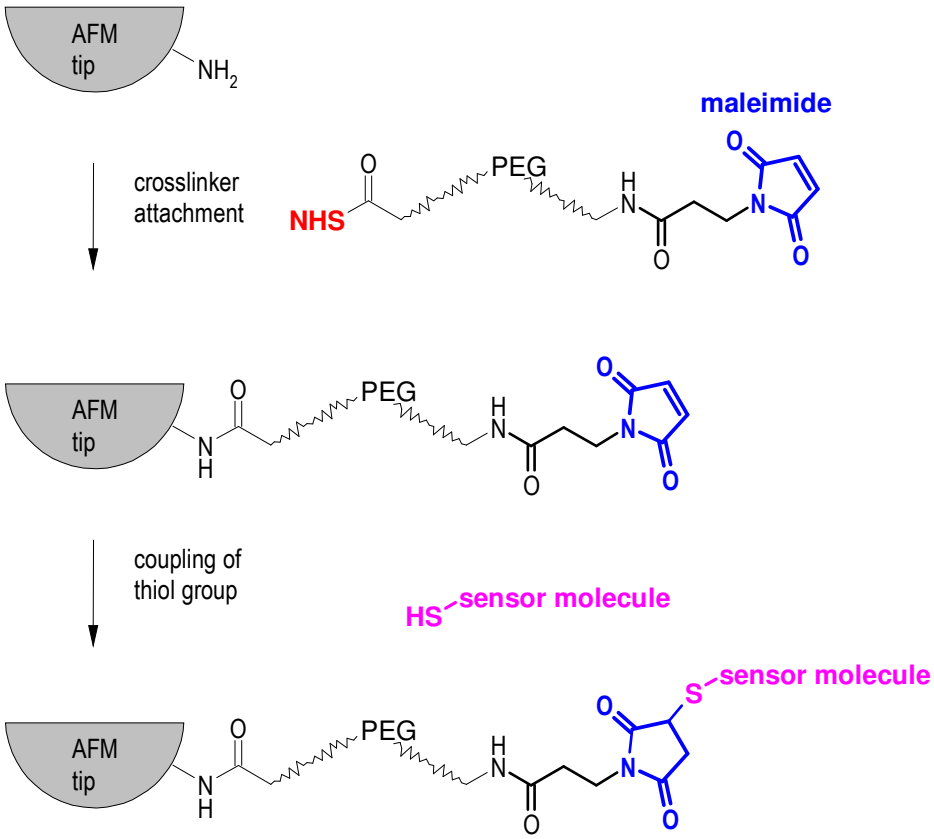

Figure 4: Reaction of amino-functionalized AFM tips with Maleimide-PEG-NHS and subsequent coupling of a sensor molecule with a thiol group (SH).

Genetically engineered proteins are commonly fused with a hexahistidine tag (His_6, i.e., a peptide with six histidine residues) on the N- or C-terminus. After over-expression in bacteria or in eucaryotic cells, the protein-of-interest can be purified from the cell extract in a single chromatographic step, using gels with immobilized Ni^{2+} ions. The same technique has been adapted for the atomic force microscope. For optimal binding, an adaptor with three immobilized Ni^{2+} ions (the so-called tris-NTA) is linked to the AFM tip by thiol-maleimide

coupling (**Figure 5**). The tris-NTA module was invented by Prof. Robert Tampé and Prof. Jacob Piehler [Lata & Piehler, 2005; Piehler et al., 2008].

Tris-NTA functions proved very useful for coupling of His_6-tagged proteins to the AFM tip (**Figure 5**). No covalent coupling is required, the tip is simply loaded with Ni^{2+} ions and then incubated in a dilute solution of the His_6-tagged protein [Rangl et al., 2013], as described in the manual "*AFM_tip_with_trisNTA*".

