Attaching Biological Molecules to AFM Probes for Nanoscale Molecular Recognition Studies

W. Travis Johnson

Agilent Technologies, Nanotechnology Measurements Division
4330 W Chandler Blvd, Chandler Arizona 85226

ABSTRACT

Atomic Force Microscopy (AFM) is an important tool to study nanoscale molecular interactions. A strong suit of AFM is its ability to measure ligand-receptor interactions with picoNewton sensitivity. These biomolecular interactions are critical factors in a variety of physiological processes; such as the initiation, modulation and termination of DNA replication, transcription, enzyme activity, infection, immune responses, tissue generation, wound healing, cell differentiation, apotopsis, and physiological responses from drugs, hormones and toxic agents. Using AFM, scientists can probe and quantify these interactions in their native environments or perform dynamic experiments *in situ* by removing or adding ions, solutes or other reagents to the sample environment. Ligand-receptor unbinding forces and several kinetic parameters can be calculated and used to infer structural information about the molecular interactions. Nanoscale bioconjugation chemistry and surface chemistry are often required in these studies because, for the AFM to resolve the mechanical forces required to separate the ligands from their targets, the ligands must be immobilized on the AFM probe and the receptors need to be immobilized on stationary substrates.

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

1 INTRODUCTION

In MRFM (molecular recognition force microscopy) studies, ligand molecules are often covalently attached to the tip of an AFM probe which transforms the AFM probe into a highly specific, single molecule biosensor [1]. In MRFM, single molecule unbinding interactions are observed and quantified individually as the ligand-functionalized AFM probe approaches and then is subsequently withdrawn away from the substrate which contains the immobilized receptor molecules. Therefore, MRFM relies heavily on surface chemistry and nanoscale bioconjugation chemistry. AFM force spectroscopy experiments such as these, can give valuable information about the structure and dynamics of single molecule unbinding events with nanoNewton precision [2]. These techniques have also been applied to gain an understanding of the intramolecular forces involved in protein unfolding [3]. Topography and recognition (TREC) imaging is another single molecule technique that is based on AFM. TREC also utilizes probe-bound ligands and immobilized receptors [4]. However, unlike force spectroscopy, TREC is a dynamic technique. In TREC, the ligand-functionalized AFM probe is scanned and oscillated over the surface that is covered with immobilized receptors. Specific interactions between the ligand molecules on the AFM probe and receptor molecules on the substrate are resolved as small changes in the probe's oscillation amplitude [5]. TREC imaging allows molecular interactions to be identified on compositionally complex samples such as biological materials. Using an AFM equipped with PicoTREC (Agilent Technologies), which resolves the signals that arise from molecular recognition events from the topography signals, the lateral positions of receptors on cells or other biological surfaces can be resolved with nanometer resolution [6, 7, 8, 9].

2 ATTACHING BIOLOGICAL MOLECULRES TO AFM PROBES

In MRFM and TREC studies it is often advantageous to attach biological molecules to a short tether that is in turn attached to the tip of the AFM probe because the tether permits the molecules to diffuse within a defined volume of space [10]. The tether also imparts upon a ligand the ability to reorient its position as it approaches the target in order that they may bind efficiently.

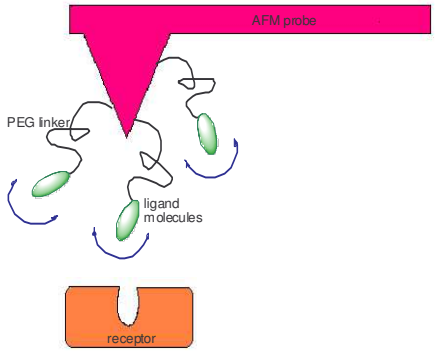

Figure 1. Ligands immobilized to an AFM probe by PEG tethers. The PEG tethers are flexible and allow the ligands to diffuse and bind with the receptor in an optimal manner.

