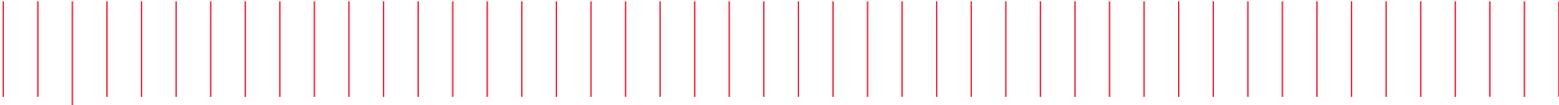


# Keysight Technologies

## Immobilizing Biological Molecules on AFM Probes for MRFM and TREC Studies

Application Note



## Introduction

Ligand molecules for a particular receptor can be attached to the tip of an AFM probe, transforming the probe into a sensitive, chemically selective biosensor for that receptor [Riener *et al.* 2003]. Molecular recognition force microscopy (MRFM) is a single-molecule AFM-based technique that relies heavily on nanoscale surface chemistry, nanoscale biochemical immobilization chemistry, and bioconjugation chemistry. In MRFM, single-molecule unbinding interactions between AFM probe-bound ligands and substrate-bound receptor pairs are observed and quantified one by one as the AFM cantilever approaches and then is subsequently withdrawn from the surface many times. The nanoNewton-scale molecular unbinding events are generally detected by measuring the optical deflection of the flexible AFM cantilever. These force spectroscopy (FS) experiments can provide valuable information about the structure and dynamics of molecular unbinding events at the single-molecule level [Noy *et al.* 1997]. In addition to intermolecular interactions, this technique has also been effectively applied to gain an understanding of the intramolecular forces involved in protein folding and polymer elongation [Allison *et al.* 2002].

Topography and recognition imaging (TREC) is another single-molecule AFM technique that utilizes probe-bound ligands and substrate-bound receptor pairs [Hinterdorfer 2004]. TREC is a dynamic force microscopy (DFM) method in which the ligand-coated AFM probe is scanned and oscillated over a biological surface in magnetic AC (MAC) Mode in order to resolve recognition maps of ligand-receptor interactions. Specific interactions between the ligand attached to the AFM probe and receptor molecules on a substrate are resolved during scanning as small changes in the MAC Mode signal [Kienberger *et al.* 2004b].

TREC imaging is a powerful technique with many potential applications because it allows a specific type of molecule to be identified in a compositionally complex sample, a characteristic that is common to many biological materials. Using a Keysight Technologies, Inc. AFM equipped with PicoTREC, which resolves the TREC AFM signals, the lateral positions of functionally active receptors on a cell or other biological surface can be resolved with nanometer resolution [Stroh *et al.* 2004a, Stroh *et al.* 2004b]. PicoTREC has been used to image, map, and analyze the chemical compositions of a variety of samples, including molecular interactions between nucleic acids and proteins [Lin *et al.* 2006], antibodies and antigens [Marcus *et al.* 2006], and small ligands and their receptors [Ebner *et al.* 2005].

There are many biochemical immobilization and bioconjugation chemistry schemes that have been applied to the investigation of ligand-receptor interactions by MRFM and TREC imaging. In such studies, biological ligands are typically bound to the tip of an AFM probe while corresponding receptor molecules or whole cells are bound to a flat substrate, such as mica, silicon, flat glass, or a gold-coated substrate. We will not concern ourselves here with the immobilization of receptors on substrates, instead we will focus our attention on the immobilization of ligands on the tips of AFM probes.

## Tethers: PEG

Single-molecule interactions have been studied with the AFM by covalently attaching ligands directly to the tip of the AFM probe [Chtcheglova 2004, Lee *et al.* 1994, Lin *et al.* 2005, Sekiguchi *et al.* 2003, Vinckier *et al.* 1998] or by absorbing molecules on the probe tip [Lehenkari *et al.* 1999, Wojcikiewicz *et al.* 2004]. This strategy often leads to a high density of ligand molecules on the tip area, though many of the ligands may not be oriented properly for optimal binding to their complementary receptors. Consequently, in most MRFM and TREC studies, it is advantageous to attach the biological ligands (nucleic acids, antibodies, small ligands, and so forth) to a tether that is in turn attached to the tip of the AFM probe. The tether imparts more freedom to the ligand than it would if it were attached directly to the tip of the AFM probe because the tether allows the ligand to diffuse within a defined volume of space [Kienberger *et al.* 2004a]. The tether also imparts to the ligand the ability to reorient its position as it approaches or comes in contact with the target so that it may bind more efficiently to a complementary receptor that has been immobilized on a substrate [Kienberger *et al.* 2000].

Polyethylene glycol (PEG) and polyethylene oxide (PEO) refer to polymers prepared from the linear polymerization of ethylene oxide. The two names are chemically synonymous, but PEG generally refers to relatively short polymers, while PEO polymers tend to be longer. Both PEG and PEO can be either liquids or low-melting solids, depending on their molecular weights. PEG is a convenient linker in many force spectroscopy applications in part because it often can be purchased functionalized with a variety of useful end groups to permit the attachment of various molecular entities.

