Attaching Antibodies to MAC Levers with the Bifunctional Amine-Amine Reactive PEG Tether, Aldehyde-PEG$_{18}$-NHS

Application Note

W. Travis Johnson Ph.D.

Introduction

Biological molecules can be attached to silicon and silicon nitride AFM probes, transforming them into sensitive, nanoscale, biochemically selective sensors. The biochemically modified AFM probes can be used to study single molecule interactions in various AFM-based techniques. One such AFM-based method that relies on the biochemically modified AFM probes is a force spectroscopy (FS) technique called molecular recognition force microscopy (MRFM) [Riener 2003, Hinterdorfer 2004]. In MRFM, distinct interactions between even just one ligand molecule, which is immobilized on the tip of the AFM probe, and complimentary receptor molecules, which are immobilized on an opposing substrate, can be investigated one by one as the biochemically modified AFM probe first approaches and then is subsequently withdrawn away from the substrate. Unbinding interactions between the ligand and a receptor can be quantified because when the AFM probe is withdrawn away from the substrate, the molecular binding complex between the ligand and the receptor cause the AFM probe cantilever to bend. The bending of the cantilever can be monitored optically. It bends in relation to its inherent spring constant and the strength of the interaction between the ligand and the receptor. As the probe is withdrawn, the cantilever continues to bend until the ligand-receptor complex dissociates. Consequently, AFM-based force spectroscopy experiments can give valuable information about the structure and dynamics of molecular unbinding events at the single molecule level [Noy 1997] and to gain an understanding of the intramolecular forces involved in protein folding and polymer elongation [Allison 2002]. Another single molecule AFM technique that utilizes immobilized ligands on an AFM probe and receptor molecules immobilize on a substrate is topography-recognition imaging (TREC). TREC is a dynamic AFM technique in which an AFM probe, which contains immobilized ligand molecules on its tip, oscillates in AC mode while it is scanned over a substrate which contains bound receptor molecules. An Agilent AFM that is equipped with PicoTREC can resolve the molecular recognition signals from the AFM surface topography signals so that the lateral positions of functionally active receptors on the substrate can be resolved with nanometer resolution [Stroh 2004a and Stroh 2004b, Kienberger 2004b]. PicoTREC has been used to map molecular binding interactions on a variety of samples. The list includes cadherin binding sites on vascular endothelial cells [Chtcheglova 2007], vascular endothelial growth factor (VEGF) receptors on endothelial cells [Lee 2007], molecular interactions between nucleic acids - protein [Lin 2006] interactions between antibodies and antigens [Bash 2006, Lohr 2007, Marcus 2006, Stroh 2004a, Stroh 2004b], interactions between virus particles and human receptors [Ebner 2007], and molecular interactions between small ligands and their receptors [Ebner 2005].

The proper orientation of ligand molecules on the tip of the probe are extremely important in MRFM. For example, as depicted in figure 1, direct covalent immobilization of ligands on the AFM probe tip [Chtcheglova 2004, Lee 1994, Lin 2005 Sekiguchi 2003, Vinckier 1998] and physisorbtion [Lehenkari 1999, Wojcikiewicz 2004] may generate high ligand densities, but these ligands may not be free to diffuse within a defined volume of space nor be able reorient and permit optimal ligand-receptor binding to occur.
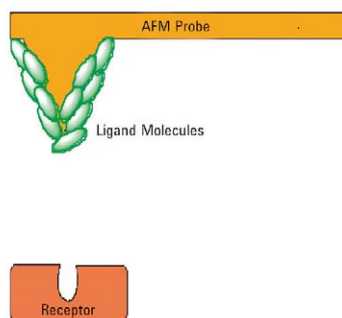

Figure 1. Direct immobilization of ligands to the tip of an AFM probe. This type of immobilization can often lead to a high density of molecules on the probe, but these ligands are generally not able to diffuse within a defined volume of space nor are they able to reorient and bind with the receptors in an optimal manner.
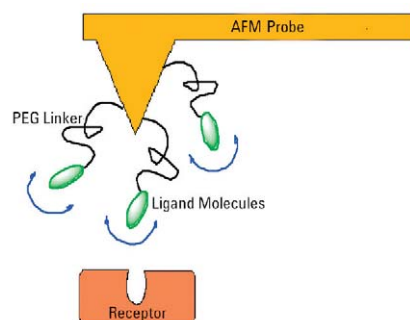
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Figure 2. Ligand immobilization with PEG linkers. The PEG linker confines the ligands within a defined volume of space and they have enough flexibility and freedom to diffuse, then reorient and bind with they complimentary receptors in an optimal manner.

