Modified AFM Probes for Nanoscale Biomolecular Recognition Studies

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ABSTRACT

Biomolecular 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 (atomic force microscopy) allows scientists to visualize, probe and analyze the structures of biological molecules in their native environments with high resolution. Organic chemistry, surface chemistry, bioconjugaton chemistry and AFM can be combined and used to locate or quantify specific biomolecular interactions. For example, in molecular recognition force microscopy (MRFM), single molecule interactions are observed and quantified one by one as an AFM probe that has been modified with a ligand or an antibody approaches and then is subsequently withdrawn away from a surface that contains binding sites. Topography and recognition imaging (TREC) also relies on AFM probes that have been modified with biomolecular entities. TREC is a dynamic AFM method in which the modified AFM probes are oscillated and scanned over a sample in order to generate a topography image of the sample's surface along with a high resolution map of the locations of biomolecular interactions. With this method, specific types of molecules can be identified in compositionally complex samples with nanometer-scale lateral resolution.

Keywords: single molecule biosensor, molecular recognition, SPM, AFM,

1 INTRODUCTION

Molecular recognition force microscopy (MRFM) is an AFM-based technique that relies heavily on probes the have been modified with ligand molecules [1, 2]. In MRFM, single molecule unbinding interactions between ligands and complimentary receptors are observed and quantified one by one as the AFM probe approaches and then is subsequently withdrawn away from the immobilized receptors. These force spectroscopy (FS) experiments can give valuable information about the structure and dynamics of molecular unbinding events at the single molecule level [3] and the technique has also been effectively applied to gain an understanding of the intramolecular forces involved in protein folding and polymer elongation [4]. Topography and recognition imaging (TREC) is another single molecule AFM technique that also utilizes immobilized ligands. TREC resolves recognition maps of ligand-receptor interactions by scanning the immobilized ligands, which are attached to an AFM probe, over the a surface. Using an AFM that is equipped with magnetic AC (MAC) Mode and PicoTREC (Agilent Technologies) to resolve the TREC AFM signals, the lateral positions of functionally active receptors can be resolved [5, 6, 7]. TREC imaging has been used to map the locations and analyze the chemical compositions of a variety of samples; including molecular interactions between nucleic acids and proteins [8], antibodies and antigens [5, 6, 9], and small ligands and their receptors [10].

Several different biochemical immobilization and bioconjugation chemistry schemes can be applied to investigations of ligand-receptor interactions by AFM. However, the use of PEG of defined chemistry and length to immobilize the molecules of interest has been shown to offer some advantages over other methods. Several short PEG tethers can be purchased functionalized with useful end groups which permit the attachment of a wide variety of interesting biological molecules and since the PEG linkers permit ligands to diffuse in defined volumes of space, ligands immobilized in such a manner are more likely to encounter receptors immobized on substrates than ligands that are attached directly to AFM probes. Furthermore, ligand-receptor interactions that involve ligands attached to PEG linkers undergo less torque than ligand-receptor interactions involving ligands attached directly to AFM probes [11], so encounters between immobilized receptors and PEG-tethered ligand molecules are more similar to encounters involving freely diffusing ligand molecules in solution. Consequently, the kinetics of ligand-receptor interactions that involve PEG tethered ligands are perhaps more applicable to kinetics involving ligand molecules in solution. PEG is a water soluble, nonadhesive polymer, so unwanted nonspecific interactions

between the ligand molecules and the AFM probe tip and between the probe tip and receptors on the substrate can be minimized [1, 11, 12]. Furthermore, monodispersive PEG linkers with well defined lengths can help resolve single molecule unbinding interactions from multiple unbinding events and help distinguish specific interactions from undesirable nonspecific interactions [13].

2 CHEMISTRY

There are three steps for immobilizing biological molecules on AFM probes: 1. AFM probe cleaning and amination, 2. PEGylation of the aminated probes, and 3. Immobilization of biological molecules on the PEGylated probes.

