Functionalization of AFM tips with native proteins or His_6-tagged proteins

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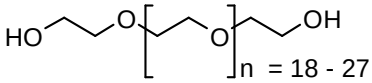
Overview

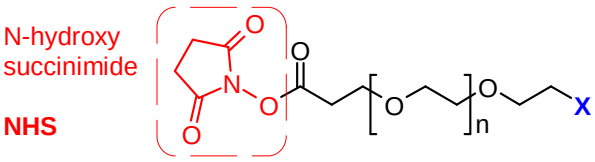
AFM measuring tips can be converted into specific biosensors when attaching specific ligand molecules to the AFM tips. For example, the AFM tip can be equipped with antibodies (DNA, hormones, drugs, etc.) in which case the AFM tip can be used to localize antigens (complementary DNA, receptors, etc.) on the sample surface with a lateral accuracy of few nanometers.

Insertion of a long, thin, flexible polymer chain between the AFM tip and the ligand has many advantages: (i) The ligand molecule can flip around and reorient rapidly, resulting in much higher probability for binding of a complementary receptor molecule on the sample surface. (ii) The elasticity of the polymer chain is much higher than that of the cantilever, thus receptor-ligand binding is easily discriminated from direct tip-surface adhesion. (iii) Polymer chains of defined length allow for rapid simultaneous imaging of Topography and RECognition sites (TREC method).

Polyethylene glycol (PEG) chains with a length of 6 – 10 nm proved to be the optimal kind of polymer for AFM tip functionalization. They have two defined end groups while the rest of the chain is chemically and physically inert.



For AFM tip functionalization we have developed a toolbox of PEG linkers all of which have a so-called **NHS**-ester function on one end and a different end group **X** on the other end. A table of all available linkers is shown in Figure 8.



When functionalizing a particular AFM tip, amino groups (NH_2) must be introduced in the first step. In the second step, the amino groups on the tip surface are reacted with the **NHS**-ester of the PEG linker, while sparing the second functional group **X** for subsequent coupling of the ligand molecule (e.g., of an antibody).

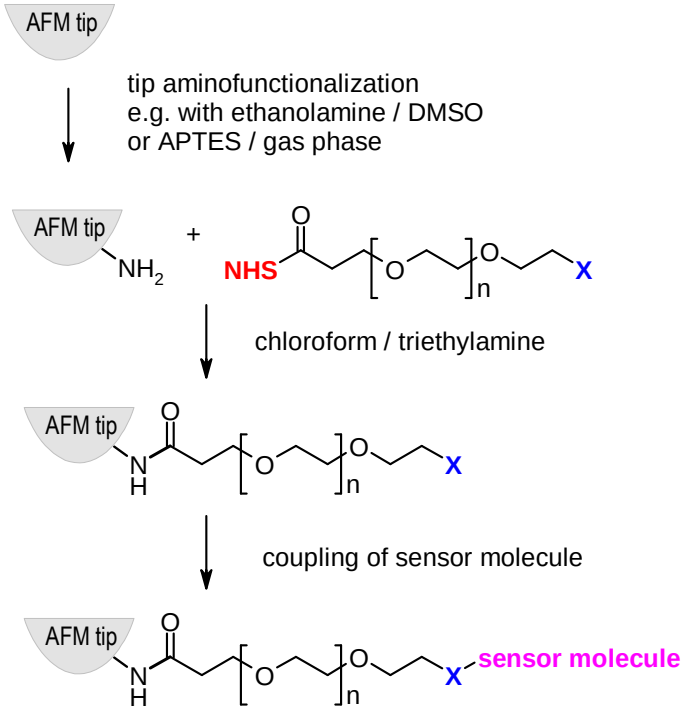


Figure 1: Three-step protocol of AFM tip functionalization: (1) Amino groups are introduced by one of the two methods shown in Figure 2. (2) A long, flexible crosslinker molecule is attached with its **NHS** ester function, resulting in a stable amide bond. Importantly, the second group **X** must not react with the amino groups on the tip surface. (3) The reactive group **X** is used for (covalent) binding of the desired **sensor molecule**.

