Amino-functionalization of AFM tips (and supports)

Hermann J. Gruber, Institute of Biophysics, Johannes Kepler University,
Gruberstrasse 40, 4020 Linz, Austria – Europe

hermann.gruber@jku.at

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5. Scope: The procedures have been optimized for AFM tip functionalization. They may work in related fields but the optimal parameters may be different. For instance, much slower coupling will occur on protein-resistant surfaces.
6. You are kindly asked for feed-back concerning errors, unexpected results, or potential hazards not foreseen at present.

AFM tip amino-functionalization

short version

for risks and details see full length procedure

Method A, with Ethanolamine Hydrochloride in DMSO

1. Wash cantilevers in chloroform (3 × 5 min), dry with nitrogen gas, continue with the next step.
2. Dissolve 3.3 g ethanolamine hydrochloride in 6.6 ml DMSO, cover with lid, heat to ~70°C for complete dissolution, let cool to room temperature.
3. Immerse Teflon block for the cantilevers and add 4 Å molecular sieves beads to cover the surrounding area (~25% of the total volume of the liquid).
4. Apply aspirator vacuum (or similar) to degas the solution and the molecular sieves beads (~ 30 min).
5. Place cantilevers on the Teflon block, cover with lid, incubate at room temperature overnight.
6. Wash cantilevers in DMSO (3 × 1 min) and ethanol (3 × 1 min)
7. Dry with a gentle stream of nitrogen gas or argon gas and store under argon in a dust box for up to 3 weeks (preferably < 1 week).

Method B, with APTES in the gas phase

1. Perform the whole procedure in a well ventilated hood.
2. Wash cantilevers in chloroform (3 × 5 min), dry with nitrogen gas, continue with the next step.
3. Flush desiccator chamber (5 L) with argon gas (through the narrow opening in the lid).
4. Place tray with 30 µL APTES and another tray with 10 µL triethylamine in the desiccator.
5. Place the cantilevers in the desiccator close to the two trays. Close lid, incubate for 2 h.
6. Remove the trays with APTES and triethylamine, flush desiccator with argon. Incubate the tips in argon for at least 2 days ("curing").
7. Store under argon in a dust box for up to 3 weeks (preferably < 1 week).

hermann.gruber@jku.at

Amino-functionalization of AFM tips (and supports)

Please, read the preceding manual *systematic_overview* for the basic concept of AFM tip functionalization with long flexible PEG linkers.

General information:

Standard aminosilanization protocols (in ethanol or other polar solvents) are inapplicable because long, sticky polymers are formed which render AFM tips extremely sticky. This problem is avoided in two alternative methods of amino-functionalization:

1. with ethanolamine hydrochloride, using DMSO as solvent
2. or with APTES vapors in the gas phase, using vapors of triethylamine as catalyst

The ethanolamine method is simpler and more suited for beginners. The APTES/gas phase method is more demanding but gives better AFM data, especially if the unbinding lengths are to be evaluated in force spectroscopy experiments.

Please, note that the above named procedures can also be applied to glass, mica, and to ultra-flat chips consisting of silicon or silicon nitride, yielding ultra-flat supports to which target molecules can be covalently bound.

Pretreatment of commercial AFM tips (cleaning, oxidation)

AFM tips consisting of silicon nitride (or silicon) are subject to spontaneous oxidation in the ambient atmosphere, resulting in a thin layer of silicon dioxide. The oxide layer exhibits silanol groups (Si-OH) that can be used for further chemical functionalization (see Figure 1). In addition, the surface is contaminated with non-polar components from the atmosphere and these are best removed by washing with chloroform.

Originally, we used piranha (see below) to ensure complete oxidation of the AFM tip surface [Riener et al., 2003]. Meanwhile, we found that washing with chloroform (3 × 5 min) is satisfactory for subsequent amino-functionalization [Ebner et al., 2007 and 2008].

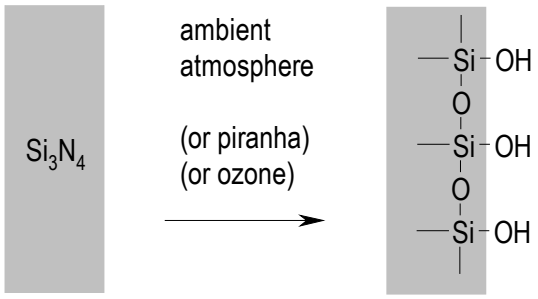


Figure 1: Oxidation of silicon nitride tips (or silicon tips). Usually the spontaneous oxidation of AFM tips by the ambient atmosphere is sufficient to generate the silanol groups (Si-OH) which are required for subsequent amino-functionalization (see **Figures 2 and 3**).

Pre-treatment without piranha:

Immerse the AFM cantilevers in chloroform (analytical grade) and incubate for 5 min. Repeat this process two more times, always using a new clean beaker with new chloroform. Finally, the AFM cantilevers are dried in a stream of nitrogen gas or argon gas.