A linker made of PEG has been shown to offer many advantages over other tethers for attaching biological molecules to AFM probes. For example, the number of PEG molecules on the tip of the probe can often be controlled in order to minimize the number of ligand-receptor binding interactions [Hinterdorfer *et al.* 1996, Riener *et al.* 2003] and PEG linkers greatly enhance the resolution of single-molecule unbinding interactions from multiple unbinding events or nonspecific interactions [Hinterdorfer *et al.* 1996]. The length of the PEG linker can be controlled, confining the volume of space about which the ligand can diffuse, so the effective concentration of ligand molecules in the experiment can be minimized and largely controlled. Since the PEG linker permits the ligand to diffuse in a defined volume of space, it is more likely to encounter receptors on the substrate. Ligand-receptor interactions that involve PEG linkers undergo less torque [Riener *et al.* 2002]. Therefore, the ligand molecules are more likely to reorient in a manner favorable to optimal binding when an encounter with an immobilized receptor occurs.

Encounters between immobilized receptors and PEG-tethered ligand molecules are often very similar to encounters involving freely diffusing ligand molecules in solution. As a consequence, the kinetics of ligand-receptor interactions that involve PEG-tethered ligands are faster and more applicable to the kinetics of ligand molecules in solution. Furthermore, PEG is a water-soluble, nonadhesive polymer, so nonspecific interactions between the ligand molecules and the AFM probe as well as between the probe tip and receptors on the substrate are minimized [Hinterdorfer *et al.* 2000, Riener *et al.* 2002, Riener *et al.* 2003].

## PEG Tether length

Short PEG tethers or linkers are often used for spacing the ligand just a few nanometers away from the AFM probe tip. Long PEG tethers allow ligand molecules to diffuse into greater volumes of space around the AFM probe tip than shorter molecules, so linker length can greatly impact the results of MRFM experiments and TREC imaging studies. For example, PEG linkers with molecular weights of 3400 to 5000 Daltons, which correspond to 25–50 nm in length, have found utility in many force spectroscopy studies [Ratto *et al.* 2004, Ray *et al.* 2007, Ros *et al.* 1998, Schumakovitch *et al.* 2002, Schwesinger *et al.* 2000], but it has been demonstrated that shorter PEG tethers, such as a PEG<sub>18</sub> (800 Daltons), offer certain advantages over longer PEG linkers. In force spectroscopy applications shorter linkers can increase single-molecule resolution and in TREC applications it is possible to achieve much higher lateral resolutions with shorter linkers. A PEG<sub>18</sub> linker with a length of 8–10 nm can achieve a lateral image resolution of 16–20 nm [Riener *et al.* 2002], thus it is certainly preferred over longer PEG linkers for TREC imaging applications.

## Overview of biomolecule immobilization steps

Figure 1 outlines a few of the important chemical steps used to immobilize biological molecules to AFM probes:

1. *Clean AFM probes.* Gentle cleaning methods are preferred (example organic solvents, UV-ozone).
2. *AFM probe activation (amination).* Most AFM probes must be activated prior to use. Silicon AFM probes should be silanized with an aminosilane (APTES) or esterified (ethanolamine). Silicon nitride AFM probes are silanized, esterified, or used as is.
3. *PEGylation.* Attach PEG linkers to activated AFM probes.
4. *Bioconjugation.* Many biological molecules must be functionalized (example thiolated oligonucleotides, antibody Fab fragments, His-tagged proteins, thiolated antibodies, and so forth). The modified biological molecules are attached to the PEG tethers.
5. *Characterization.* The number of ligand molecules on the surface of the AFM probe must be determined.

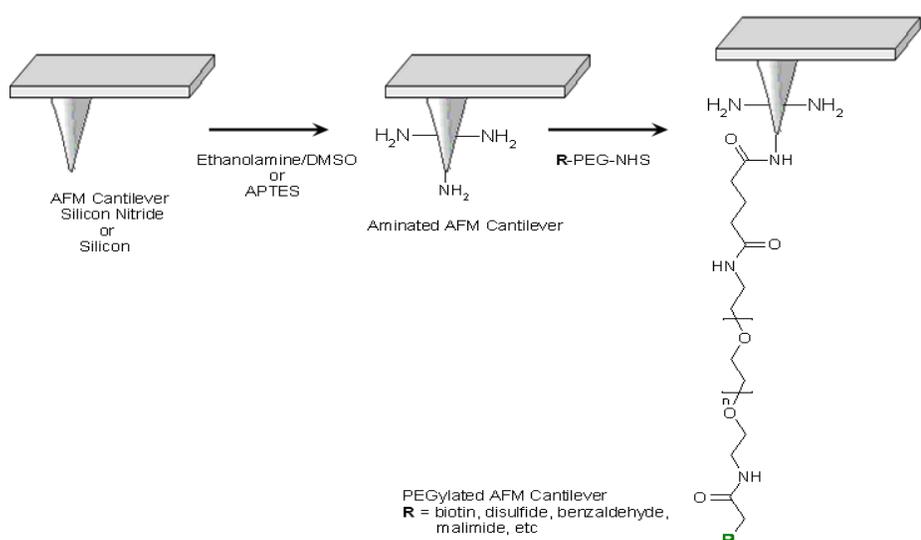


Figure 1. AFM probes can be aminated and then PEGylated with heterobifunctional PEG linkers and used directly or further conjugated with proteins for AFM molecular recognition studies.