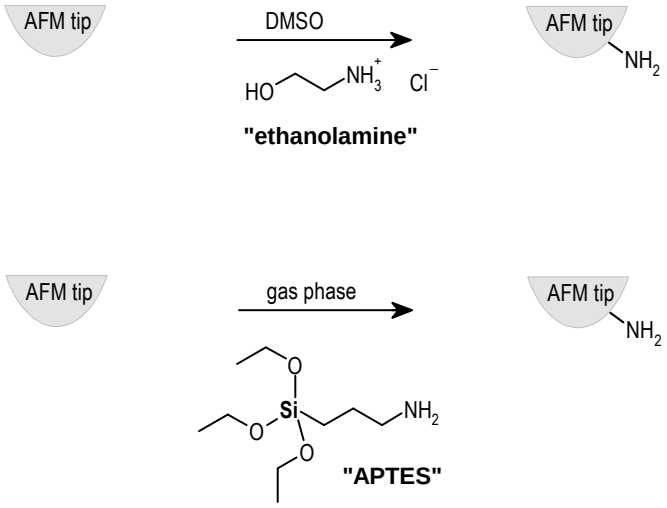


Figure 2: Introduction of amino groups (NH_2) on AFM tips. The two procedures shown above solve two problems at the same time: (1) A relatively low density of amino groups is generated on the tip surface which is just right for doing single molecule force microscopy after tip functionalization with PEG linkers. (2) The tip does not become sticky when using one of the above two procedures.

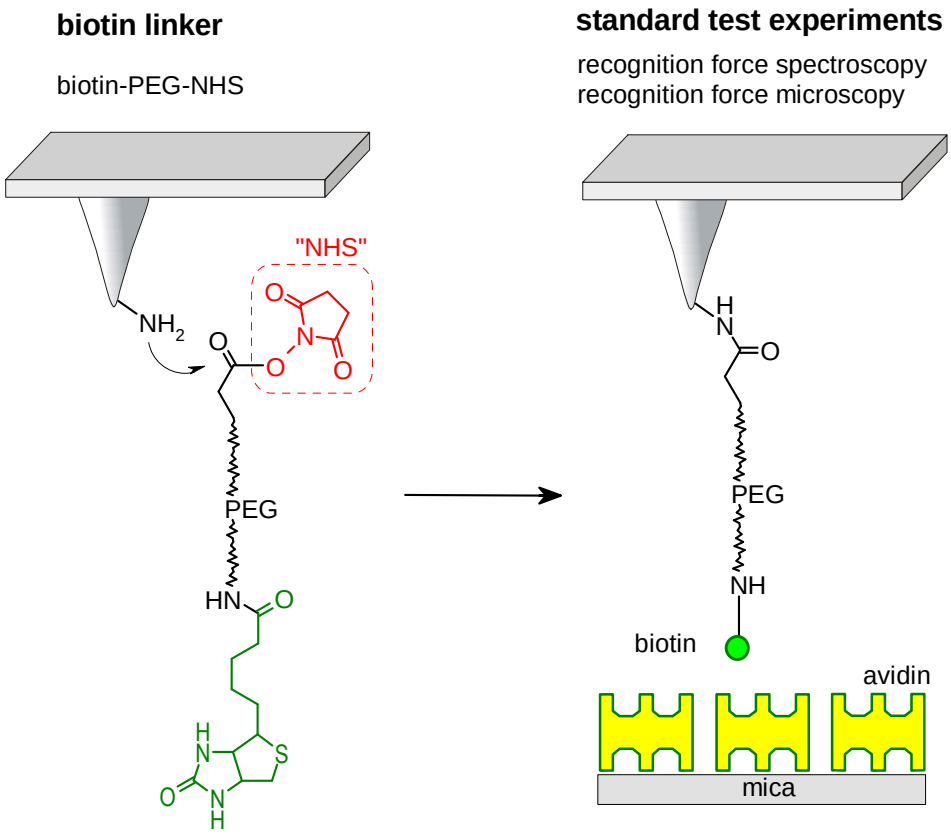


Figure 3: AFM tip functionalization with biotin-PEG-NHS. For testing purposes, we have synthesized a PEG linker which already contains a biospecific ligand in position **X** (see Figure 1). The most obvious ligand for this purpose is biotin (i) because it binds to avidin with exceptionally high affinity, and (ii) because avidin can be immobilized on mica within few minutes. An unbinding force of 50-60 pN is observed at the usual loading rates [*Anal. Chim. Acta 479, 59*].