Figure 3. Outline of the synthetic route followed by Ebner *et al* to synthesize the selective, bifunctional amine-amine linker, aldehyde-PEG$_{18}$-NHS.

Consequently, it has been demonstrated in most MRFM and TREC imaging studies that it is advantageous to attach ligand molecules to the end of a flexible PEG (polyethylene glycol) tether that is in turn covalently attached to the tip of the AFM probe (figure 2). The flexible tether gives the ligand molecules the ability to reorient their positions as they approach or come in contact with the target so that they can bind more efficiently to their complimentary receptors on the sub-strate [Kienberger 2000]. Not only does a PEG tether give ligands more degrees of freedom than they might otherwise have had they been immobilized directly to the surface of the probe, but the linker also allows ligands to diffuse within a well defined volume of space, the size of which is relative to the length of the linker [Kienberger 2004a] so the effective concentration of ligand molecules and their locations can be controlled.

Chemistry
The PEG linker, aldehyde-PEG$_{18}$-NHS, can be synthesized by those skilled in the art of organic chemistry [Ebner 2007] or purchased from one of many vendors that will perform custom synthesis for a fee. Figure 3 summarizes the synthetic route that Edner et al followed to synthesize aldehyde-PEG$_{18}$-NHS.

The linker has been used to immobilize antibodies [Bonanni 2005, Ebner 2007, Chtcheglova 2007] and virus particles [Ebner 2007] on AFM probes for MRFM and TREC imaging without any protein pre-activation steps or post-act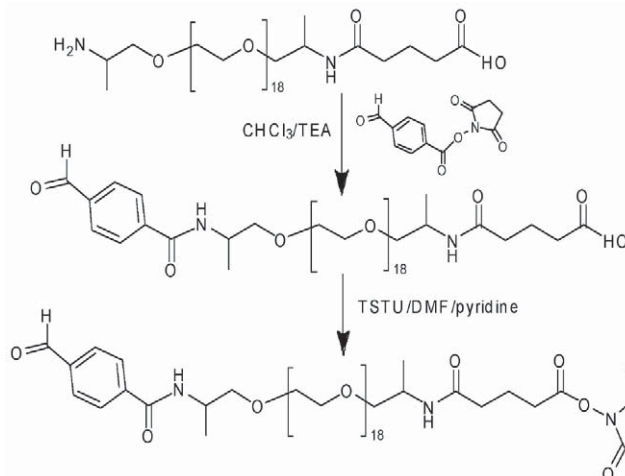ivation purification steps. In addition, this PEG linker may also be useful to immobilize other entities that possess free amine groups, including lysine residues or aminated nucleic acids, to AFM probes.

Preview of Biomolecular Immobilization Steps
The steps for biomolecular immobilization using aldehyde-PEG$_{18}$-NHS can be generalized as follows:
1. AFM probe cleaning and amination
2. PEGylation of the aminated probe
3. Immobilization of antibodies to the PEGylated probes.

MAC levers and many other AFM probes must be cleaned in a gentle manner that will not degrade the paramagnetic film or the reflective coating on the backside of the cantilevers [Chtcheglova 2004, Lohr 2007, Vig 1993]. The cleaned probes can be aminated with an alkoxyaminosilane (APTES) or ethanolamine [Ebner 2007b], PEGylated with aldehyde-PEG$_{18}$-NHS [Bonanni 2005, Ebner 2007, Chtcheglova 2007] and conjugated with the antibodies.

It is important to note that any antibodies that are to be immobilized on aldehyde-PEG AFM probes must be free from extraneous primary amines, ammonium ions and amine buffers (including Tris), BSA, gelatin, azide or any other protein "stabilizing agents" or contaminants that are commonly found in commercial antibody preparations. This is because these materials can interfere with the protein immobilization reactions. If there is any doubt about the purity of any antibodies, they should be purified, using for example a PD10 column from GE Healthcare, HPLC or dialysis, before use. It is very important that the antibody preparations be free from any protein-based stabilizing agents, such as BSA or gelatin, because large proteins may be extremely difficult or impossible to remove from small amounts of antibodies. Furthermore, in order to ensure that the antibodies are active, a functional assay [Ebner 2007b, Reiner 2003] should be performed before proceeding with the immobilization chemistry.

