Keysight Technologies

Attaching Antibodies to AFM Probes with the Sulfhydryl Reactive PEG Tether, NHS-PEG_{18}-PDP

Application Note

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Introduction

AFM probes can be transformed into sensitive, chemically selective biosensors by attaching ligand molecules to the tips of the probes. Single-molecule molecular recognition force microscopy (MRFM) is an AFM-based technique that relies heavily on probes that have been modified with ligand molecules [Riener *et al.* 2003, Hinterdorfer 2004]. In MRFM, single-molecule unbinding interactions between ligands and complementary receptors that are immobilized on a substrate are observed and quantified one by one as the AFM cantilever approaches and subsequently withdraws from the substrate. 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]. The 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 ligands on an AFM probe and complementary receptors on a substrate. TREC resolves recognition maps of ligand-receptor interactions by scanning an AFM probe, which contains immobilized ligands, over a substrate, which contains receptors, in magnetic AC (MAC) Mode. Using a Keysight AFM equipped with PicoTREC, which resolves the TREC AFM signals, the lateral positions of functionally active receptors can be resolved with nanometer resolution [Stroh *et al.* 2004a, Stroh *et al.* 2004b, Kienberger *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, Stroh et al. 2004a, Stroh *et al.* 2004b], 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, such as a MAC lever, while corresponding receptor molecules are bound to a flat substrate, such as mica, silicon, flat glass, or a gold-coated substrate.

This protocol describes the attachment of antibodies to AFM probes via short polyethylene glycol (PEG) linkers. The heterobifunctional, amine and sulfhydryl reactive PEG linker, PDP-PEG_{18}-NHS, should be synthesized in an organic chemistry laboratory [Haselgruber *et al.* 1995, Kamruzzahan *et al.* 2006] or purchased from a vendor that performs custom synthesis. The AFM probes will be cleaned, activated with an alkoxy aminosilane, PEGylated with PDP-PEG_{18}-NHS, and then conjugated with the antibody.

The antibodies will be activated with sulfhydryl groups so that they can couple effectively with the sulfhydryl reactive PEG linker. With minor modifications, the methods described in this protocol may also be applicable to other sulfhydryl reactive NHS-PEG linkers, including NHS-PEG_{12}-maleimide and NHS-PEG_{24}-maleimide (both of which are available from Quanta BioDesign of Powell, Ohio USA) and NHS-PEG_{27}-maleimide and NHS-PEG_{29}-SS-Pyr (available from PolyPure of Oslo, Norway) as well as other commercially available or custom-synthesized sulfhydryl reactive NHS-PEG_n linkers. However, it should be noted that longer or shorter linkers may not be as well suited to TREC imaging as PEG_{18} [Kamruzzahan *et al.* 2006].

The NHS ester of S-acetylthiopropionate (SATP reagent from Pierce Biotechnology) is useful to introduce sulfhydryl groups into proteins, peptides, and other molecules, so that they can be conjugated with linkers that contain sulfhydryl reactive groups, including NHS-PEG-PDP. 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 four to five sulfhydryl groups per mole of antibody (MW approximately 150 kDa). The sulfhydryl groups are introduced in a protected form. After the reaction between the antibody and SATP, a desalting column is used to purify the sulfhydryl-modified antibody from excess reagent and other byproducts of the reaction. Later, the antibody will be treated with hydroxylamine to deacylate (deprotect) the sulfhydryl group before it is coupled with an AFM probe that has been modified with a PDP-PEG18 linker.

Before proceeding, ensure that the antibody is active, stable, and extremely pure. It 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. If there are any doubts about the purity of the antibody, it should be purified before use. It is recommended that an ELISA be performed on the antibody in order to ensure activity.

Materials

- *N*-succinimidyl-S-acetylthiopropionate (SATP reagent, Pierce Biotechnology)
- PD 10 column (GE Healthcare)
- Aminopropyltriethoxysilane (APTES packaged under argon, SureSeal, Sigma Aldrich)
- DMSO (Sigma Aldrich)
- Trizma Base (Sigma Aldrich)
- Methylene chloride (Sigma Aldrich)
- Ethanol (Sigma Aldrich)
- Hydroxylamine-HCl (Sigma Aldrich)
- NHS-PEG18-PDP (for synthetic method, see Haselgruber *et al.* 1995, Kamruzzahan *et al.* 2006)
- Sterile PBS buffer (0.1 M phosphate, 0.15 M NaCl, 1 mM EDTA, pH 7.4)
- Argon
- Type IV AFM probes (Keysight Technologies)
- Purified antibody
- Sterile 18 Mega ohm water

Special Equipment

- UV-Vis spectrophotometer
- UV-ozone cleaner
- Small vacuum desiccator jar
- pH meter

Chemical Reactions

Activation of Antibody with SATP

The NHS ester moiety of SATP is easily hydrolyzed in the presence of moisture, which will make the reagent nonreactive, so it should be stored in the presence of desiccant at -20 to -80°C. After removing the container from the freezer, it should be equilibrated to room temperature for 30 minutes before opening in order to avoid condensation. Dilute the antibody to a concentration of 60 µM (for an antibody of MW 150,000 Da, 60 µM = 9 mg/ml) in sterile PBS buffer.