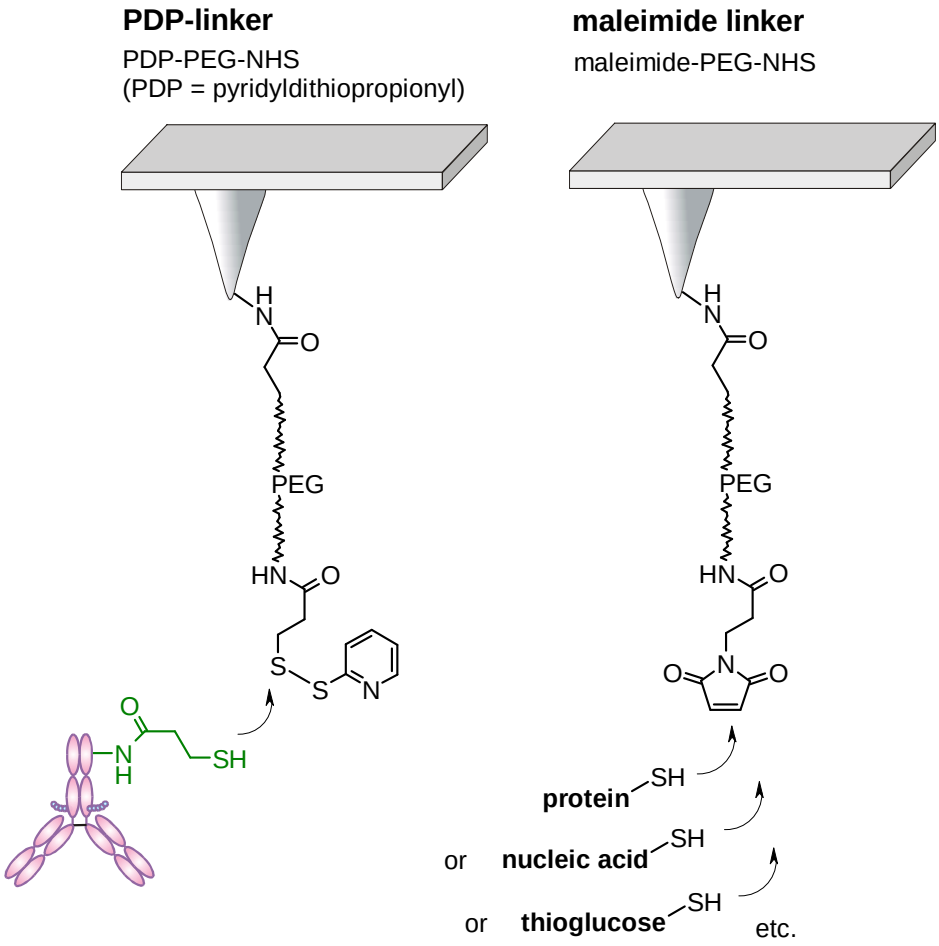


Figure 4: AFM tip functionalization with SH-containing ligand molecules. The maleimide linker (on the right) has many advantages over the PDP linker (on the left):

- Coupling of SH groups is much faster than with the PDP-linker, therefore a much lower ligand concentration can be used.
- Coupling of SH groups towards maleimide is irreversible. In case of the PDP linker (see upper left), the initially coupled ligand can be cleaved off by a second ligand molecule.
- Both thiols and disulfides can be coupled to maleimides (when including TCEP), therefore oxidation of ligand-SH into ligand-S-S-ligand does not prohibit coupling to maleimide.