## Individual steps

### 1. Clean AFM probes

Many contaminants can become physisorbed to AFM probes during manufacture, shipping, storage, or handling. Therefore, all AFM probes must be thoroughly cleaned before attempting any surface chemistry. Strong acids, such as nitric acid,  $H_2SO_4$ , HF, or piranha, are often used for this purpose [Chtcheglova 2004, Hinterdorfer *et al.* 1996, Hinterdorfer *et al.* 1998, Lin *et al.* 2005, Riener *et al.* 2003, Ros *et al.* 1998]. However, harsh conditions can damage many probes. For example, optically reflective metal coatings, such as gold or aluminum, on the backside of cantilevers can be damaged by acid. Minor damage to the reflective coating will lead to an increase in electronics noise over the deflection signal. Even relatively minor damage to the reflective coating can render an AFM probe unusable. In addition, MAC levers, which are necessary for TREC, contain a paramagnetic film that is easily removed in harsh conditions. Consequently, relatively gentle cleaning methods, including solvent rinses [Riener *et al.* 2003, Schumakovitch *et al.* 2002], plasma cleaning [Lee *et al.* 2007], or ozone cleaning procedures [Chtcheglova 2004, Lohr *et al.* 2007, Vig 1993] are recommended. Vig 1993 describes the method for 24 ozone cleaning semiconductor surfaces in greater detail.

### 2. AFM probe activation

Most AFM probes are made from silicon or silicon nitride. Passive absorption has been used to immobilize biological entities to these AFM probes either directly [Lehenkari *et al.* 1999] or by incubating the AFM probe with a layer of biotinylated BSA, which then is bound to another biotinylated entity via a streptavidin intermediary [Wojcikiewicz *et al.* 2004]. Although seemingly simple and straightforward, absorption can add significant mass to the tip of the probe, so it may be too bulky for TREC applications and other MRFM experiments that require high resolution and accuracy. In other cases, the tips of AFM probes are coated with a layer of gold, which can form a strong bond with thiolated molecules [Burns 2004, Hugel *et al.* 2005, Lee *et al.* 1994]. Unfortunately, gold adds approximately 20 nm or more to the probe tip radius, which often results in a loss of resolution and accuracy.

Other methods of immobilization have proven particularly useful in AFM applications. Silicon nitride films are typically produced by the nitridation of silane materials using ammonia, which produces a mixed film containing Si-NH<sub>2</sub> groups whose density is determined by the pH of the process [Lee *et al.* 1996]. Consequently, PEG linkers with amine reactive end groups can often be anchored directly to silicon nitride cantilevers without any activation [Riener *et al.* 2003, Chtcheglova 2004]. Also, when both silicon and silicon nitride surfaces are exposed to air or water they will naturally oxidize. Therefore, the surface silicon and silicon nitride materials are covered in an oxide layer that contains numerous reactive SiOH entities. The pKa of SiOH is approximately 6.8 [Hubbard 2002], so it is slightly acidic, and various reactions have been developed to cover surface oxides in a thin layer of amine groups. Silicon and silicon nitride AFM probes can be easily esterified using ethanolamine in dry DMSO to add a layer of amine groups to the surface [Hinterdorfer *et al.* 1996, Raab *et al.* 1999, Ray *et al.* 2007, Riener *et al.* 2003]. Molecular sieves are added to the reaction mixture in order to remove water and drive equilibrium in favor of esterification.

Aminosilanes are also commonly used to prepare aminated AFM probes. Traditional reactions of alkoxy aminosilanes in solvents such as toluene, ethanol, or acetone are common [Lin *et al.* 2005, Ros *et al.* 1998, Schumakovitch *et al.* 2002, Schwesinger *et al.* 2000, Vinckier *et al.* 1998]. However, one drawback of this technique relates to the propensity of alkoxy aminosilanes to form polymers in solution, which adds bulk and roughness to the AFM probe [Lee *et al.* 1996]. This drawback can largely be circumvented by performing silanization reactions in the vapor phase using freshly distilled aminopropyltriethoxysilane (APTES) [Lohr *et al.* 2007, Kamruzzahan *et al.* 2006, Ratto *et al.* 2004, Riener *et al.* 2003, Stroth *et al.* 2004b, Wang *et al.* 2002, Lee *et al.* 2007].

### 3. PEGylation

PEG-tethering reactions (PEGylation) are a critical step in immobilizing biological molecules to AFM probes. Tethers can be synthesized or purchased with various end groups to bind to activated AFM probes and anchor a multitude of conjugate biological entities, including antibodies, peptides, and nucleic acids. Table 1 lists some useful, commonly used, relatively short X-PEG-Y linkers that have been effectively utilized in MRFM and TREC studies. The table also includes the linkers' functional end groups, references that describe their synthesis or where they can be purchased.