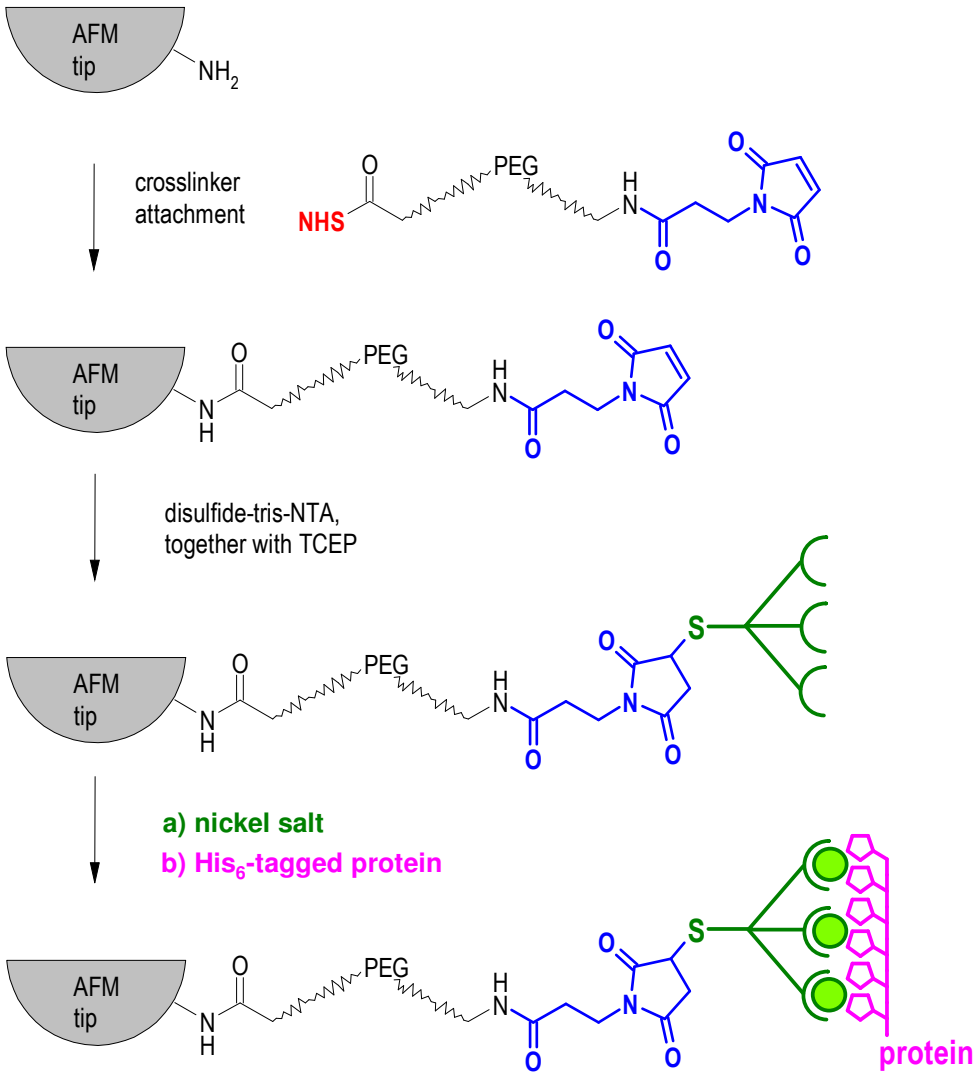

Figure 5: AFM tip functionalization with tris-NTA groups which are loaded with Ni^{2+} ions for subsequent binding of His_6-tagged proteins.

Glycoproteins usually contain sialic acid residues which possess so-called vicinal diols (two adjacent OH groups). Such vicinal diols can easily be cleaved by reaction with sodium periodate ($NaIO_4$), resulting in an aldehyde function which possesses high reactivity for hydrazide functions. The method is very useful for the coupling of antibodies to AFM tips with uniform, defined geometry (**Figure 6**). Antibodies carry two small oligosaccharides next

to the central hinge region. Periodate treatment generates aldehyde groups in the oligosaccharides which provide for coupling to a hydrazide-functionalized AFM tip (**Figure 6**). In an older version of the procedure, we first reacted the oxidized antibody with the small hydrazide module (PDPH, **Figure 6**) and subsequently coupled it to a thiol-reactive AFM tip (see *Figure 5* in the manual "*simple_introduction*") [Zhu et al., 2010]. Meanwhile, we developed the simpler method shown in **Figure 6**. The methodical details of **Figure 6** are described in the manual "*AFM_tip_with_hydrazide*".