The left part of the upper figure shows the conventional method of antibody linking to AFM tips which has prevailed from 1995 [*PNAS 93, 3477*] until 2007 [*Bioconjugate Chem. 18, 1176*]. The draw-back of this method was the absence of free SH groups (cysteines) in antibodies. Thus, each batch of antibody had to be pre-modified with a reagent (SATP) to introduce free SH groups. This problem was eliminated when the PDP linker was replaced by the aldehyde or acetal linker (see Figure 5).

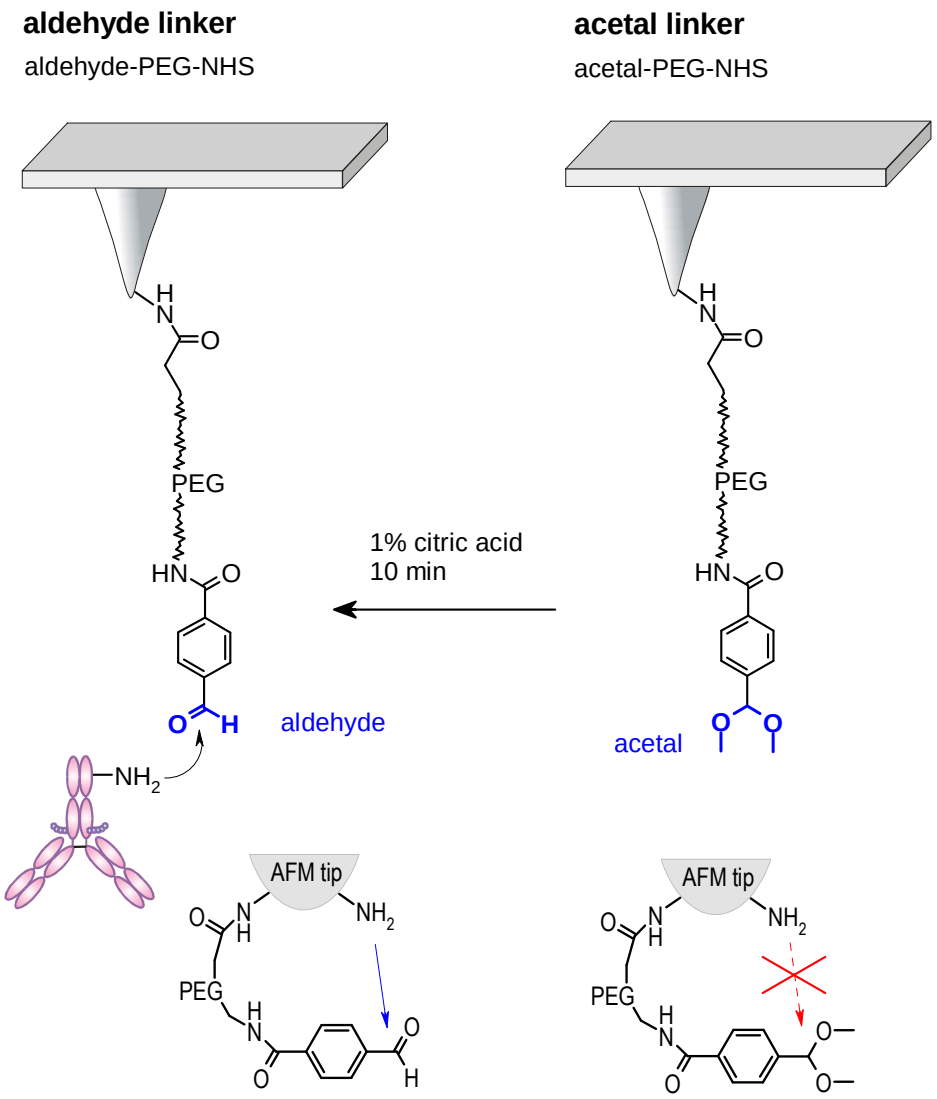


Figure 5: AFM tip functionalization with native proteins. The first step of AFM tip functionalization is generation of NH_2 groups. At the same time, NH_2 groups are also the most abundant coupling functions of most biomolecules [J. Exp. Med. 125, 823]. From this follows that we need a crosslinker with two NH_2-reactive end groups. Unfortunately, such a linker can also crosslink two adjacent NH_2 groups on the tip surface (see lower left of the upper figure). This can be prevented in two ways:

(1) The linker **aldehyde-PEG-NHS** (see Figure 8) has two NH_2-reactive end groups: Reaction of NH_2 groups with the NHS ester function is much faster than reaction of the aldehyde group, therefore this linker will first form an amide bond with the tip surface. High concentrations of aldehyde-PEG-NHS must be used to ensure that all NH_2 groups on the tip surface are consumed by the NHS-esters before the undesired loop formation begins.