2.1 AFM Probe Cleaning and Amination

The tips of the AFM probes must be thoroughly cleaned to remove contaminants that may have physisorbed to the probes during manufacture, shipping, storage or handling. Strong acids such as nitric acid, H_2SO_4, HF or piranha are often used for this purpose [1, 13, 16, 19, 20, 21] but such harsh conditions can damage some AFM probes. For example, MAC levers, which are required for TREC imaging, contain a paramagnetic film which can be easily damaged in harsh conditions. Furthermore, gold or aluminum reflective coatings are on the backsides of many probes and the metal coatings can be damaged by harsh conditions. Consequently, relatively gentle cleaning methods, including solvent rinses [1, 17], plasma cleaning [22] or ozone cleaning procedures [19, 23, 24] are required.

Silicon and silicon nitride surfaces are covered in a native oxide layer that contains numerous reactive silanol (Si-OH) groups which can be modified in much the same manner as carboxylic acids. The pKa of the silanol group is approximately 6.8 [25]. Various reactions have been developed to add a thin layer of amine groups to oxidize silicon. For example, silicon and silicon nitride surfaces can be esterified using ethanolamine in dry DMSO [1, 13, 15, 26]. When heat and molecular sieves are added, water can be removed from the reaction and equilibrium is driven in favor of esterification; much as azeotrophic distillation of alcohols and water drives equilibrium in favor of esterification with carboxylic acids. Silanols react with alkoxysilanes in organic solvents such as toluene, ethanol or acetone [16, 17, 18, 21, 27], so alkoxyaminosilanes can also be used to prepare aminated AFM probes. Unfortunately, alkoxysilanes easily form polymers in solution [28] which can add bulk and roughness to AFM probe tips; decreasing AFM imaging resolution. However, by performing alkoxysilane reactions in the vapor phase under an inert atmosphere and with very pure aminopropyltriethoxysilane (APTES) or by performing aminations by esterification with ethanolamine, it has been demonstrated that the sharpness and smoothness of AFM probes can usually be maintained [1, 6, 14, 22, 23, 29, 30].

2.2 PEGylation of Aminated Probes

Heterobifunctional PEG tethers may be purchased or synthesized with a variety of end groups that permit their attachment to aminated AFM probes and to allow the immobilization of interesting biological entities. Homobifunctional amine-PEG-amine and heterobifunctional amine-PEG-COOH linkers are usually utilized as intermediates in the synthesis of other tethers with specific reactive functional groups. For example, starting with amine-PEG_{18}-amine, Hasegrubler et al described the synthesis of a PEG_{18} tether with a *N*-hydroxysuccinimide (NHS) ester on one terminus and a 2-pyridyldithio (PDP) group on the other terminus [31]. Such NHS ester modified PEGs can be immobilized on APTES or ethanolamine activated AFM probes [11, 32, 33]. Sulfhydryl containing proteins [29, 34] or thiolated oligonucleotides [8, 17] can be immobilized directly onto the NHS-PEG-PDP or NHS-PEG-maleimide linkers. The NHS-PEG_{18}-PDP linker has served to immobilize ligands in several successful MRFM and TREC imaging experiments [5, 6, 11, 20, 22, 29, 34, 35, 36, 37]. Genetically engineered His6 tagged proteins can be tethered to AFM probes via PEG linkers containing nitrilotriacetic acid (NTA) end groups [11, 32, 38, 39, 40, 41]. The synthesis and application of a PEG linker that contains an amine reactive aldehyde group terminus has been described [33]. The NHS-PEG-aldehyde linker was used to immobilize antibodies [33, 42, 43] and virus particles [33] on AFM probes for MRFM and TREC imaging. Antibodies and other biological molecules that have free amine groups can be anchored to the PEG-aldehyde linker without protein preactivation and purification steps. Various other reactive PEG linkers can also be synthesized by those skilled in the art of organic chemistry.

2.3 Immobilizing Biological Molecules on PEGylated AFM Probes

A virtually endless list of biological molecules can be attached to AFM probes to quantify ligand-receptor interactions in MRFM studies or to map molecular interactions with PicoTREC, but before proceeding with any bioconjugation reaction, it is wise to ensure that the protein is active, stable and extremely pure. For example, the proteins that are to be attached to nucleophile-reactive PEG tethers should be free of ammonium ions (including amine buffers such as Tris), BSA, gelatin, azide and any other protein "stabilizing agents" or contaminants because these materials can interfere with the coupling reactions. It is also recommended that functional assays be performed on all antibodies before performing the bioconjugation reactions in order to ensure that the antibodies are functional and react specifically with their particular antigens.