Homobifunctional amine-PEG-amine linkers and heterobifunctional amine-PEG-COOH linkers are utilized as intermediates in the synthesis of various tethers that are reactive for specific functional groups. Haselgruber *et al.* 1995 describes the synthesis of a PEG<sub>18</sub> tether from amine-PEG-amine that contains an N-hydroxysuccinimide (NHS) ester on one terminus and a 2-pyridyldithio (PDP) group on the other terminus. The NHS-PEG<sub>18</sub>-PDP tether was originally synthesized to facilitate bioconjugation reactions between antibodies and liposomes, but it has also served as the tether for many MRFM and TREC imaging experiments.

Since the NHS ester group is very reactive towards amines, such as the ones found on APTES or ethanolamine-activated AFM probes, it is often used as a PEG end group to enable PEG attachment to AFM probes [Riener *et al.* 2002, Kienberger *et al.* 2000b, Ebner *et al.* 2007]. Protein molecules have also been anchored directly to AFM probes by homobifunctional NHS-PEG-NHS linkers [Ratto *et al.* 2004].

Both PEG-PDP and PEG-maleimide are reactive towards sulfhydryl groups. Cystine-terminated peptides and proteins [Hinterdorfer *et al.* 2000, Kamruzzahan *et al.* 2006] as well as sulfhydryl-modified oligonucleotides [Lee *et al.* 1996, Lin *et al.* 2006, Schumakovitch *et al.* 2002] can be linked directly to PEG-PDP and PEG-maleimide linkers. Reactive sulfhydryl groups are also made available by the reduction of proteins that

PEG <sub>n</sub>	End Group X	End Group Y	Commercial Source/Reference
PEG11	-COOH	-biotin	Polypure (Oslo Norway)
PEG12	-NHS	-biotin	Quanta BioDesign (Powell, Ohio USA)
PEG12	-NHS	-maleimide	Quanta BioDesign (Powell, Ohio USA)
PEG18	-NH <sub>2</sub>	-NH <sub>2</sub>	Polypure (Oslo Norway), Sigma Aldrich (St. Louis, MO USA)
PEG18	-NHS	-PDP	Haselgruber <i>et al.</i> 1995, Riener <i>et al.</i> 2002
PEG18	-NHS	-biotin	Riener <i>et al.</i> 2002, Riener <i>et al.</i> 2003
PEG18	-NHS	-His6	Riener <i>et al.</i> 2002, Kienberger <i>et al.</i> 2000b
PEG18	-NHS	-NTA	Riener <i>et al.</i> 2002
PEG18	-NHS	-aldehyde	Ebner <i>et al.</i> 2007
PEG27	-NHS	-biotin	Polypure (Oslo Norway)
PEG27	-NHS	-maleimide	Polypure (Oslo Norway)

Table 1. Common X-PEG<sub>n</sub>-Y Molecules and Commercial Sources or Synthesis References

contain disulfide bonds. Antibodies can be reduced with DTT to generate Fab fragments [Hinterdorfer *et al.* 2000, Raab *et al.* 1999, Riener *et al.* 2002]. Most proteins can be conjugated to sulfhydryl reactive reagents to facilitate their attachment to PEG-PDP and PEG-maleimide linkers [Bash *et al.* 2006, Baumgartner *et al.* 2000a, Baumgartner *et al.* 2000b, Hinterdorfer *et al.* 1998, Hinterdorfer *et al.* 2002, Kamruzzahan *et al.* 2006, Lee *et al.* 2007, Riener *et al.* 2002, Stroh *et al.* 2004a, Stroh *et al.* 2004b].

Genetically engineered His6-tagged proteins can be tethered to AFM probes via PEG linkers containing nitrilotriacetic acid (NTA) end groups [Kienberger *et al.* 2000a, Kienberger *et al.* 2000b, Kienberger *et al.* 2005, Nevo *et al.* 2003, Riener *et al.* 2001, Riener *et al.* 2002]. In addition, biotinylated PEG linkers (PEG-biotin) can be utilized in molecular recognition applications involving avidin or streptavidin without further modification [Ebner *et al.* 2005, Riener *et al.* 2001, Riener *et al.* 2002, Riener *et al.* 2003].

The synthesis of a new PEG linker that contains an amine reactive aldehyde terminus was recently described [Ebner *et al.* 2007]. The aldehyde group of NHS-PEG-aldehyde can be reacted directly with protein molecules (such as antibodies) that contain lysine groups available near the protein's surface [Bonanni *et al.* 2005, Ebner *et al.* 2007, Chtcheglova *et al.* 2007] or even with virus particles [Ebner *et al.* 2007]. Antibodies or other biological molecules that have free amine groups may be anchored to PEG-aldehyde without laborious protein preactivation and purification steps.

Various other PEG molecules with assorted functional groups can either be synthesized by those skilled in the art of organic chemistry or by contracting with a vendor that will perform custom PEG molecule synthesis for a fee (e.g., Sensopath Technologies of Bozeman, MT USA).

