Flexible attachment of antibodies and other sensor molecules to the measuring tips of force microscopes

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Summary:

Force microscopes can image single molecules by mechanical scanning of a surface with a sharp tip. At Johannes Kepler University (Linz, Austria, Europe) we synthesized flexible chain molecules by which antibodies (and other biomolecules) can be tethered to the tip surface like a dog on a leash. When the antibody finds an antigen on a sample surface, it pulls on the leash and the antigen can be localized with nanometer precision on the sample surface.

The working principle of a force microscope is similar to that of a vinyl record player (Figure 1). A cantilever with a sharp tip scans over the surface. Individual molecules (shown as green oval shapes) are detected as "mountains" by the deflection of the cantilever arm. The deflection is magnified by a laser beam. The computer reconstructs the topographic image from the deflections of the laser beam.

Figure 1: Schematic of a force microscope.
The left side of Figure 2 shows the conventional use of force microscopes for recording of topographic images. The cantilever with the tip is moved over the sample surface, preferably with a small oscillation which helps to minimize the contact force.

The right side of Figure 2 depicts a microscope tip which has been upgraded into a specific sensor by flexible attachment of an antibody molecule. The tip is still able to record topographic images, as shown on the lower left. Bright spots correspond to individual protein molecules on a flat surface. – At the same time a "recognition image" is recorded. Here the dark spots indicate antigens which are recognized by the tip-linked antibody.

Figure 2: Recording of topographic images (left) and of recognition images (right) in a force microscope.

Key publication:
Long, flexible linker molecules have been synthesized from polyethylene glycol (PEG) chains of defined length (Figure 3). The extended chain has a similar length as one arm of an antibody molecule. All linkers have one NHS ester function (see Figure 3) which rapidly couples to amino groups on the tip surface (see Figure 4). The second end of the PEG chain does not react with the tip surface, therefore it sits on the free-tangling end of the PEG chain.

Attachment of Biotin-PEG-NHS to the tip results in a surface-bound chain with a terminal biotin. Its use is explained in Figure 4.

The linker Maleimide-PEG-NHS (product of Polypure, Oslo, Norway) allows to couple SH-containing molecules to the tip surface, as explained in Figure 5. The method is ideal for DNA and for genetically engineered proteins. It can also be used to introduce other coupling functions, e.g. for His6-tagged proteins or for glycoproteins.

The linker Acetal-PEG-NHS is the "workhorse" for easy attachment of antibodies and a large number of other biomolecules to measuring tips (see Figure 6). It couples lysine residues which are abundant on the surface of most proteins (e.g. 80-90 per antibody).

The linker Azide-PEG-NHS provides for linking of ligands containing a C≡C triple bond.

Figure 3: Linker molecules for flexible attachment of sensor molecules to measuring tips. Linkers with $n = 18$ have a slightly different structure than shown here (see manual "general_overview").
Biotin-PEG-NHS is used for model experiments and for characterization of new engineered (strept)avidin constructs (Figure 4). Hereby, biotin serves as substitute for an antibody. Biotin has a very high affinity for avidin, a protein from egg white. Avidin can easily be immobilized on freshly cleaved mica by electrostatic adsorption. The tip with the biotin-PEG chain is then used to detect avidin molecules on the mica surface and to measure the binding forces between avidin and biotin.

Model experiments with tip-linked biotin and mica-bound avidin are essential for beginners who learn "tip chemistry" and recognition force microscopy. Similar experiments are useful when exploring new measuring modes in the force microscope.

**Figure 4:** Flexible attachment of vitamin H (= biotin) to the measuring tip.

**Key publications:**


The linker Maleimide-PEG-NHS is first reacted with an amino group on the tip, yielding a stable amide bond (Figure 5). Hereby, maleimide does not react with NH$_2$, ensuring unilateral attachment of the PEG chain. In the second step, a biomolecule with a free SH group is coupled to the maleimide on the outer end of the PEG chain.

Antibodies do not contain free SH groups. For a special study (Figure 7) we introduced an SH group on the short carbohydrate chain in the center of the antibody, thereby ensuring equal pulling forces on both lower arms of the antibody. The method is also ideal for coupling of DNA and of small molecules containing SH groups.

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**Figure 5:** Flexible attachment of SH-containing biomolecules to measuring tips.

**Key publications:**


The most general method for protein attachment to AFM tips is shown in Figure 6. The NHS ester function of Acetal-PEG-NHS reacts with the amino group on the tip surface, yielding an amide bond. Hereby it is important that all amino groups on the tip surface are consumed by such amide bond formation.

In the second step, the tip is immersed in 1% citric acid for 10 min, resulting in the conversion of all acetal groups into aromatic aldehyde groups. In the third step, a lysine residue (-NH$_2$) of a protein reacts with the aldehyde on the free-tangling end of the PEG chain. Usually NaCNBH$_3$ is included for conversion of the initially formed C=N bond into a stable CH-NH bond.

Figure 5: Flexible attachment of proteins to measuring tips.

Key publications:


**Figure 7** summarizes an advanced study where an antibody against 5-methylcytosine was used to measure the distances between adjacent methylcytosines on single-stranded DNA (ssDNA) chains. The DNA was unilaterally coupled to a mica surface and the antibody was linked to the tip in a symmetric way (see **Figure 5**).

In the force microscope, the antibody is allowed to bind to two methylcytosine residues on one DNA strand (stage 1 in **Figure 7**). Then the tip is retracted from the mica surface. This causes stretching of the DNA between the mica surface and one arm of the antibody (stage 2). Between stage 2 and 3 the stressed arm of the antibody releases its methylcytosine. Further retraction of the tip causes stretching of the DNA segment between mica and the second arm of the antibody (stage 3), until the second arm is also ripped off the DNA (stage 4).

![Diagram of antibody binding and stretching](image)

**Figure 7**: Measurement of the distances between adjacent methylcytosine residues on DNA.

Figure 8 summarizes a study where the linker Acetal-PEG-NHS was used for flexible attachment of ATP to the measuring tip. This tip was used to characterize ATP binding to both sides of the so-called uncoupling protein 1 (UCP1) in the inner membranes of mitochondria. Binding of ATP from the cytoplasmic side (c-side) causes deep insertion and block of proton flux through the channel, while binding from the matrix side (m-side) does not.

![Figure 8](image)

**Figure 8:** ATP binding to the cytosolic side (c-side) of mitochondrial uncoupling protein causes block of H⁺ flow, while ATP binding to the matrix side (m-side) does not.