Native sulfhydryl groups in proteins can be utilized to link them to PEG-maleimide or PEG-PDP tethers on

aminated AFM probes. However, free sulfhydryl groups may not always be available, in which case surface disulfides must be reduced to prepare them for bioconjugation. At issue in many cases is the fact that disulfide reduction comes with an inherently high risk of protein inactivation [26, 44]. Fortunately, a more gentle approach involving the NHS ester of S-acetylthioacetic propionic acid (SATP reagent from Pierce Biotechnology, Rockford, IL) can be used to introduce active sulfhydryl groups onto proteins, peptides and other molecules so that they can be conjugated with PEG-maleimide or PEG-PDP tethers [5, 6, 11, 20, 22, 29, 34, 35, 36, 37]. The reaction between the SATP reagent and an amine group on the surface of a protein creates a stable, covalent amide bond that generally preserves the protein's inherent activity. The level of sulfhydryl incorporation may be controlled by varying the molar ratios of SATP and antibody, but higher levels of acylation generally correspond to greater degrees of protein inactivation. Thiolated oligonucleotides are readily available from oligonucleotide primer and probe vendors, so these can also be covalently conjugated with PEG-maleimide modified AFM probes [35].

A more straightforward approach for immobilizing proteins and other biological molecules on AFM probes involves the use of a novel NHS-PEG-aldehyde linker. The proteins that are to be immobilized with this linker do not require disulfide reduction, preactivation with SATP or other reagents [33, 42, 43]. The NHS ester portion of the PEG linker reacts quickly with aminated AFM probes but the reaction of amines with the aldehyde moiety at the other end of the linker is not so kinetically favorable, so the aldehyde remains intact and available to react with lysine residues on the protein of interest. The reaction between and amine and an aldehyde generates a Schiff's base intermediate which is easily hydrolyzed, but the intermediate can be stabilized as equilibrium is driven to the right with a specific Schiff's base reducing agent (such as $NaCNBH_3$). Ebner et al used the NHS-PEG-aldehyde linker to immobilize antibodies and, interestingly, human rhino virus particles to AFM probes. Subsequently, the modified probes were used to study specific interactions between the virus particles and human receptor proteins [33].

It is usually advantageous to optimize the density of ligand molecules on AFM probes to control the number of molecules that can have access to receptors immobilized on a substrate. The methods described above often result in ligand densities ranging from 200-500 molecules per μm^2, which is well suited for many single molecule unbinding studies. For example, an AFM probe with a tip radius of 20-50 nm and 200-500 molecules per μm^2 translates into approximately one molecule per effective tip area [13]. At this density, just one ligand on the AFM probe may have access to immobilized receptors at any given time.

Although modified AFM probes are often extremely difficult to characterize and evaluate by means other than their use in actual MRFM or TREC imaging experiments, several different methods to determine the density of ligands on AFM probes have been developed [34]. These methods utilize relatively larger silicon or silicon nitride substrates that are treated with identical chemical conditions as the AFM probes and they are based on (a) direct fluorescence, (b) fluorescently labeled secondary antibodies and (c) a horse radish peroxidase (HRP) based assay [13, 20].

3 CONCLUSION

Bioconjugation chemistry and surface immobilization chemistry can enhance the power and utility of AFM. Biomolecular 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 allows scientists to visualize, probe and analyze the structures of biological molecules in their native environments with high resolution and without the need for extraneous labels or tags. Organic chemistry, inorganic surface chemistries, bioconjugaton chemistry and AFM force spectroscopy and/or imaging methods can be combined with AFM methods and used to locate or quantify specific biomolecular interactions. Nanoscale chemistries and AFM have been been exploited to study ligand receptor interactions such as biotin-avidin [1, 10, 11, 41, 45], nucleotide-nucleotide interactions [28, 46], DNA-protein interactions [8], interactions between cell surface receptors and adhesion proteins [22, 36, 37, 43, 47, 48], antibody-antigen interactions [6, 13, 20, 23, 26, 34, 35, 39, 49] and interactions between virus particles and human virus receptors [33].

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