#### 4. Bioconjugation

A virtually endless list of biological molecules can be attached to AFM probes via PEG tethers in order to map out molecular interactions with PicoTREC or to quantify ligand-receptor interactions in MRFM studies. Before proceeding with any reaction involving an antibody or other protein, it is important to first ensure that the protein is active, stable, and extremely pure. For example, all proteins that are to be attached to X-PEG-Y tethers should be free of ammonium ions and amine buffers (including Tris), BSA, gelatin, azide or any other protein "stabilizing agents" or contaminants because these materials will interfere with the coupling reactions. In order to ensure activity, it is recommended that an ELISA be performed on all antibodies before performing the chemical reac-

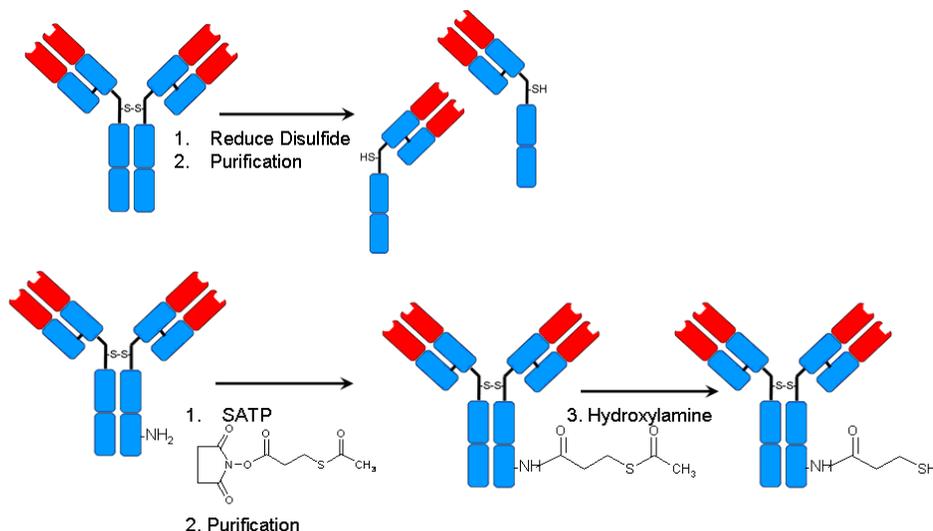


Figure 2. Antibodies and other proteins generally must be thiolated prior to conjugation with PDP-PEG linkers. (A) Reduction of disulfides is a well-known pathway that is, unfortunately, not compatible with all proteins, including many antibodies. (B) Conversion of lysine residues to active sulfhydryl groups with SATP reagent provides a path that often preserves protein function, activity, and integrity.

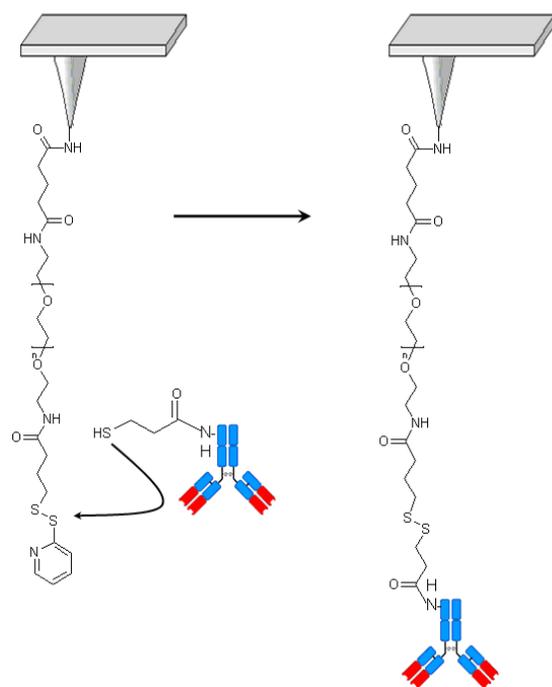


Figure 3. Proteins with active sulfhydryl groups can be conjugated to PEG-PDP linkers.

tions. Native sulfhydryl groups on proteins, peptides, and other compounds can often be utilized to link them to PEG-maleimide or PEG-PDP tethers that have been anchored to aminated AFM probes. However, in many cases, free sulfhydryl groups are not available near the surface of the molecules of interest, so the molecule must be activated with a thiolating reagent or reduced with a reagent such as DTT in order to prepare them for bioconjugation or immobilization (Figure 2A). Unfortunately, reduction of disulfides with DTT also runs a risk of protein inactivation [Raab *et al.* 1999, Yoshitake *et al.* 1979]. The NHS ester of S-acetylthioacetic and propionic acid (SATP reagent from Pierce Biotechnology, Rockford, IL USA) can be used for the introduction of active sulfhydryls into proteins, peptides, and other molecules so that they can be conjugated with PEG-maleimide or PEG-PDP tethers (Figure 2B). The reaction between the NHS ester of the SATP reagent and an amine group on the surface of a protein creates a stable, covalent amide bond. The reaction utilizes a 10:1 molar ratio of SATP reagent to protein and yields approximately five moles of sulfhydryl groups per mole of antibody or other protein (MW approximately 150 kDa).

The level of sulfhydryl incorporation may be altered by varying the molar ratios of SATP and antibody. When larger molar excesses of SATP reagent are utilized, more complete acylation of primary amine groups can occur. However, these higher levels of acylation generally correspond to a greater risk of protein inactivation. A desalting column is generally used to purify SATP-modified proteins from excess reagent and other byproducts of the reaction. Deprotection (deacylation) with hydroxylamine generates the reactive sulfhydryl group for final conjugation reactions with PDP-PEG or maleimide-PEG modified AFM probes (Figure 3).