(2) The linker **acetal-PEG-NHS** (see Figure 8) has a masked aldehyde function (called "acetal group") which cannot react with NH_2 functions. Thus, all NH_2 groups on the tips react with NHS-ester functions only. Subsequently, the tip is immersed in 1% citric acid for 10 min

whereby all acetal groups are converted into aldehyde functions. The citric acid treatment is compatible with all commercially available AFM tips. Old-style MAC levers did not survive this treatment but they are no longer available anyway.

In conclusion, the new linker acetal-PEG-NHS is the "work horse" for tip functionalization with proteins. Its use is straightforward, low concentrations of this linker are sufficient for efficient tip functionalization, there is no need to pre-derivatize the protein (as in Figure 3), and 10 – 50 pmol of protein is sufficient for one batch of cantilever functionalization.

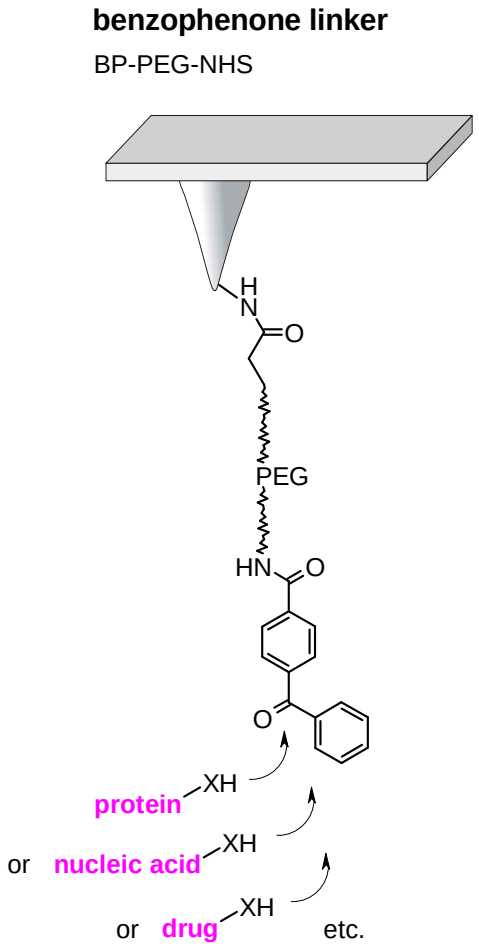


Figure 6: Photocoupling of biomolecules or synthetic molecules to AFM tips. The benzophenone linker provides for the most general method of AFM tip functionalization. Benzophenone is to be preferred over all other photo-reactive groups (azides) because it can be excited by light many times without getting de-activated. Moreover, the hydrophobic nature of benzophenone greatly favors transient interaction with biomolecules and ensures high coupling efficiency.