- Please, note that chloroform is toxic and acts as co-carcinogen. Work in a well ventilated hood. Avoid breathing chloroform vapors. Strictly avoid contact with your skin! Latex gloves protect for 1-2 seconds only and must immediately be removed from the hand in case of splashing. Use chloroform-resistant gloves or exert extreme care!
- Please, transfer used chloroform in the "organic waste" which is subsequently picked up by a professional waste disposal company.

Procedure with piranha:

First, the "pre-treatment without piranha" is performed as described above. Only then the treatment with piranha can be applied. **Please, do not use this procedure unless there is a strong need for it**!

- Please, note that piranha is extremely dangerous: It is potentially explosive, extremely corrosive, and it will destroy gloves, clothes, skin, and body tissue within seconds.
- Protect yourself (body, face, hands) as much as possible and work in a well ventilated hood, with the front shield pulled down as much as possible.
- Please, note that here we can use a milder form of piranha, with a 1:19 ratio (v/v) of 30% aqueous hydrogen peroxide (H_2O_2) and 98% sulfuric acid (H_2SO_4), not the usual ratio of 3:7 (v/v)!
- Use the minimal volume possible, e.g., a mixture of 0.1 mL 30% H_2O_2 and 1.9 mL H_2SO_4 in a 10 mL beaker
- The order of mixing is extremely critical. First, aqueous H_2O_2 is pipetted into the beaker and only then sulfuric acid may be added. The reverse order would cause instantaneous boiling and splashing or even explosion!
- Used piranha must remain in the hood until it is deactivated (at least overnight), making sure that no colleagues can come into contact with it. Subsequently, the small quantity of 2 mL can be poured into a large quantity of water and flushed down the drain.

Piranha treatment is not compatible with MAC levers, it destroys the magnetic coating!

Some laboratories use ozone cleaners for oxidation of impurities and of silicon nitride. If so, this procedure must be performed in a well-ventilated hood. Subsequently, the cantilevers are washed in ethanol (3 × 5 min), in water (3 × 5 min) and dried with a stream of nitrogen gas.

Amino-functionalization with ethanolamine

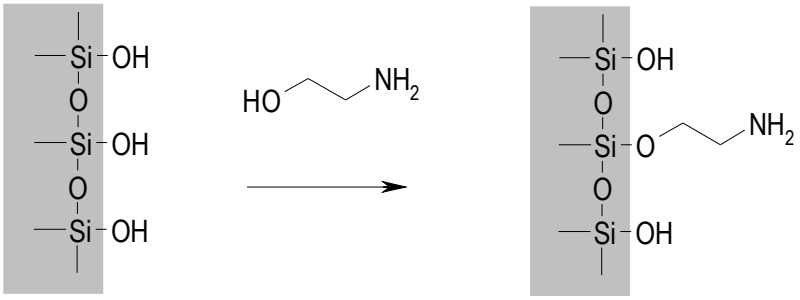
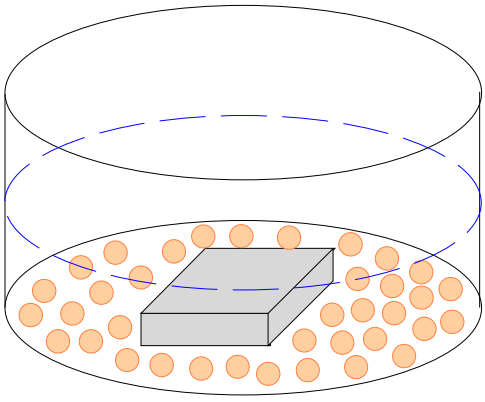


Figure 2:. The coupling reaction of ethanolamine (hydrochloride) with oxidized silicon nitride surfaces works reliably [Riener et al., 2003; Ebner et al., 2007]. The above scheme shows the most plausible chemical mechanism [Ebner et al., 2008].

Ethanolamine hydrochloride (3.3 g) is dissolved in DMSO (6.6 mL) by gentle heating to ~70°C in a crystallization dish (see below). Subsequently, a Teflon block is immersed in the center and molecular sieve beads (4 Å) are added around the Teflon block (about 10% of the volume of the solution). The solution is allowed to cool to room temperature. Dissolved air is removed by degassing in a desiccator (or vacuum chamber) at aspirator vacuum for 30 min. A membrane pump may be used, instead. Do not use an oil pump because DMSO will evaporate and contaminate the pump oil. The cantilevers are placed on the Teflon block and incubated in this solution overnight. The cantilevers are washed in DMSO (3 × 1 min) and ethanol (3 × 1 min), and dried with a gentle stream of nitrogen gas or argon gas. If the tips are not used immediately, they are stored in a desiccator under argon atmosphere for up to 3 weeks (preferably less than 1 week). The Teflon block is extensively washed in ethanol, dried with a stream of nitrogen gas and stored in a dust-free box.