NSTI-Nanotech 2008, www.nsti.org, ISBN 978-1-4200-8503-7 Vol. 1

Short polyethylene glycol (PEG) linkers are used in many force spectroscopy applications. PEG is a water soluble, nonadhesive polymer, so, nonspecific interactions can be minimized [1, 11]. Many PEG tethers can be purchased functionalized with variety of useful end groups to permit the attachment of a wide range of molecules. The number of PEG molecules on the tip of the probe can often be controlled. When the number of ligand-receptor binding interactions are minimized, single molecule interactions can be resolved [1, 12]. PEG linkers permit probe-bound molecules to diffuse within in a well defined volume of space, so that the ligands may be more likely to encounter and bind to receptors on the substrate. The steps involved in biological molecule immobilization to AFM probes are: 1. Cleaning: Gentle cleaning methods are preferred (organic solvents, UV-ozone); 2. AFM Probe Activation (amination): Silicon and silicon nitride AFM probes should be silanized with an amniosilane (APTES) or esterified (ethanolamine); 3. PEGylation: Bifunctional PEG linkers are attached to the activated AFM probes.; 4. Bioconjugation: The biological molecules are attached to the PEG tethers and 5. Characterization: The number of molecules on the surface of the AFM probe must be determined.

2.1 AFM Probe Activation

When both silicon and silicon nitride surfaces are exposed to air or water they will naturally oxidize so that the surfaces of silicon and silicon nitride materials are covered in a thin oxide layer that contains numerous reactive SiOH groups. The pKa of SiOH is approximately 6.8 [13], 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 esterified using ethanolamine in dry DMSO to add a layer of amine groups [1, 12, 14, 15].

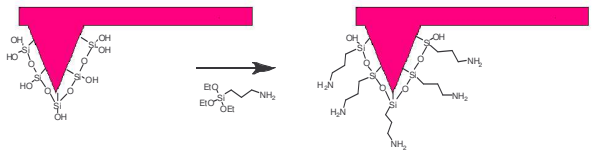

Figure 2. Activation (amination) of AFM probes with aminopropyltriethoxysilane (APTES)

Alkoxy aminosilanes may also be used to prepare aminated AFM probes. Traditional reactions of in solvents such as toluene ethanol or acetone [16, 17, 18, 19, 20] should be avoided because alkoxy aminosilanes have a propensity to form polymers in solution. This can add bulk and roughness to the tip of the AFM probe [21]. However, by performing AFM probe amination reactions in the vapor phase using freshly distilled APTES (aminopropyltriethoxysilane), tip smoothness and tip sharpness can be generally be maintained [1, 7, 9, 22, 23, 24, 25].

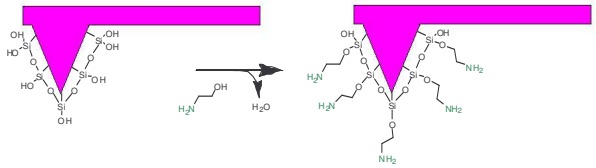

Figure 3. Amination of AFM probes with ethanolamine

2.2 PEGylation and Bioconjugation

PEG tethers can be synthesized or purchased with various end groups so that they can be attached to activated AFM probes and anchor many 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/or TREC studies. References that describe their synthesis or commercial sources, where they can be purchased, are also included in the table.

The NHS ester group is very reactive towards the amine groups on activated AFM probes so NHS-PEG linkers can be attached directly to these probes [11, 26, 27]. Both PEG-PDP and PEG-maleimide are reactive towards sulfhydryl groups. Consequently, cystine terminated peptides and proteins [23] or sulfhydryl modified oligonucleotides [28] can be linked directly to PEG-PDP and PEG-maleimide tethers on AFM probes. In addition, many proteins can be modified with sulfhydryl reactive reagents to facilitate their attachment to immobilized PEG-PDP and PEG-maleimide tethers [8, 9, 11, 22, 23, 29, 30, 31, 32, 33]. The synthesis of a PEG tether possessing an amine reactive aldehyde group at one terminus was recently described [27]. The aldehyde group of this tether can be reacted directly with protein molecules that contain lysine groups available near the protein's surface [6, 27, 34] or even with virus particles [27]. Antibodies or other biological molecules that have free amine groups may be anchored to the PEG-aldehyde tether without laborious protein preactivation and purification steps. Various other PEG molecules with assorted of 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 synthesis.