Immediately before use, dissolve 5 mg of SATP (*N*-succinimidyl-S-acetylthiopropionate; Pierce Biotechnology) in 0.3 mL of DMSO to give a 68 mM solution of SATP. Quickly add 10 µL (approximately 680 nmoles) of the SATP reagent solution to 1 mL (approximately 60 nmoles) of the antibody solution [Lohr *et al.* 2007]. With the aid of a mechanical vortex apparatus, mix the solution and incubate at room temperature for 30 minutes. (Figure 1.)

Purification of SATP-modified Antibody

After 30 minutes, separate the SATP-modified antibody from the unreacted and hydrolyzed SATP reagent using a PD 10 column (GE Healthcare). Elute the column with PBS EDTA buffer at a flow rate of 1 mL/min and collect 0.5 mL fractions. It can be assumed that no large protein molecules will be taken into the matrix of the column and the void volume of the PD 10 column is equal to 2.5 mL; therefore, antibodies should elute from the column after approximately 2.5 mL. Unreacted and hydrolyzed SATP reagent and other small molecules should be retained in the column matrix. Any proteins in the eluted fractions will absorb light at 280 nm, so they will be detectable with the aid of a UV-Vis spectrophotometer. Any fractions lacking absorbance at 280 nm can be discarded. The fractions absorbing at 280 nm are combined and the concentration

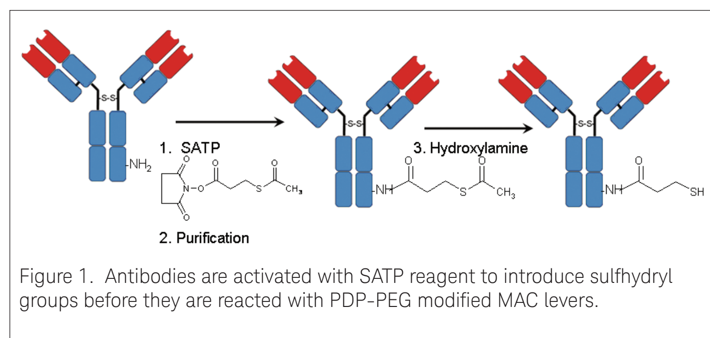

Figure 1. Antibodies are activated with SATP reagent to introduce sulfhydryl groups before they are reacted with PDP-PEG modified MAC levers.

of protein calculated [Layne 1957, Stoscheck 1990]. The number of SATP groups per antibody should be approximately 4:1, which can be experimentally determined by Ellman's procedure [Ellman 1958]. The SATP-modified antibody solution should be aliquoted into 75–85 µg portions and stored at -80°C.

Cleaning AFM Probes

Surface contaminants, which are commonly acquired during manufacturing, shipping, storage, and handling of AFM probes, can severely impact surface chemistry. Any contaminants must be removed so that they do not interfere with the coupling reactions. AFM probes contain a paramagnetic coating that is easily dissolved in strong acids and bases and many AC and contact mode cantilevers contain a reflective metal coating that can easily be damaged in harsh conditions. Therefore, gentle cleaning procedures are required. The AFM probes must first be rinsed in organic solvent to remove oils and gross contaminants and then be cleaned in a UV-ozone cleaner to remove organic and other oxidizable surface contaminants [Lohr *et al.* 2007, Stroh *et al.* 2004b, Vig 1993].

Five AFM probes are soaked in methylene chloride with their tips facing upwards for 1 hour. After 1 hour, the AFM probes are carefully rinsed under a stream of methylene chloride and placed in a UV-ozone cleaner, again with their tips facing upwards. The UV-ozone cleaner is turned on and the tips are exposed to ozone for 1 hour. Vig 1993 describes the method for ozone cleaning in great detail. After one hour, the AFM probes are removed and immediately used in the next step.

Aminofunctionalization of AFM Probes using APTES (3-aminopropy-triethoxysilane)

The tips of AFM probes are composed of silicon or silicon nitride. Both silicon and silicon nitride surfaces are covered in a natural oxide layer, so they contain numerous reactive SiOH groups. Methods have been developed to cover the oxide surface of silicon, silicon nitride, glass, and quartz materials with amine groups. One method that has been used successfully to coat the tips of AFM probes and other AFM probes with a uniform layer of amine groups is esterification using ethanolamine [Hinterdorfer *et al.* 1996, Raab *et al.* 1999, Ray *et al.* 2007, Riener *et al.* 2003].