hermann.gruber@jku.at

Amino-functionalization with APTES in the gas phase

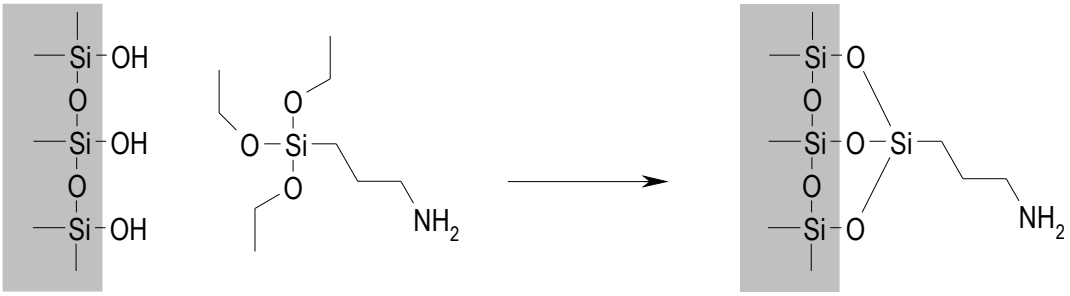


Figure 3. Gas phase silanization allows depositing monomeric APTES molecules while avoiding the formation of large, sticky clusters [Riener et al., 2003; Ebner et al., 2007]. The above scheme shows the conventional view of APTES binding. An alternative hypothesis is discussed elsewhere [Ebner et al., 2008].

The reagent (aminopropyltriethoxysilane, APTES) must not contain any aggregates; therefore it must be used immediately after purchase. Alternatively, it can be re-distilled under vacuum. Fortunately, new (or re-distilled) APTES can be kept "fresh" **if** small aliquots are sealed in crimp vials under argon gas and stored at -25°C for no longer than 12 months. The lining of the crimp cap must consist of Teflon-coated silicon, not of Teflon-coated rubber! Rubber would become inelastic at -25°C which would cause a leak. The crimp pliers must carefully be adjusted so as to apply sufficient pressure for sealing, yet not so much pressure that the Teflon coating is ruptured.

For gas phase silanization, a desiccator (5 L) must be purchased which uses a large silicone O-ring as a seal between lid and jar. The conventional vacuum grease seal must not be used. The desiccator is flooded with argon gas through the top opening in the lid in order to remove air and moisture. Then, two small plastic trays (e.g. the lids of Eppendorf reaction vials) are placed inside the desiccator. APTES (30 µL) is pipetted into one tray and triethylamine (10 µL) into the other tray. The AFM tips are placed in the vicinity of the two trays on a clean inert surface (e.g. Teflon), and the desiccator lid is put onto the jar. After 120 min of incubation, the trays with APTES and triethylamine are removed, the desiccator is again flooded with argon gas for 5 min, and the tips are left inside at room temperature for 2 days in order to ‘‘cure’’ the APTES coating [Ebner et al., 2007 and 2008].

Literature particularly addressing amino-functionalization of AFM tips (and supports).

1. Riener, C. K., Stroh, C. M., Ebner, A., Klampfl, C., Gall, A. A., Romanin, C., Lyubchenko, Y. L., Hinterdorfer, P., and Gruber, H. J. (2003) Simple test system for single molecule recognition force microscopy. *Anal. Chim. Acta 479,* 59-75.

2. Ebner, A., Hinterdorfer, P., and Gruber, H. J. (2007) Comparison of different aminofunctionalization strategies for attachment of single antibodies to AFM cantilevers. *Ultramicroscopy 107*, 922-927.

3. Ebner, A., Wildling, L., Zhu, R., Rankl, C., Haselgrübler, T., Hinterdorfer, P., and Gruber, H. J. (2008) Functionalization of probe tips and supports for single molecule recognition force microscopy. *Top. Curr. Chem. Volume 285: STM and AFM Studies on (Bio)molecular Systems* (Samori, B., Ed.) pp 29-76, Chapter 2, Springer Verlag, Berlin-Heidelberg.

hermann.gruber@jku.at

Risk and Safety

	**Aminopropyltriethoxysilane** (APTES) = 3-(triethoxysilyl)propylamine, mutagenic, irritant, R22-34; S26-36/37/39-45
	**Chloroform** ($CHCl_3$): toxic, co-carcinogenic, H302-H315-H351-H373, P281, R22-38-40-48/20/22; S36/37
-	**Dimethylsulfoxide** (DMSO): R36/37/38, S23-26-36
	**Ethanol**: flammable, R11, S7-16
	**Ethanolamine hydrochloride**: H315-H319-H335, P261-P305 + P351 + P338, R36/37/38, S26-36/37/39
	**Hydrogen peroxide** (H_2O_2) 30% solution, caustic, irritant, H302-H318, P280-P305 + P351 + P338, R22-41 (Europe), S26-39 (Europe)
	**Sulfuric acid** (H_2SO_4) 98% solution, caustic, H314, P280-P305 + P351 + P338-P310, R35 (Europe), S26-30-45 (Europe)
	**Triethylamine** (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

- 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.