Cleaning AFM Probes
The AFM probes must be thoroughly cleaned to remove surface contaminants that may have physisorbed during manufacture, shipping, storage or handling before they are chemically modified. Strong acids such as nitric acid, H$_2$SO$_4$, HF or piranha are often used for cleaning silicon and silicon nitride substrates, including some AFM probes [Chtcheglova 2004, Hinterdorfer 1996, Hinterdorfer 1998, Lin 2005, Riener 2003, Ros 1998]. However, harsh conditions such as these will damage delicate MAC levers that are necessary for TREC imaging, and it can remove the reflective coating from other AFM probes. Consequently, relatively more gentle cleaning methods such as ozone cleaning [Chtcheglova 2004, Lohr 2007, Vig 1993] must be used to clean MAC levers and many other AFM probes. The MAC levers should be rinsed first in an organic solvent to remove gross contaminants before being placed in a UV-ozone cleaner. After the tips of the probes are cleaned with ozone, they should be used immediately in the subsequent amination reactions. Vig 1993 describes ozone cleaning in great detail [Vig 1993].
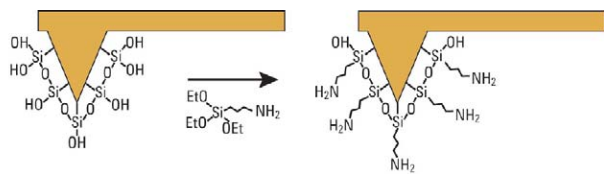
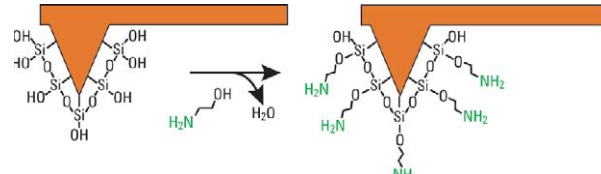Figure 4. Amination of MAC levers with aminopropyltriethoxysilane (APTES)

Figure 5. Amination of MAC levers with ethanolamine

Aminating AFM Probes

For a review of various methods for modifying AFM probes with amine groups, the reader is referred to a fine article by Ebner et al [Ebner 2007b]. The tips of MAC levers and most other AFM probes are composed of silicon or silicon nitride, both of which are covered in a native oxide layer, so they contain numerous reactive siloxy (SiOH) groups that conveniently can be covalently modified. However, it should be noted that it is critical that the sharpness of the AFM probes be maintained. Alkoxysilanes are commonly utilized to introduce chemically reactive functional groups onto silicon and silicon nitride surfaces. But, alkoxysilanes can conjugate with one another, forming polymers in solution and clusters of polymers on siloxy surfaces. Consequentially, alkoxysilanes in solution can easily dull the tips of the probes [Lee 1996], and alkoxysilane reactions in solvent should be avoided. Fortunately, aminopropyltriethoxysilane (APTES) is an alkoxyaminosilane that can be applied to AFM probes in the vapor phase. Applying this reagent in the vapor phase circumvents the negative issues associated with alkoxysilane reactions in solution. A uniform, well defined layer of amine groups on the surface of the AFM probe tips is the result. This preferred method involves exposing the AFM probes to vapors from freshly distilled APTES under an inert argon atmosphere [Lohr 2007, Kamruzzahan 2006, Ratto 2004, Riener 2003, Stroh 2004b, Wang 2002, Lee 2007].

Another amination method that is compatible with MAC levers and can be used to introduce a smooth, uniform layer of amine groups onto the probe tip surface is esterification using ethanolamine [Ebner 2007, Kamruzzahan 2006, Hinterdorfer 1996, Raab 1999, Ray 2007, Riener 2003].