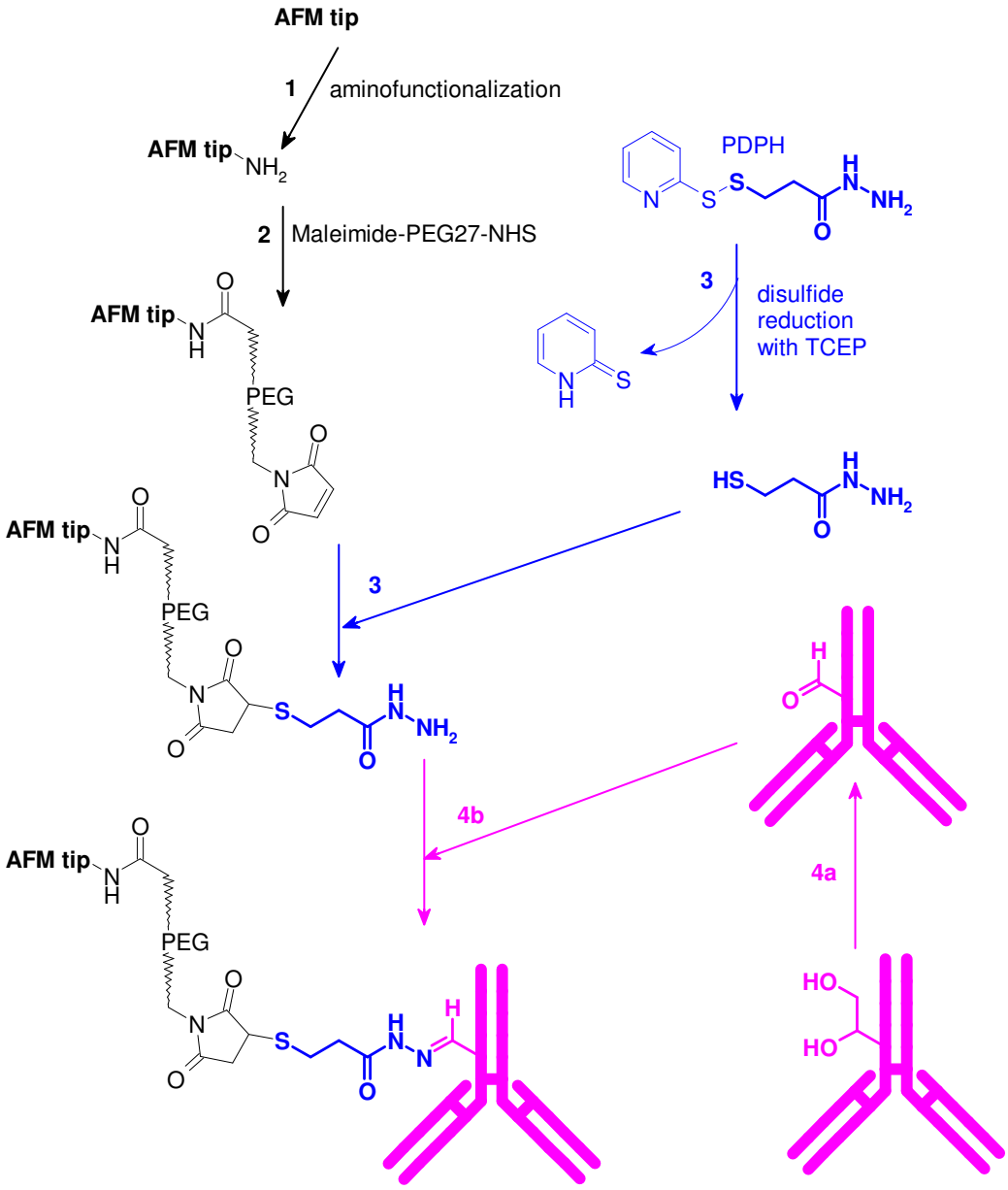

Figure 6: Four-step protocol for AFM tip functionalization with periodate-treated antibodies. (1) Aminofunctionalization with APTES or ethanolamine hydrochloride. (2) Coupling of Maleimide-PEG27-NHS. (3) The one-pot reaction with PDPH and TCEP extends the maleimide group with a hydrazide function. (4) Coupling of periodate-treated **and** rigorously dialyzed antibody via stable hydrazone linkage.

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In 2001, the term "click chemistry" was first coined by K. B. Sharpless for "spring-loaded reactions" which spontaneously occur with high specificity and high efficiency in unproblematic solvents, such as water [Kolb et al., 2001]. The most prominent example of click chemistry is the cycloaddition reaction between an azide and an alkyne (C≡C bonds), as shown in **Figure 7**.