2.3 Characterization

After the AFM probes have been functionalized with the PEG linkers and the biological molecules, it is imperative that steps be taken to determine the density of the ligand molecules on the AFM probe. The methods described above generally will result in approximately one molecule per effective tip area [12], so just one ligand on the AFM probe may have access to the receptors on the substrate at any given time. The Hinterdorfer lab in Linz

Austria has developed several methods to calculate ligand density on AFM probes [33]. The methods utilize relatively larger silicon or silicon nitride substrates which are treated in parallel under identical reaction conditions along with the AFM probes. The methods are based on (a) direct fluorescence, (b) fluorescently labeled secondary antibodies and (c) horse radish peroxidase (HRP) [12, 32].

3 CONCLUSION

Biomolecular interactions are critical to most biological phenomena. AFM offers unique advantages over many other tools for the study of biological process at the nanometer scale so it has become an important tool. For example, AFM allows scientists to visualize, probe, and analyze biological interactions in their native environments with unprecedented resolution and without the need for extraneous labels or tags. As described above, bioconjugation chemistry and surface chemistry can enhance the power and utility of AFM by allowing specific biomolecular interactions to be quantified and/or located on biological surfaces.

REFERENCES

[1] Riener et al *Analytica Chimica Acta* 479 (2003) 59-75

[2] Noy et al *Annu. Rev. Mater. Sci.* 27 (1997). 381–421

[3] Allison et al *Curr. Opinion Biotechn.* 13, (2002) 47–51

[4] Hinterdorfer, P *Handbook of Nanotechnology* (Ed.: B. Bushan), Springer Verlag, Heidelberg (2004) 475–494

[5] Kienberger et al *Biol. Proc. Online* 6, (2004) 120–128

[6] Chtcheglova et al *Biophysical Journal* 93 (2007) L11-L13

[7] Lee et al *PNAS USA* 104(23) (2007) 9609–9614

[8] Stroh et al *Biophysical Journal* 87 (2004a) 1981-1990

[9] Stroh et al *PNAS* 101, 34 (2004) 2503–12507

[10] Kienberger et al *BIOforum Europe* 06 (2004a) 66-68

[11] Reiner et al *Recent Research Developments in Bioconjugate Chemistry* 1 (2002) 133-149

[12] Hinterdorfer et al *PNAS USA* 93 (1996) 3477–3481

[13] Hubbard Ed. *Encyclopedia of Surface and Colloid Science* Vol 1 (2002) CRC Press. P. 469

[14] Raab et al *Nature Biotechnology* 17 (1999) 902-905

[15] Ray et al *J. Phys. Chem. B* 111 (2007) 1963-1974

[16] Lin et al *Emerging Information Technology Conference Proceedings*, IEEE Aug. (2005)

[17] Ros et al *PNAS USA* 95 (1998) 7402–7405

[18] Schumakovitch et al *Biophysical Journal* 82 (2002) 517–521

[19] Schwisinger et al *PNAS USA* 97(18) (2000) 9972–9977

[20] Vinckier et al *Biophysical Journal* 74 (1998) 3256-3263

[21] Lee et al *Science* (1996) 266, 771-773

[22] Lohr et al *Methods* 41 (2007) 333–341

[23] Kamruzzahan et al *Bioconjugate Chemistry* 17, 6 (2006) 1473-1481

[24] Ratto et al *Biophysical Journal* 86 (2004) 2430–2437

[25] Wang et al *Biophysical Journal* 83 (2002) 3619–3625

[26] Kienberger et al *Single Mol.* 1 (2000) 59–65

[27] Ebner et al *Bioconjugate Chem.* 18 (2007) 1176-1184

[28] Lin et al *Biophysical Journal* 90 (2006) 4236-4238

[29] Bash et al *FEBS Letters* 580 (2006) 4757–4761

[30] Baumgartner et al *PNAS USA* 97(8) (2000) 4005–4010

[31] Baumgartner et al *Single Mol.* 1, 2 (2000) 119-122

[32] Hinterdorfer et al *Nanobiology* 4 (1998) 177-188

[33] Hinterdorfer et al *Colloids and Surfaces B: Biointerfaces* 23 (2002) 115–123

[34] Bonanni et al *Biophysical Journal* 89 (2005) 2783-2791

[35] Kienberger et al *Single Mol.* 1 (2000) 123–128