

# Functionalization of AFM tips with Maleimide Linkers

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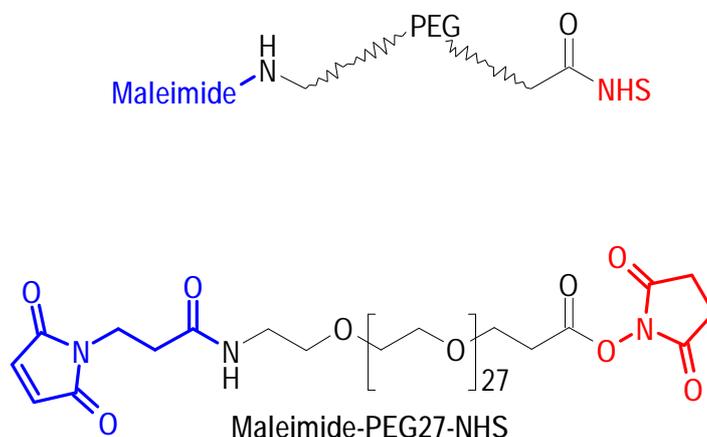
**AFM tips with Maleimide**  
**short version**  
**for risks and details see full length procedure**

1. Aminofunctionalization of the cantilever(s) (see [02\\_AFM\\_tip\\_aminofunctionalization](#)).
2. Dissolve 1 portion of Maleimide-PEG-NHS (1 mg) in chloroform (0.5 mL), transfer the solution into the reaction chamber, add triethylamine (30  $\mu$ L) and mix.
3. Immediately place cantilever(s) in the reaction chamber, cover the chamber and incubate for 2 h.
4. Wash with chloroform ( $3 \times 10$  min), dry with nitrogen gas. Continue with next step.
5. Place cantilever(s) on Parafilm in a polystyrene Petri dish.
6. Pre-mix the following components:
  - 100  $\mu$ L thiol component (e.g., 1  $\mu$ M protein, 10-100  $\mu$ M oligonucleotide, or 1-4 mM of a small molecule) in any kind of buffer (not containing amines or 2-mercaptoethanol or dithiothreitol or dithioerythrol or glutathione)
  - 2  $\mu$ L EDTA (100 mM, pH 7.5)
  - 5  $\mu$ L Hepes (1 M, pH 7.5)
  - 2  $\mu$ L TCEP hydrochloride (100 mM)
  - 2  $\mu$ L Hepes (1 M, pH 9.6) – or more if the thiol component is in a strongly acidic solution
7. Pipet the mixture onto the cantilever(s), cover with lid, incubate for 2-4 h
8. Wash in PBS or any other buffer of choice ( $3 \times 5$  min)
9. Mount cantilever in AFM setup (or store in a 24 well plate under PBS at 4°C for 1-2 weeks)

## Functionalization of AFM tips with Maleimide Linkers

Please, read the preceding manual [01\\_AFM\\_tip\\_functionalization\\_in\\_general](#) for the basic concept of AFM tip functionalization with long flexible PEG linkers.

Please, read the manual [02\\_AFM\\_tip\\_aminofunctionalization](#) for the details on how amino groups are generated on the AFM tip surface.