In the last decade, this reaction has become very popular in bioconjugation, labeling, and immobilization, therefore we have incorporated this method in our repertoire of AFM tip functionalization [Zhu et al., 2016] (**Figure 7**). The major advantage is that synthetic ligands can easily be equipped with alkyne groups and reacted with the azide-tip, without getting into conflict with the chemical functions of the ligand molecule.

Details of the method are found in the manual "*AFM_tip_click_chemistry*".

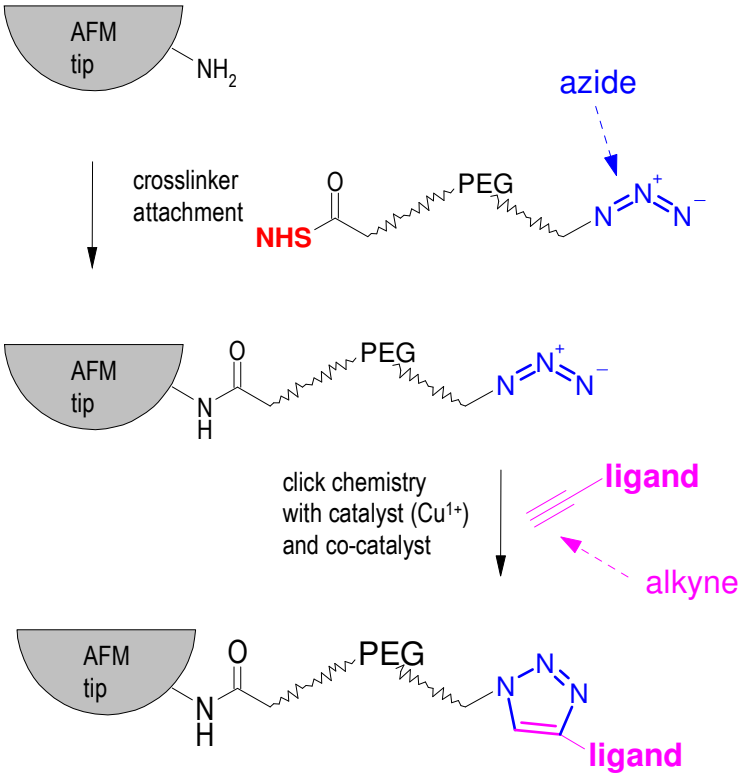

Figure 7: Reaction of amino-functionalized AFM tips with Azide-PEG-NHS and subsequent coupling of an alkyne-derivatized ligand via click chemistry. Please, note that coupling is performed with the help of a catalyst (Cu^{2+} and ascorbate) and accelerated by a co-catalyst.

For the training of beginners [Riener et al., 2003a], as well as for testing of new AFM modes [Ebner et al., 2005] and for characterization of new (strept)avidin mutants or constructs [Rangl et al., 2014], we developed a simple test system in which the sensor molecule (**biotin**) is already part of the PEG linker (see **Figure 8**).