Thiolated oligonucleotides are generated by automated solid phase synthesis and are readily available from many oligonucleotide vendors. The oligonucleotides can be conjugated to sulfhydryl reactive PEG linkers, such as PEG-maleimide in a manner similar to sulfhydryl reactive proteins (Figure 4).

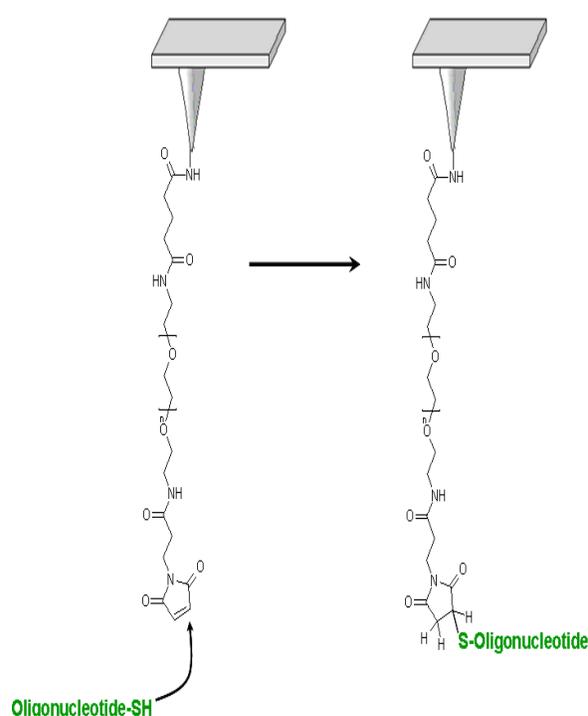


Figure 4. Thiolated nucleic acids can be conjugated to PEG-maleimide linkers so that nucleotide-nucleotide or nucleotide-protein interactions can be studied with the AFM.

As shown in Figure 5, antibodies or other proteins can often be attached directly to the PEG-aldehyde linker after the NHS-PEG-aldehyde linker has been immobilized on an aminated AFM probe [Bonanni *et al.* 2005, Chtcheglova *et al.* 2007, Ebner *et al.* 2007]. The proteins do not require any preactivation steps. Ebner *et al.* 2007 also attached a human rhino virus particle to the PEG-aldehyde linker in order to investigate interactions between the virus and human receptor proteins. The reaction between an amine and an aldehyde generates a Schiff's base intermediate that is easily hydrolyzed. However, the intermediate can easily be stabilized by the addition of a Schiff's base-specific, gentle reducing agent, sodium cyanoborohydride ( $\text{NaCNBH}_3$ ). Many proteins of interest have been genetically engineered to contain His tags. His-tagged protein molecules can be easily linked to PEG-NTA linkers and utilized in MRFM experiments [Riener *et al.* 2001, Riener *et al.* 2002]. manner in which the proteins are conjugated to the AFM probes. HRP antibodies conjugated to the ligands are used to label the ligands on the surface and the enzyme activity is measured in a spectrophotometer. Enzyme densities can be calculated by calibrating the system with anti-rabbit HRP antibodies in solution.

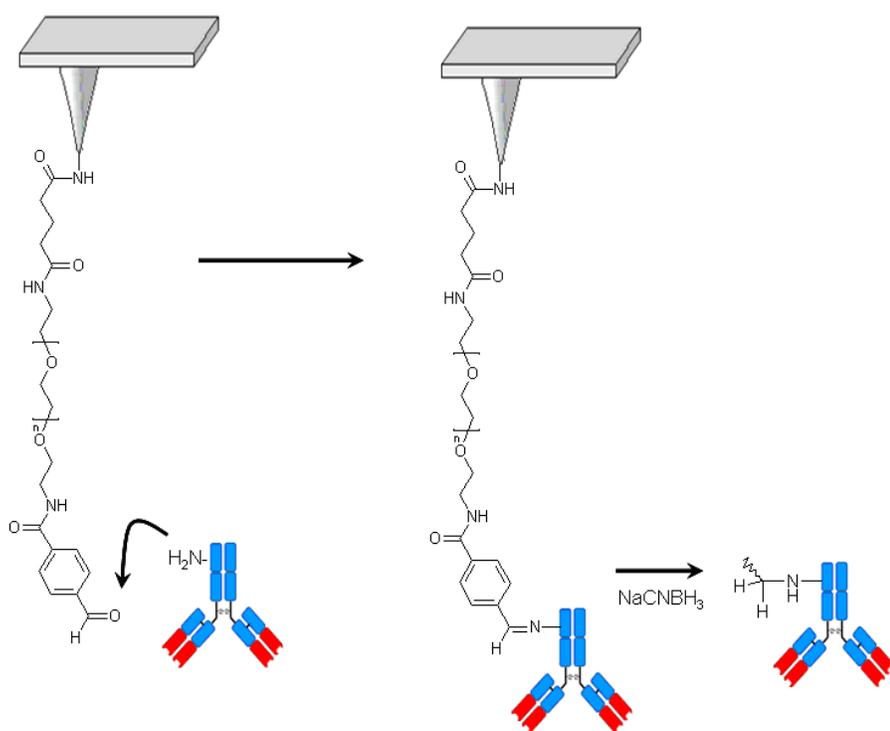


Figure 5. Unmodified proteins can be conjugated directly to PEG-aldehyde linkers via lysine residues on the surface of the protein. Chemical activation and final purification of the protein are not necessary.