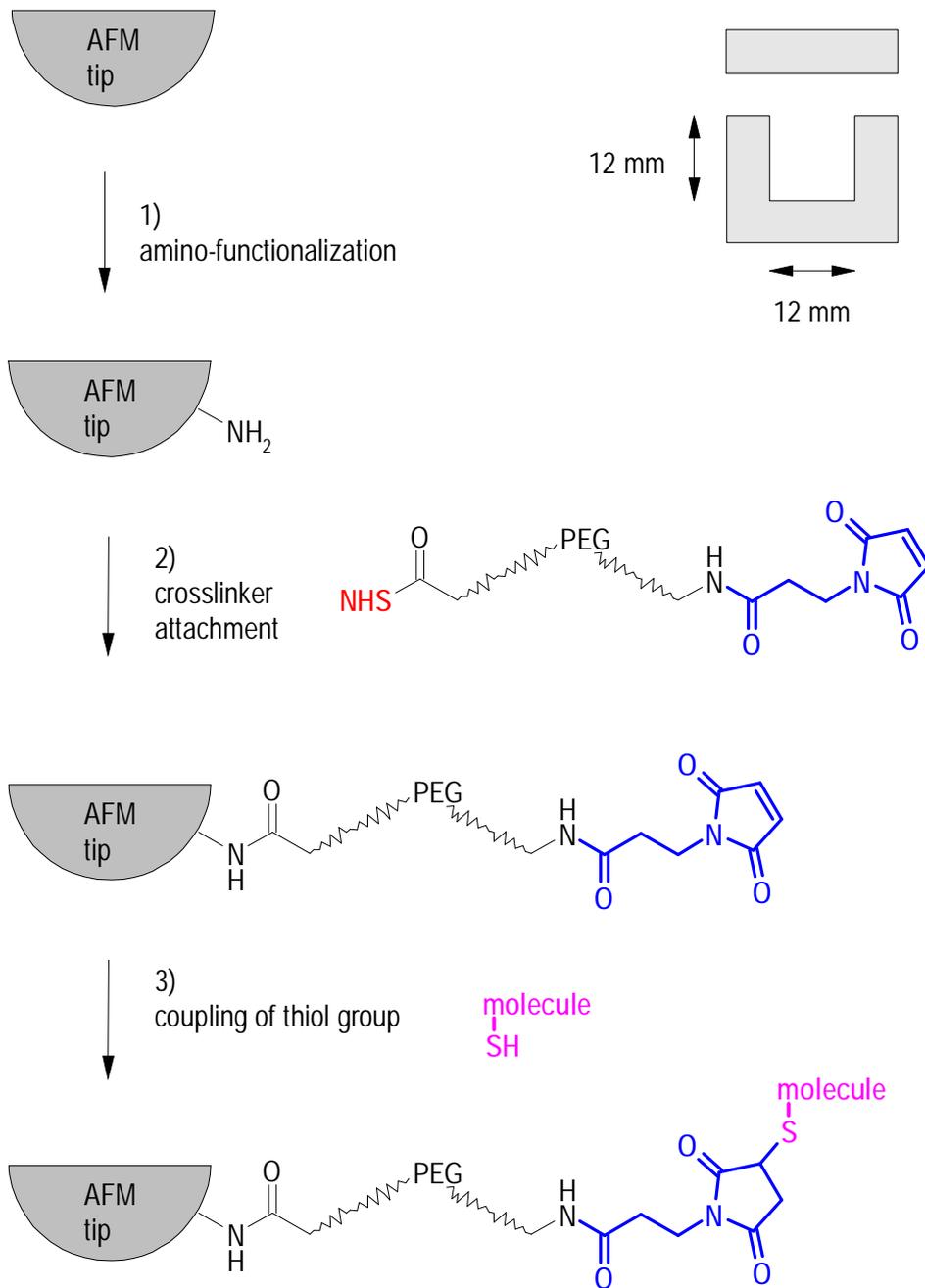
**Figure 1:** Structure of the Maleimide-PEG27-NHS which is used to tether thiol groups (e.g., proteins with cysteines) to amino-functionalized AFM tips (see Figure 2).

As depicted in Figure 2, the first step of tip functionalization is the generation of amino groups on the tip surface, the second step is the reaction of the amino groups with the NHS ester function of the PEG linker (Maleimide-PEG-NHS), and the third step is coupling of the sensor molecule.

The disadvantage of the maleimide linker is that the sensor molecule must have a free thiol group (SH) which is absent in most extracellular proteins, such as antibodies.

Coupling of thiols to maleimides has significant advantages:

- Protein mutants with one free cysteine can be coupled to maleimides in a site-specific way.
- In contrast to disulfide coupling [Kamruzzahan et al. (2006) *Bioconjugate Chem.* 17, 1473-1481], coupling to maleimides is much faster and not reversed by excess of thiol ligand in the solution.
- Low micromolar concentrations of thiol-modified oligonucleotides can be coupled to maleimide-functionalized tips in an overnight reaction.<sup>2</sup>
- Coupling can be performed in presence of TCEP which converts disulfides (= oxidized thiols) into reactive thiol functions.<sup>3</sup> This is possible because TCEP does not react with maleimide, in contrast to other reducing agents, such as 2-mercaptoethanol or DTT which are not compatible with maleimide coupling.



**Figure 2:** Reaction of amino-functionalized AFM tips with Maleimide-PEG-NHS and subsequent coupling of a molecule with a thiol group (SH).

The experimental details for the **first step** are described in the manual [02\\_AFM\\_tip\\_aminofunctionalization](#).