The sample surface with the complementary "receptor molecules" is easily prepared by incubating mica in 15 mM NaCl with avidin (0.1 mg/mL, 15 min) which results in a dense monolayer of avidin, as needed in force spectroscopy experiments [Riener et al., 2003a].

Shorter times and lower concentrations of avidin lead to a low density of avidin molecules, as needed for TREC experiments [Ebner et al., 2005]. The AFM experiments can be performed in 150 mM NaCl, only the adsorption process of avidin must be performed at low NaCl concentration. The details of the method are found in the manual "*AFM_tip_with_biotin*".

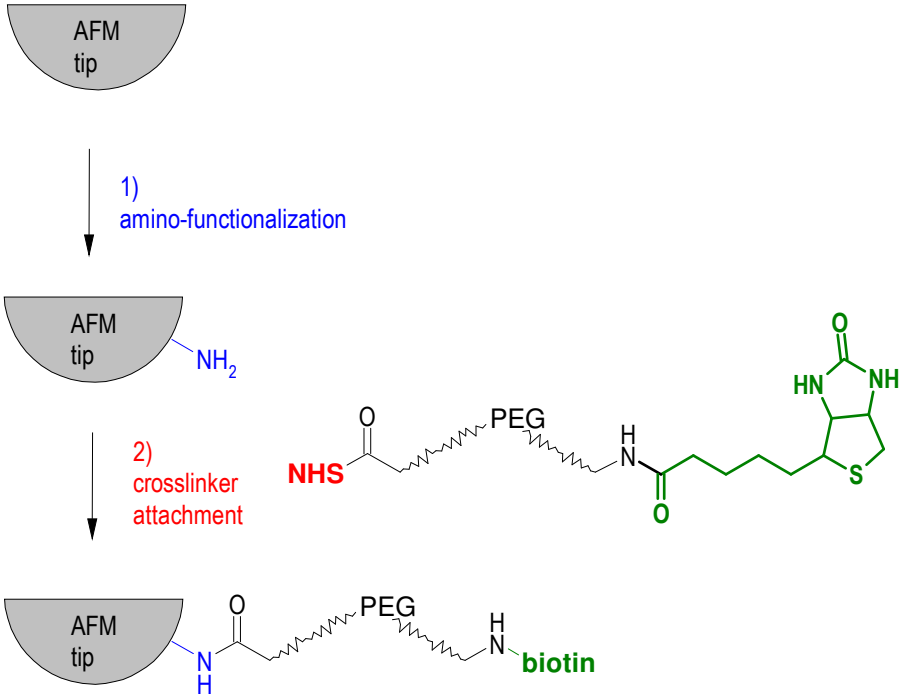

Figure 8: Reaction of amino-functionalized AFM tips with Biotin-PEG-NHS results in tips with flexibly linked biotin that has a high affinity for immobilized avidin or streptavidin.

Force spectroscopy studies of interacting biomolecules require that the "sensor molecule" (e.g., an antibody) is attached to the AFM tip, while the complementary target molecule (e.g., an antigen) is immobilized on an ultra-flat support, such as mica or a silicon (nitride) chip.

Occasionally, the target molecule can be immobilized on mica by simple adsorption [Riener et al., 2003a and 2003b]. In most studies cited here, the target molecules were immobilized by the same strategies as used for AFM tip functionalization (compare **Figure 9A** with **Figure 2**).

Sometimes we used a very short crosslinker (EGS, [Rangl et al., 2014]) for the immobilization of target molecules on aminosilanized mica. The disadvantage of EGS is that it has two NHS ester functions, therefore it can form many loops between adjacent amino groups on the solid surface.

Figure 9B offers a new coupling scheme which is closely analogous to the acetal linker scheme (Figure 3), except that here we use a very short acetal linker ("SDMB") which lacks the long PEG chain used for AFM tip functionalization. The linker works very well [Posch, S., et al., manuscript in preparation].