### 5. Characterization

After the AFM probes have been functionalized with the PEG linkers and ligand molecules, it is imperative that steps be taken to determine the density of the ligand molecules on the AFM probe. In most MRFM and TREC studies, it is usually advantageous to optimize the density of ligand molecules on the tip of the AFM probe that can have access to the receptors. The methods described here generally result in a ligand density of 200–500 molecules per  $\mu\text{m}^2$ , which has been found to be well suited for many single-molecule unbinding studies because (for an AFM probe with a tip radius of 20–50 nm) this corresponds to approximately one molecule per effective tip area [Hinterdorfer *et al.* 1996]. Consequently, in many cases, just one ligand on the AFM probe will have access to the receptors on the surface at any given time. Unfortunately, modified AFM probes are extremely difficult to characterize and evaluate by any means other than functional assays such as MRFM or TREC imaging with PicoTREC. However, the Hinterdorfer lab in Linz, Austria, has developed three different methods to determine ligand density [Hinter-

dorfer *et al.* 2002] utilizing relatively larger silicon or silicon nitride substrates (approximately 1 cm<sup>2</sup>) that are treated in parallel under identical reaction conditions along with the AFM probes. The methods developed in the Hinterdorfer lab are based on (a) direct fluorescence, (b) fluorescently labeled secondary antibodies, and (c) a horse radish peroxidase (HRP) based assay [Hinterdorfer *et al.* 1996, Hinterdorfer *et al.* 1998].

(a) The ligands are fluorescently labeled and attached to the silicon or silicon nitride substrates in the exact manner in which the proteins are conjugated to the AFM probes. The substrates are then placed under a widefield epifluorescence microscope. The fluorescence intensity is measured and the surface densities of the ligands are calculated after calibration.

(b) The ligands are attached to the silicon or silicon nitride substrates in the exact manner in which the proteins are conjugated to the AFM probes and also to primary antibodies. Fluorescently labeled secondary antibodies are then used to label the surface-bound primary antibodies. The number of fluorescent molecules on the surface is determined as for (a) above.

(c) The ligands are attached to the silicon or silicon nitride substrates in the exact manner in which the proteins are conjugated to the AFM probes. HRP antibodies conjugated to the ligands are used to label the ligands on the surface and the enzyme activity is measured in a spectrophotometer. Enzyme densities can be calculated by calibrating the system with anti-rabbit HRP antibodies in solution.

## Conclusion

Biomolecular interactions are processes that are critical to most biological phenomena. For example, molecular interactions are responsible for the initiation, modulation, and termination of DNA replication, RNA transcription, enzyme activity, infection, immune response, tissue generation, wound healing, cellular differentiation, programmed cell death, and the activities of drugs, hormones, or toxic substances. AFM offers unique advantages over many other tools for the study of biological processes at the nanometer scale and has become an important tool for studying the nanomechanical properties of biological samples.

AFM allows scientists to visualize, probe, and analyze the structures of biological molecules in their native environments with unprecedented resolution and without the need for extraneous labels or tags. It does not always require rigorous sample preparation techniques, which are often required for high-resolution imaging studies using electron microscopy. However, as described above, bioconjugation chemistry and surface immobilization chemistry certainly enhance the power and utility of AFM. For example, organic and inorganic surface chemistries, bioconjugation chemistry, and AFM force spectroscopy and imaging methods have been combined and used to quantify and map specific biomolecular interactions between a variety of biological molecules of scientific interest and physiological importance.

Nanoscale chemistries have been exploited to study ligand-receptor interactions such as the prototypical ligand-receptor interaction, biotin-avidin [Ebner *et al.* 2005, Riener *et al.* 2001, Riener *et al.* 2002, Riener *et al.* 2003, Wong *et al.* 1997], nucleotide-nucleotide interactions [Lee *et al.* 1994, Lee *et al.* 1996], DNA-protein interactions [Lin *et al.* 2006], interactions with cell surface receptors and adhesion proteins [Baumgartner *et al.* 2000a, Baumgartner *et al.* 2000b, Chtcheglova *et al.* 2007, Lee *et al.* 2007, Lehenkari *et al.* 1999, VanVilet *et al.* 2006], antibody-antigen recognition [Bash *et al.* 2006, Hinterdorfer *et al.* 1996, Hinterdorfer *et al.* 1998, Hinterdorfer *et al.* 2002, Raab *et al.* 1999, Stroh *et al.* 2004b, Kienberger *et al.* 2005, Kienberger *et al.* 2006, Lohr *et al.* 2007], interactions between virus particles and human virus receptors [Ebner *et al.* 2007], and many other molecules of biological importance.