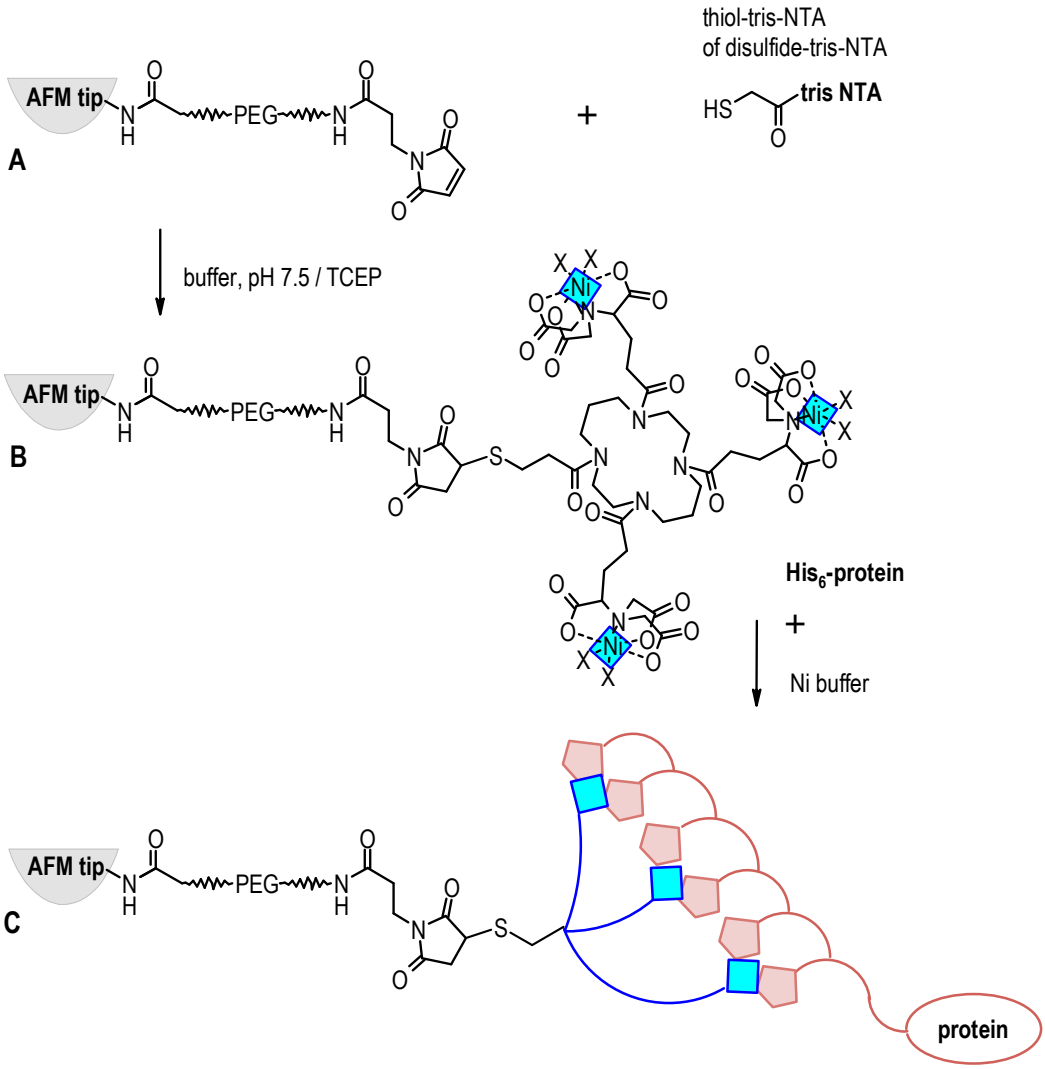


Figure 7: AFM-tip functionalization with His_6-tagged proteins. His_6-tagged proteins can be attached to the PEG linker with uniform orientation when using an adaptor molecule (termed "tris-NTA") which presents three semi-complexed Ni^{2+} ions to which the six histidine residues of a His_6 tag bind with very high stability (few % dissociation per hour [J. Am. Chem. Soc. 131, 5478]). Please, note that the use of tris-NTA is covered by a patent (US 2008/0038750 A1). For improvements and adaptations of this technique, we have collaborated with the inventors (Prof. Robert Tampé, Biocenter, Frankfurt am Main, Germany, tampe@em.uni-frankfurt.de).