**For the second step**, the contents of one crimp-sealed vial of Maleimide-PEG-NHS (1 mg) is dissolved in chloroform (0.5 mL) and the solution is transferred into a tiny reaction chamber. Such a chamber can be prepared by drilling a circular hole with a perfectly flat bottom into a

small Teflon block (see sketch in Figure 2). Alternatively, a graduated 10 mL measuring cylinder can be cut between the 1 and 2 mL marks and smoothed with a hot flame. Triethylamine (30  $\mu$ L) is added and mixed into the solution. The amino-functionalized tips are immersed in this solution and the reaction chamber is covered (with a suitable Teflon disk, or an inverted small glass beaker, or with aluminum foil) to minimize evaporation of chloroform and triethylamine. After two hours the tips are washed in chloroform (3  $\times$  10 min incubation) and dried with nitrogen gas. **The maleimide-functionalized cantilevers have never been stored in our laboratory but always used for the next step within ~1 hour.**

**Before the third step.** stock solutions of EDTA (100 mM, pH 7.4), TCEP hydrochloride (100 mM) and Hepes (1 M, pH 7.5 and pH 9.6) are prepared, as described in the list of Materials (see below). Small aliquots can be stored at -20°C and thawed for daily use.

**For the third step.** a piece of Parafilm is pressed onto the inner surface of a polystyrene Petri dish and several cantilevers are placed onto the Parafilm in a radial arrangement, with the tips in the center and facing upwards. The tipped ends must be so close together that all of them can be wetted simultaneously when adding one 100  $\mu$ l droplet of aqueous solution. This addition, however, is only done after sample mixing (see next paragraph).

Then, 100  $\mu$ L of aqueous sample with the thiol component is pipetted into a reaction vial (1.5 mL or 2 mL) or a polystyrene tube (4 – 5 mL). Parameters such as thiol concentration, buffer components, and reaction times are addressed in the **Notes** (see below). 2  $\mu$ L 100 mM EDTA (pH 7.5) is added and the sample is mixed with a 100  $\mu$ L digital pipette. 5  $\mu$ L of 1 M Hepes (pH 7.5) is added and the sample is mixed. This step introduces high buffering capacity, as needed for the next steps. 2  $\mu$ L of 100 mM TCEP hydrochloride is added and the sample is mixed. Hereby the pH drops from 7.5 to 7.2. Finally, 2  $\mu$ L of 1 M Hepes (pH 9.6) is added. Hereby the pH is raised to about 7.6.

The sample mixture is transferred onto the cantilever tips, the Petri dish is covered with the lid and the reaction is allowed to proceed at room temperature for 2 h (or longer, see **Notes**). Subsequently, the cantilevers are washed in PBS (3  $\times$  5 min) and stored in a 24 well plate under PBS at 4°C for up to 1-2 weeks.

#### **Notes:**

- The concentration necessary for reaction of all maleimide functions on the tip can vary **from 1  $\mu$ M to 4 mM**. If the thiol does not pre-adsorb to the tip [Kamruzzahan et al. (2006) *Bioconjugate Chem.* 17, 1473-1481] then 4 mM thiol component is required to ensure completion of the reaction within 1 hour [Linda Wildling, Ph.D. thesis at Johannes Kepler University Linz]. If the thiol component is a protein, then 1  $\mu$ M or even less will be sufficient to ensure completion of tip functionalization within 1 hour [Kamruzzahan et al. (2006) *Bioconjugate Chem.* 17, 1473-1481]. In case of oligonucleotides, completion of the reaction was reportedly achieved by overnight coupling at 25  $\mu$ M oligonucleotides concentration,<sup>2</sup> even at pH 6.5 where coupling is about 10  $\times$  slower than at pH 7.5. If you don't know the proper conditions, use a high concentration of thiol. If the thiol component is expensive or comes in a dilute sample, use 10-100  $\mu$ M and react for long times under argon.
- If a thiol protein (with a free cysteine) is to be coupled, then no other protein should be present, even if the other protein has no thiol function. If abundant, then the second

protein will dominate in the pre-adsorption process and the thiol protein has little chance to undergo the desired coupling reaction [Kamruzzahan et al. (2006) *Bioconjugate Chem.* 17, 1473-1481].