Alkoxy aminosilanes are also commonly used to prepare aminated AFM probes. Traditional reactions of alkoxy aminosilanes in solvents such as toluene, ethanol, or acetone are commonly utilized [Lin *et al.* 2005, Ros *et al.* 1998, Schumakovitch *et al.* 2002, Schwesinger *et al.* 2000, Vinckier *et al.* 1998] for this purpose, but alkoxy aminosilanes can easily form polymers in solution, which can add unnecessary bulk and roughness to the tip of the AFM probe [Lee *et al.* 1996]. An alkoxy aminosilane reaction that takes place in the vapor phase circumvents the negative issues associated with silanization in solution and results in a uniform layer of amine groups on the surface of the AFM probes. The method utilizes freshly distilled aminopropyltriethoxysilane (APTES) under an inert argon atmosphere [Lohr *et al.* 2007, Kamruzzahan *et al.* 2006, Ratto *et al.* 2004, Riener *et al.* 2003, Stroh *et al.* 2004b, Wang *et al.* 2002, Lee *et al.* 2007].

The freshly cleaned AFM probes are removed from the UV-ozone cleaner and immediately placed in a Petri dish in the bottom of a clean, dry, small glass vacuum desiccator jar along with the lids from two 1.5 mL microcentrifuge tubes. The jar is purged with argon for 10 minutes and 10 µL APTES (3-aminopropy-triethoxysilane; Sigma Aldrich) and 10 µL N,N-diisopropylethylamine are quickly added to the microcentrifuge lids. The jar is purged with argon for 2 more minutes, sealed, and allowed to stand for 1 hour. The AFM probes should not be removed from the vacuum desiccator jar until the next step.

Conjugation of Disulfide-PEG-NHS Linkers to the Amine Functionalized AFM Probes

Dry methylene chloride (0.5 mL, Sigma Aldrich), triethylamine (7 µL, Sigma Aldrich), and 7.5 mg of PDP-PEG-NHS linker [Haselgruber *et al.* 1995, Hinterdorfer *et al.* 1998, Kamruzzahan *et al.* 2006, Stroh *et al.* 2004a, Stroh *et al.* 2004b] are added to a small glass vial that has been dried in an oven. The vial is sealed and gently inverted several times until all of the solid substances have dissolved. After the AFM probes have been in the vacuum desiccator jar with the APTES for 1 hour, they are removed and immediately and carefully added to the vial containing the PDP-PEG-NHS linker with their tips facing upwards. The vial is sealed and allowed to stand in a cool, dark location for 2 hours.

After 2 hours, the AFM probes are removed from the vial and carefully rinsed with methylene chloride, ethanol, and then 18 Mega ohm water and placed with their tips facing upwards in a Petri dish filled with PBS buffer. (Figure 2.)

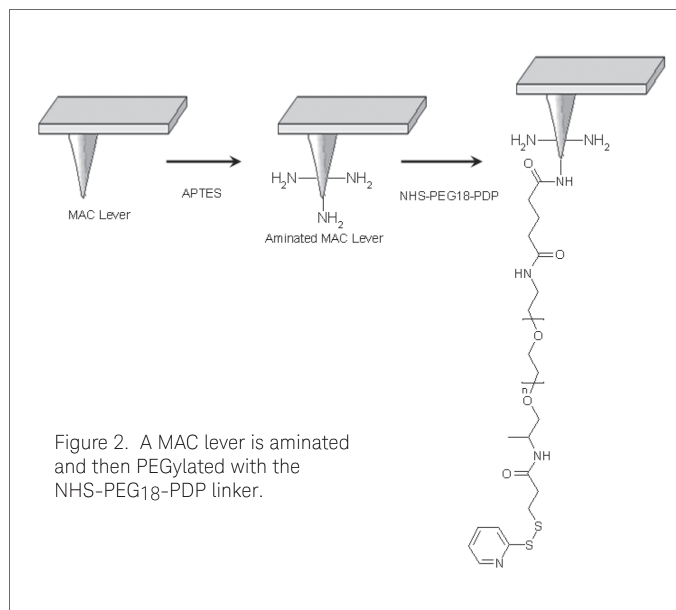

Figure 2. A MAC lever is aminated and then PEGylated with the NHS-PEG$_{18}$-PDP linker.