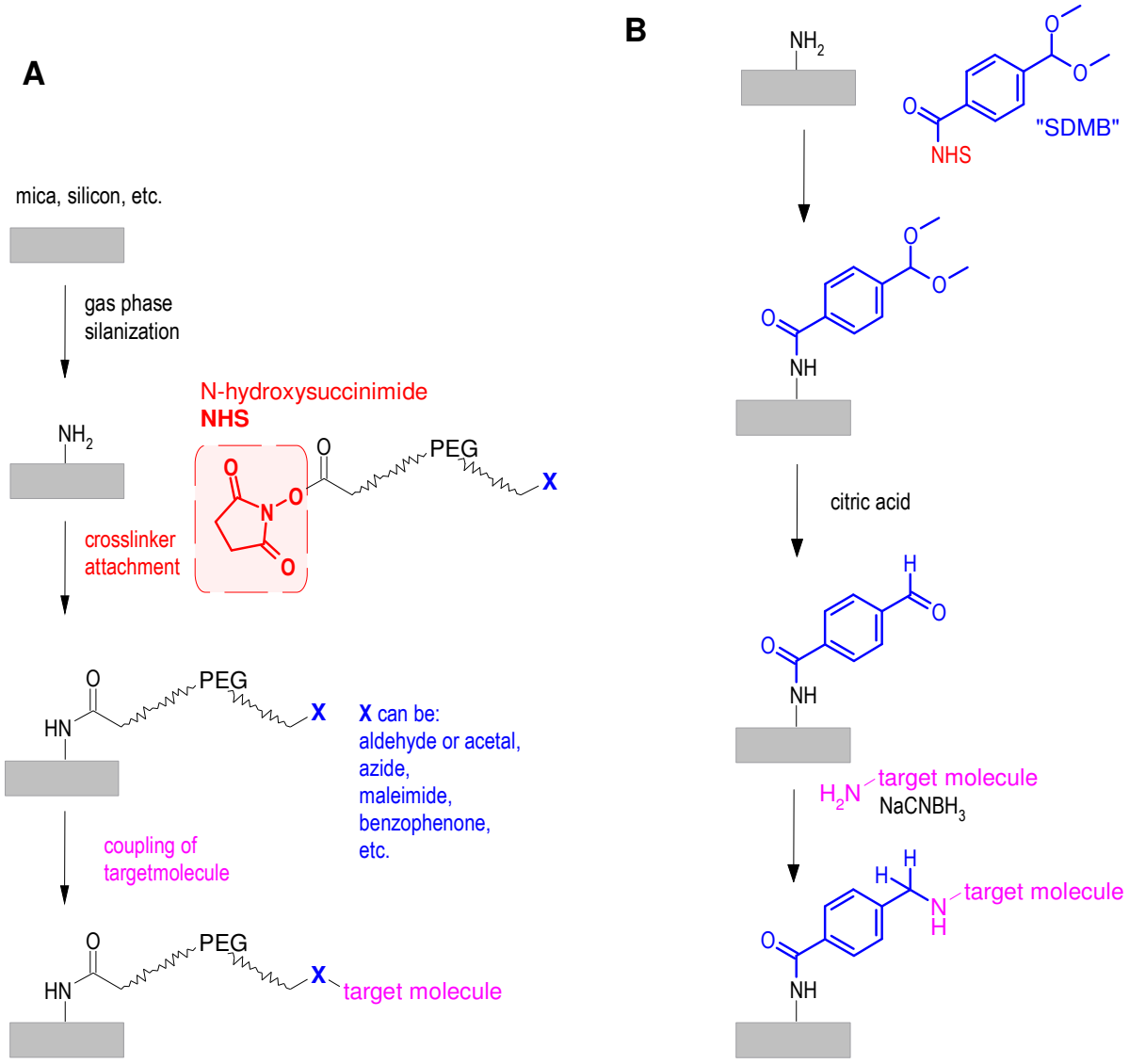

Figure 9: Covalent immobilization of target molecules on ultra-flat supports like mica or silicon (nitride) chips for characterization of the mechanism by which they interact with a sensor molecule on the AFM tip. **A**: The methods described for AFM tip functionalization via PEG chains (**Figures 2-8**) can equally be used for flexible tethering of target molecules to an ultra-flat support. **B**: Alternatively, the target molecule can be immobilized by the short linker SDMB which is chemically analogous to the long linker Acetal-PEG-NHS (**Figure 3**).