- The concentration of TCEP in the above reaction mixture is 2 mM which is sufficient to reduce 2 mM disulfide into 4 mM thiol component. If higher thiol concentrations than 4 mM are used and if all of the thiol is suspected to be oxidized, only then more than 2  $\mu$ L of 100 mM TCEP hydrochloride should be applied. In any case, the volumes of 100 mM TCEP hydrochloride (pH ~ 2) and of 1 M Hepes (pH 9.6) **must be upscaled by the same factor** to ensure the proper pH value in the reaction mixture.
- The order of the pipetting steps is critical and must not be changed. For instance, if TCEP is mixed with Hepes (pH 9.6) in absence of sample, then TCEP may quickly oxidize and become inactive. The sequence of pipetting steps has been optimized to minimize the adverse effect of air on TCEP and on the thiol component!
- EDTA is necessary (and sufficient) to prevent re-oxidation during the coupling procedure on a time scale of few hours. For longer reaction times it is advantageous to react under argon atmosphere, in addition.
- Buffers with high concentrations of primary amines (Tris, glycine) are unfavorable for maleimide linkers because the maleimide can also react with primary or secondary amines at high concentrations. Dialysis of the protein sample for a few hours solves this problem because residual concentrations of amines are not too critical.
- The maleimide linker method is not applicable if the protein sample contains reducing agents (such 2-mercaptoethanol or dithiothreitol). In this case, the protein sample (50-100  $\mu$ L) must be dialyzed, preferably with a "Side-A-Lyzer MINI Dialysis Device" from Pierce, with a molecular weight cut-off (MWCO) of 10K or 20K. Dialysis must be very rigorous (against 500 mL buffer for ~36 h, with several buffer changes in between).
- The maleimide linker method is incompatible with protein samples containing ammonium bicarbonate or ammonium acetate. In these cases, rigorous dialysis (as described in the previous paragraph) is required to remove the ammonium ions.
- It is not a problem if the thiol component is oxidized into a disulfide (e.g., during dialysis) because TCEP is added during coupling. TCEP converts thiols into disulfides within seconds. At the same time TCEP does not react with maleimides (except at much higher concentrations than used here). Consequently, a slight excess of TCEP over the thiol component ensures complete reduction without adverse side effects.
- Pipetting of chloroform and triethylamine should be performed with glass pipettes and a pipette bulb (or with a graduated glass capillary in case of triethylamine) and not with the plastic tips of digital pipettes because the plastic tips may release polymeric material in chloroform and triethylamine. – Never use your mouth for pipetting, especially not with such toxic liquids as chloroform and triethylamine.
- The ideal tools for accurate and easy pipetting of chloroform and triethylamine are gas-tight Hamilton syringes. A 500  $\mu$ L syringe is suggested for chloroform (part no. 81230, RN-type) and a 50  $\mu$ L syringe for triethylamine (part no. 80230, RN-type).

Two removable needles with a blunt ends (part no. 7780-02) need to be purchased in addition.

- Immediately after pipetting, these syringes should be washed free of chloroform or triethylamine. Use nitrile or rubber gloves (not latex) for the cleaning procedure. First, isopropanol (same as 2-propanol) is pulled into the syringe and disposed into the solvent waste (3 ×). Then, the piston is removed and carefully rinsed with isopropanol. The barrel filled with isopropanol several times from a squeeze bottle and the contents poured into the solvent waste. Finally, the barrel is again filled with isopropanol and the piston is inserted to push the contents into the solvent waste bottle. This step is repeated twice. Subsequently, the syringe must be dried by one of two alternative methods:
  - Diethyl ether can be pulled into the syringe and pushed into the solvent waste. Then the piston is moved up and down (15-20 ×).
  - The outsides of the barrel and the piston are dried with soft paper tissue or with a stream of gas. The barrel interior is dried with nitrogen gas from a Pasteur pipette which is inserted into the barrel. Then, the needle is inserted into the tip of the Pasteur pipette until the glass base of the syringe needle softly touches the tip of the Pasteur pipette. Now the gas flow is forced through the needle which causes evaporation of isopropanol from the interior of the needle (takes about 1 min).
- Be careful when using Pasteur pipettes which are connected to a nitrogen gas tank via silicon tubing. **Make sure that the needle valve is closed when you open the main valve of the gas tank! Slowly open the needle valve! Always hold (or fix) the Pasteur pipette and not the silicon tubing.** In this way, only the soft tubing may jump off the pipette if the nitrogen flow is opened too quickly. In the opposite case, the Pasteur pipette may become a dangerous weapon hurting yourself or your colleague.
- In spite of the small quantities of chloroform and triethylamine, the reactions should be performed in a well ventilated hood.
- Strictly avoid contact of the organic solvents and of triethylamine with your skin. Latex gloves cannot protect you for more than 1-2 seconds. Nitrile gloves provide slightly longer protection. In case of splashing the gloves must be immediately removed from the hands.

## Materials

100 mM EDTA is prepared by weighing 931 mg of disodium EDTA dihydrate (EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$ ) into a beaker (25 mL) and adding water to a final volume of ~20 mL. A stir bar is added and the suspension is stirred while dropwise adding NaOH (first 20%, towards the end 2%) to adjust a pH of 7.4. The solution is transferred into a volumetric flask (25 mL) or a graduated cylinder and water is added to give a final volume of 25 mL. Aliquots of different size (2 mL, 200  $\mu\text{L}$ , 20  $\mu\text{L}$ ) are stored at  $-20^\circ\text{C}$  for up to several years.