SATP-Modified Antibodies

SATP Deacetylation Buffer
SATP deacetylation buffer is made by mixing 184 mg hydroxylamine-HCl (Sigma Aldrich), 600 mg Trizma Base (Sigma Aldrich), 100 mL of sterile 18 Mega ohm water, and adjusting the pH of the solution to 7.5.

Deacetylation of SATP-modified Antibodies and Coupling of Antibodies to PDP-PEG AFM Probes
A small sheet of the parafilm is pressed into a sterile polystyrene Petri dish with the paper backing facing up so that it just covers and sticks to the bottom of the dish. The paper backing is removed to provide a clean surface and the Petri dish is covered to prevent contamination.

A 75 µg aliquot of the purified SATP-modified antibody solution (approximately 10 µL) is removed from the freezer and allowed to thaw. The SATP deacetylation buffer solution (100 µL) is added to SATP-modified antibody and the solution is mixed using a mechanical vortex apparatus and allowed to stand for 1 hour.

After 1 hour, a pair of tweezers are cleaned with ethanol and a lint-free lab tissue. Using the clean tweezers, the disulfide-PEG functionalized AFM cantilevers are placed in the parafilm-coated Petri dish with their tips pointed upwards and inwards in a circular manner similar to the shape formed by the spokes of a wagon wheel. Still using the tweezers, each AFM cantilever is gently pressed down onto the surface of the parafilm so that it lightly adheres to the paraffin. It is important that the AFM cantilevers adhere firmly to the parafilm to prevent them from floating away when the SATP-modified antibody solution is added in the next step. It is also important to work quickly (so the PEG linkers do not dry out) and carefully (in order to avoid breaking the AFM probes).

When all of the AFM probes are arranged in the Petri dish, 50 µL of the SATP-modified antibody in deacetylation buffer is added to the center of the circle formed by the AFM cantilevers so that the solution just touches the tips of the cantilevers. The Petri dish is covered to prevent evaporation and allowed to stand for 1 hour.

After the AFM probes have reacted with the SATP-modified antibody for 1 hour, they are rinsed in PBS buffer and placed in a Petri dish containing PBS buffer. The AFM probes are now ready to be utilized in MRFM or TREC experiments [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]. It is strongly recommended to use the modified AFM probes immediately, but they can often be stored for 2–3 days at 2–8°C under sterile conditions without significant loss of activity. (Figure 3.)

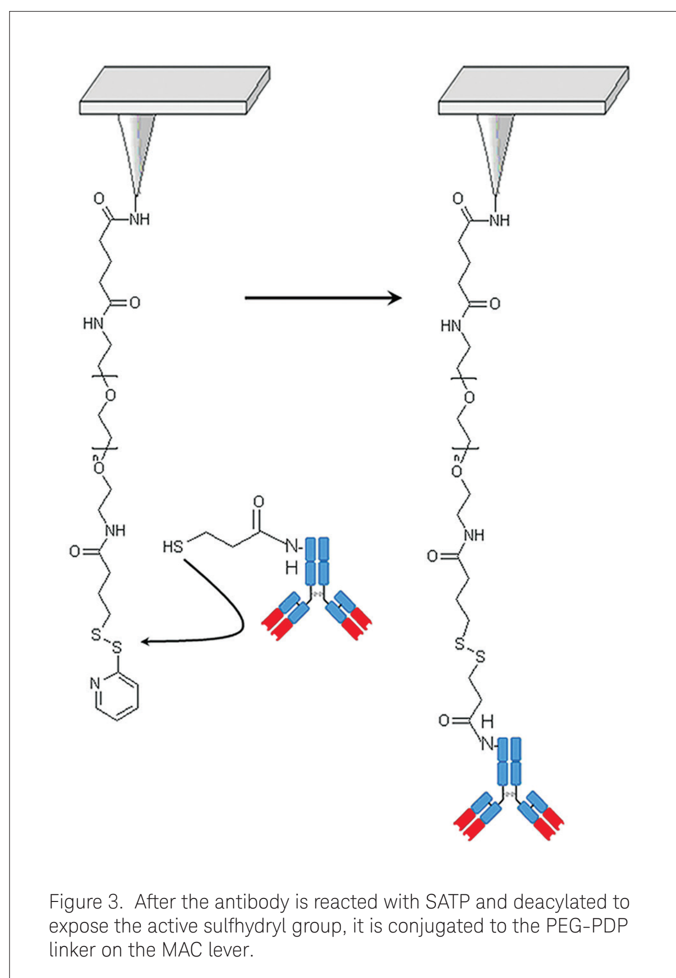

Figure 3. After the antibody is reacted with SATP and deacylated to expose the active sulfhydryl group, it is conjugated to the PEG-PDP linker on the MAC lever.

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