PEGylation and Antibodies Immobilization

The aminated MAC levers are PEGylated using aldehyde-PEG$_{18}$-NHS [Ebner 2007]. Both the NHS ester and the benzaldehyde functional groups of this linker are reactive with amines, but the NHS ester is considerably more reactive towards amines than the aldehyde. Therefore, this amine-amine reactive bifunctional linker can be immobilized on aminated probes by the NHS ester terminus, leaving the benzaldehyde terminus at the other end free to react subsequently with lysine residues on proteins or amine groups on other biological entities. Consequently, antibodies can be immobilized on AFM probes using the aldehyde-PEG$_{18}$-NHS linker in a straightforward manner. The PEGylation reaction is performed on aminated probes using dry methylene chloride. The purified antibodies can be relatively quickly and easily immobilized on the PEGylated AFM probes without any additional chemical reactions or modifications involving the antibodies (figure 6).

Chemical reactions between primary amines and aldehydes generate unstable Schiff's base intermediates, which are easily hydrolyzed, but Schiff's base intermediates can be stabilized by the addition of a specific Schiff's base reducing agent such as sodium cyanoborohydride (NaCNBH$_3$). An added benefit is, when the reducing agent is added to the reaction *in situ*, it drives equilibrium towards the formation of the stable amine, which results in greater immobilization yields. The modified MAC levers can be utilized directly in MRFM and/or TREC experiments [Bash 2006, Baumgartner 2000a, Baumgartner 2000b, Chtcheglova 2007, Hinterdorfer 1998, Hinterdorfer 2002, Hinterdorfer 2004, Hinterdorfer 2006, Kamruzzaha 2006, Kienberger 2004, Kienberger 2005, Lee 2007, Lin 2006, Lohr 2007, Marcus 2006, Riener 2002, Stroh 2004a, Stroh 2004b, VanVilet 2006]. It is strongly recommended to use any antibody modified AFM probes immediately to ensure that no activity is lost, but they can often be stored for 2-3 days at 2-8°C under sterile conditions without significant loss of activity.
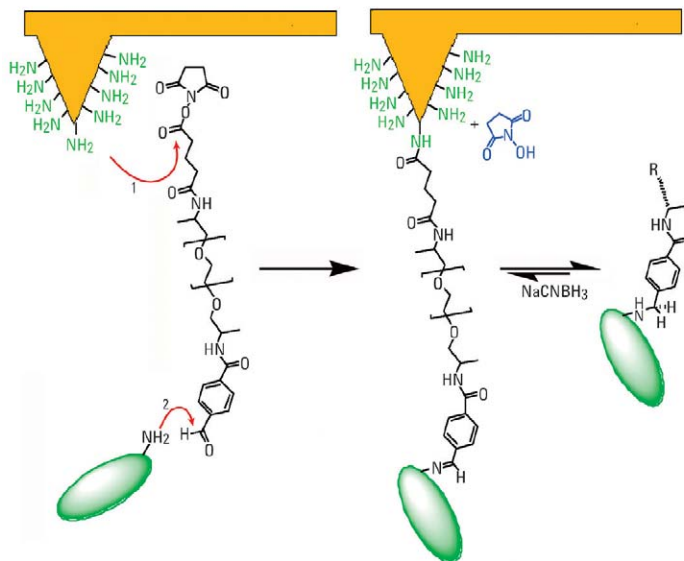

Figure 6. Immobilizing antibodies on AFM probes using the amine-amine reactive linker aldhyde-PEG$_{18}$-NHS (1). The PEG linker is immobilized on aminated AFM probes by the NHS ester terminus (2). Lysine residues on the antibodies react with the benzaldehyde moiety of the immobilized linker. The reaction between the aldehyde and the amine forms a Schiff's base intermediate that is reduced using the selective Schiff's base reducing reagent, sodium cyanoborohydride (NaCNH$_3$), to form a stable amine linkage.

Conclusion

In MRFM and TREC imaging studies, biological molecules, such as antibodies, are attached to the end of a flexible PEG (polyethylene glycol) linker that is in turn attached to the tip of the AFM probe. The PEG linker gives the biological molecules the ability to reorient their positions as they come in contact with receptor sites on the substrate, so that they can bind more efficiently to the receptors. The amine-amine reactive PEG linker, aldehyde-PEG_{18}-NHS linker, has a significant advantage over PEG linkers that were used in earlier MRFM and TREC imaging studies because no protein pre-activation steps or post-activation purification steps are required, so antibodies can be immobilized on AFM probes in a more simple, relatively more straightforward manner

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