## References

- Allison *et al.* *Curr. Opin. Biotechnol.* 13 (2002) 47–51.
- Bash *et al.* *FEBS Letters* 580 (2006) 4757–4761.
- Baumgartner *et al.* *PNAS USA* 97(8) (2000a) 4005–4010.
- Baumgartner *et al.* *Single Mol.* 1, 2 (2000b) 119–122.
- Bonanni *et al.* *Biophysical Journal* 89 (2005) 2783–2791.
- Burns, D. J. M.S. Thesis, Dept. of Mech. Eng., Massachusetts Institute of Technology (2004).
- Chitchevlova, L. A. Ph.D. Thesis, École Polytechnique Fédérale De Lausanne, Université Strasbourg I, France (2004)
- Chitchevlova *et al.* *Biophysical Journal* 93 (2007) L11–L13.
- Ebner *et al.* *Chem. Phys. Chem.* 6 (2005) 897–900.
- Ebner *et al.* *Bioconjugate Chem.* 18 (2007) 1176–1184.
- Haselgruber *et al.* *Bioconjugate Chem.* 6 (1995) 242–248.
- Hinterdorfer *et al.* *PNAS USA* 93 (1996) 3477–3481.
- Hinterdorfer *et al.* *Nanobiology* 4 (1998) 177–188.
- Hinterdorfer *et al.* *Single Mol.* 1(2) (2000) 99–103.
- Hinterdorfer *et al.* *Colloids and Surfaces B: Biointerfaces* 23 (2002) 115–123.
- Hinterdorfer, P. *Handbook of Nanotechnology* (Ed.: B. Bushan), Springer Verlag, Heidelberg (2004) 475–494.
- Hinterdorfer and Dufrene. *Nature Methods* 3 (2006) 347–355. <Not mentioned in this application note>
- Hubbard Ed. *Encyclopedia of Surface and Colloid Science* Vol. 1 (2002) CRC Press. P. 469.
- Hugel *et al.* *Physical Review Letters* 94 (2005) 048301-1 to 048301-4.
- Kamruzzahan *et al.* *Bioconjugate Chemistry* 17, 6 (2006) 1473–1481.
- Kienberger *et al.* *Single Mol.* 1 (2000a) 123–128.
- Kienberger *et al.* *Single Mol.* 1 (2000b) 59–65.
- Kienberger *et al.* *BIOforum Europe* 06 (2004a) 66–68.
- Kienberger *et al.* *Biol. Proc. Online* 6 (2004b) 120–128.
- Kienberger *et al.* *J. Mol. Biol.* 347 (2005) 597–606.
- Kienberger *et al.* *Acc. Chem. Res.* 39(1) (2006) 29–36.
- Lee *et al.* *Science* 266 (1994) 771–773.
- Lee *et al.* *Science* 266 (1996) 771–773.
- Lee *et al.* *PNAS USA* 104(23) (2007) 9609–9614.
- Lehenkari *et al.* *Biochemical and Biophysical Research Communications* 259 (1999) 645–650.
- Lin *et al.* *Emerging Information Technology Conference Proceedings IEEE Aug.* (2005).
- Lin *et al.* *Biophysical Journal* 90 (2006) 4236–4238.
- Liu *et al.* *Langmuir* 15 (1999) 8547–8548. <Not mentioned in this application note>
- Lohr *et al.* *Methods* 41 (2007) 333–341.
- Marcus *et al.* *Biochemical and Biophysical Research Communications* 342 (2006) 1123–1129.
- Nevo *et al.* *Nature Structural Biology* 10 (2003) 553–557.
- Noy *et al.* *Annu. Rev. Mater. Sci.* 27 (1997) 381–421.
- Raab *et al.* *Nature Biotechnology* 17 (1999) 902–905.
- Ratto *et al.* *Biophysical Journal* 86 (2004) 2430–2437.
- Ray *et al.* *J. Phys. Chem. B* 111 (2007) 1963–1974.
- Riener *et al.* *Single Mol.* 2(2) (2001) 127–128.
- Riener *et al.* *Recent Research Developments in Bioconjugate Chemistry* 1 (2002) 133–149.
- Riener *et al.* *Analytica Chimica Acta* 479 (2003) 59–75.
- Ros *et al.* *PNAS USA* 95 (1998) 7402–7405.
- Schumakovitch *et al.* *Biophysical Journal* 82 (2002) 517–521.
- Schwesinger *et al.* *PNAS USA* 97(18) (2000) 9972–9977.
- Sekiguchi *et al.* *Biophysical Journal* 85 (2003) 484–490.
- Stroh *et al.* *Biophysical Journal* 87 (2004a) 1981–1990. Stroh *et al.* *PNAS* 101, 34 (2004b) 12503–12507.
- VanVilet *et al.* *Nanotoday* 1(3) (2006) 18–25.
- Vig, J. R. in *Handbook of Semiconductor Cleaning Technology* (Ed. by Kern, W.) Noyes Pub., NJ (1993) 233–273.
- Vinckier *et al.* *Biophysical Journal* 74 (1998) 3256–3263.
- Wang *et al.* *Biophysical Journal* 83 (2002) 3619–3625.
- Wojcikiewicz *et al.* *Biol. Proc. Online* 6(1) (2004) 1–9.
- Wong *et al.* *Science* 275(5301) (1997) 820–822.
- Yoshitake *et al.* *Eur. J. Biochem.* 101 (1979) 395–399.

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