It is not enough to use the proper chemistry for coupling of sensor molecules to AFM tips. Equally important is efficient coupling on a time scale of 1-2 hours, even at low protein concentrations (0.1-1 µM).

The coupling reaction shown in **Figure 10** [Kamruzzahan et al., 2006] gave us the opportunity to stage the reaction in a spectrophotometer and to monitor the reaction rate by release of a colored by-product (the initially released HS-pyridine tautomerizes into 2-thiopyridone, ε_{343} = 8080 M^{-1} cm^{-1}). In the spectrophotometer the reaction occurs by statistical collisions between diffusing molecules and here the kinetic is so slow that extrapolation of the same mechanism for the AFM tip indicates coupling times of one month at 1 µM protein concentration [Kamruzzahan et al., 2006]!

The contradiction between slow coupling in solution and fast coupling on the AFM tip was reconciled by assuming weak pre-adsorption of the protein to the AFM tip surface. One adsorbed antibody that "sits" next to a crosslinker has a formal concentration of 3 mM within the 6 nm hemisphere where the crosslinker can diffuse (**Figure 10**). Obviously pre-adsorption enhances the local concentration from 0.1-1µM to 3mM, thereby accelerating the coupling process by a factor of 10^3 to 10^4, as compared to reaction in homogeneous solution.

Kinetic experiments on sticky surfaces [Hahn et al., 2007] *versus* protein-resistant surfaces [Hölzl et al., 2007] showed that the same hypothesis also applies to aldehyde coupling of proteins. The hypothesis has important consequences for AFM tip chemistry (see manual "*acceleration_of_coupling_by_preadsorption*").

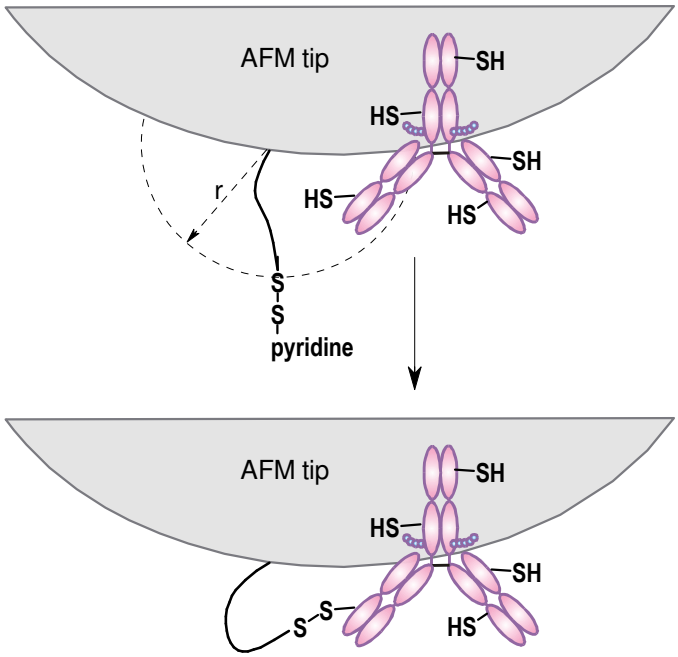

Figure 10: Hypothesis for acceleration of antibody linking to tip-bound pyridyl-S-S-PEG tentacles, due to pre-adsorption of protein next to the PEG linker. The free end of a 6 nm long, surface-bound PEG linker can reach all locations in a hemisphere with r = 6 nm. One particle per hemisphere (450 nm^3) corresponds to a formal concentration of 3 mM [Kamruzzahan et al., 2006]. Tip radius, antibody dimensions, and PEG length were roughly drawn to scale.

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