1 M HEPES (pH 7.5) is prepared by dissolving HEPES (free acid) in water at a concentration of 238.3 g/L. As an example, 11.915 g HEPES is weighed into a 50 mL graduated cylinder

and water is added to give a final volume of 50 mL. The suspension is mixed until the solid is completely dissolved. Alternatively, a 1 M stock solution of Hepes acid can also be purchased, e.g., for Sigma-Aldrich. Subsequently, the 1 M stock solution is transferred to a beaker and stirred while adding 20% NaOH (5 M) until the pH reaches the final value 7.5. At this point, the true Hepes concentration will be lower than 1 M but this fact is ignored. Half of the total solution is immediately used for preparation of 1 M Hepes (pH 9.6, see next paragraph) while afterwards the remainder of 1 M Hepes (pH 7.5) is divided into aliquots of different size (2 mL, 200  $\mu$ L, and 20  $\mu$ L) and stored at -20°C for up to several years.

1 M Hepes (pH 9.6) is prepared from half of the unused solution of 1 M Hepes (pH 7.5, see previous paragraph) by continuing the addition of 20% NaOH until pH 9.6 has been reached. Aliquots of different size (2 mL, 200  $\mu$ L, and 20  $\mu$ L) are stored at -20°C for up to several years.

100 mM TCEP hydrochloride is prepared by weighing about 100 mg into a beaker and adding water to give a final concentration of 28.7 mg/mL. If the weight of the solid is exactly 100 mg then 3.47 mL of water is added. Aliquots of different size are prepared (e.g. 1 mL, 100  $\mu$ L, and 10  $\mu$ L) and stored at -20°C for up to several years. The high stability of TCEP in this stock solution is due to the low pH (~2) which prevents oxidation of TCEP in presence of air.

#### Literature describing maleimide-functionalized AFM tips:

1. Lin, L., Wang, H., Liu, Y., Yan, H., and Lindsay, S. (2006) Recognition Imaging with a DNA Aptamer. *Proc. Natl. Acad. Sci. U.S.A.* 90, 4236-4238.
2. Strunz, T., Oroszlan, K., Schafer, R., and Güntherodt, H. J. (1999) Dynamic force spectroscopy of single DNA molecules. *Proc. Natl. Acad. Sci. U.S.A.* 96, 11277-82.
3. Wildling, L., Rankl, C., Haselgrubler, T., Gruber, H. J., Holy, M., Newman, A. H., Zou, M. F., Zhu, R., Freissmuth, M., Sitte, H. H., and Hinterdorfer, P. (2012) Probing binding pocket of serotonin transporter by single molecular force spectroscopy on living cells. *J. Biol. Chem.* 287, 105-113.
4. Yoshimura, S. H., Takahashi, H., Otsuka, S., and Takeyasu, K. (2006) Development of glutathione-coupled cantilever for the single-molecule force measurement by scanning force microscopy. *FEBS Lett.* 580, 3961-3965.

## Risk and Safety

	<p><b>Chloroform</b> (CHCl<sub>3</sub>): toxic, co-carcinogenic, H302-H315-H351-H373, P281, R22-38-40-48/20/22; S36/37</p>
	<p><b>Diethyl ether</b>, extremely flammable, narcotic, forms explosive peroxides when allowed to evaporate, H224-H302-H336, P210-P261, R12-19-22-66-67 (Europe), S9-16-29-33 (Europe)</p>
<p>-</p>	<p><b>EDTA disodium salt</b>, toxic if ingested or when the dust would be inhaled, not dangerous under normal working conditions</p>
<p>-</p>	<p><b>Hepes, free acid</b>, irritant if ingested or when the dust would be inhaled, not dangerous under normal working conditions</p>
	<p><b>Isopropanol = 2-propanol</b>: flammable, H225-H319-H336, P210-P261-P305 + P351 + P338, R11-36-67 (Europe), S7-16-24/25-26 (Europe)</p>
	<p><b>Triethylamine</b> (TEA): flammable, irritant, caustic, H225-H302-H312-H314-H332, P210-P280-P305 + P351 + P338-P310, R20/21/22-35, S3-6-26-29-36/37/39-45</p>
	<p><b>Tris(carboxyethyl)phosphine hydrochloride</b> (TCEP hydrochloride): H314, P280-P305 + P351 + P338-P310, R34 (Europe), S26-27-36/37/39 (Europe)</p>