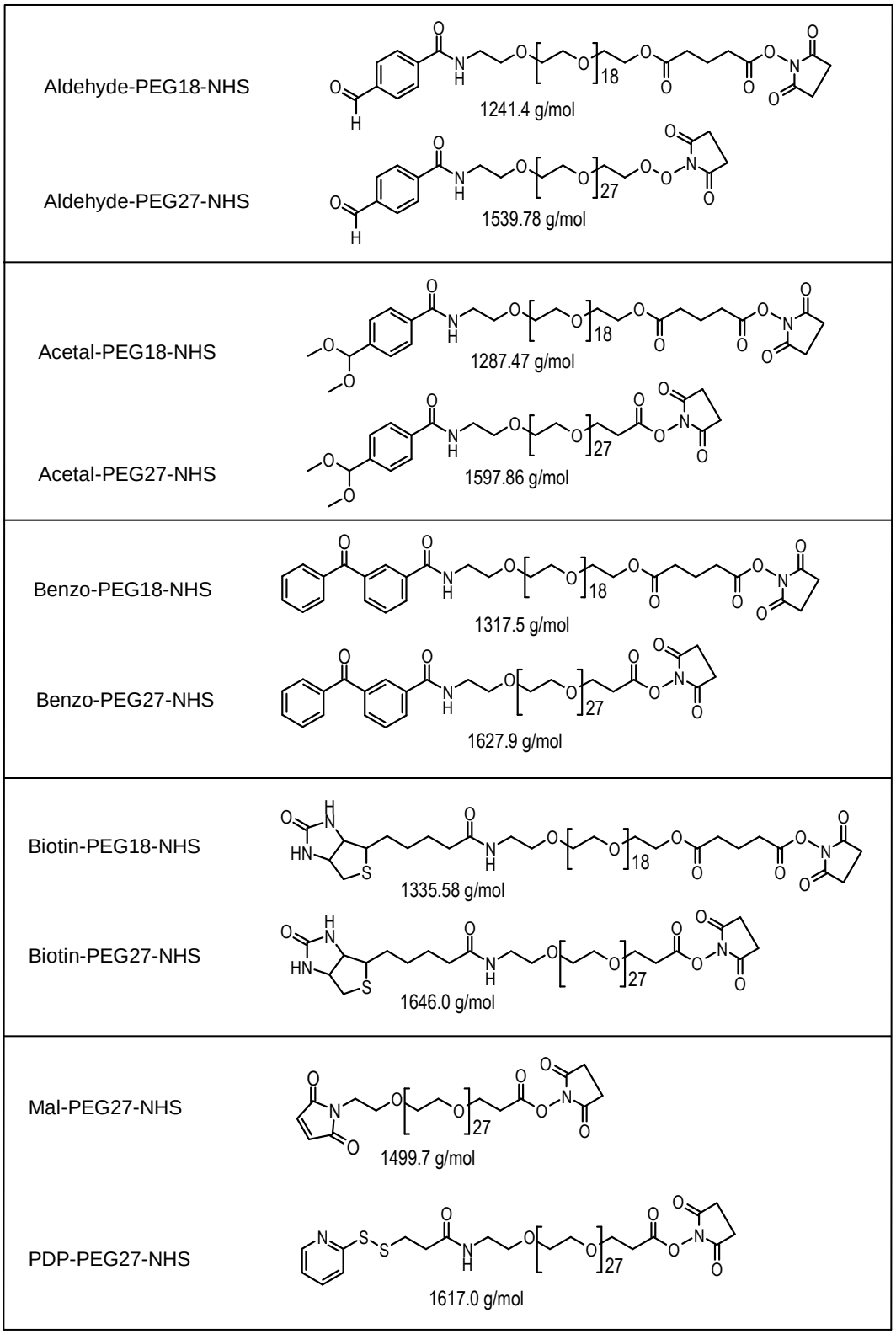


Figure 8: List of polyethylene glycol (PEG) linkers available for AFM tip functionalization. PEG18 chains have an extended length of ~6 nm, PEG27 chains ~10 nm.

Literature addressing AFM tip functionalization:

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9. Tang, J., Ebner, A., Kraxberger, B., Leitner, M., Hykollari, A., Kepplinger, C., Grunwald, C., Gruber, H. J., Tampé, R., Sleytr, U. B., Ilk, N., and Hinterdorfer, P.* (2009) Detection of metal binding sites on functional S-layer using single molecule Force spectroscopy. *J. Struct. Biol. 168*, 17-22.