

# Chemical Functionalization of AFM Cantilevers

by  
Sunyoung Lee

B.S., Chemical Engineering  
Seoul National University, 2002

Submitted to the Department of Materials Science and Engineering  
in partial fulfillment of the requirements for the degree of

Master of Science in Materials Science and Engineering  
at the  
**MASSACHUSETTS INSTITUTE OF TECHNOLOGY**

September 2005

© Massachusetts Institute of Technology 2005, All rights reserved.

Author.....

Department of Materials Science and Engineering

August 24, 2005

Certified by.....

Professor Krystyn J. Van Vliet

Lord Foundation Assistant Professor of Materials Science and Engineering

Thesis Supervisor

Certified by.....

Professor Angela M. Belcher

John Chipman Career Development Professor of Materials Science and

Engineering and Biological Engineering

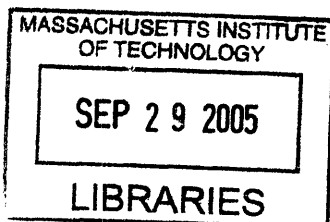
Thesis Reader

Accepted by.....

Professor Gerbrand Ceder

R. P. Simmons Professor of Materials Science and Engineering

Chair, Department Committee on Graduate Students



ARCHIVES

# Chemical Functionalization of AFM Cantilevers

by

Sunyoung Lee

Submitted to the Department of Materials Science and Engineering  
on August 24, 2005, in partial fulfillment of the  
requirements for the degree of  
Master of Science in Materials Science and Engineering

## Abstract

Atomic force microscopy (AFM) has been a powerful instrument that provides nanoscale imaging of surface features, mainly of rigid metal or ceramic surfaces that can be insulators as well as conductors. Since it has been demonstrated that AFM could be used in aqueous environment such as in water or various buffers from which physiological condition can be maintained, the scope of the application of this imaging technique has been expanded to soft biological materials.

In addition, the main usage of AFM has been to image the material and provide the shape of surface, which has also been diversified to molecular-recognition imaging – functional force imaging through force spectroscopy and modification of AFM cantilevers. By immobilizing of certain molecules at the end of AFM cantilever, specific molecules or functionalities can be detected by the combination of intrinsic feature of AFM and chemical modification technique of AFM cantilever. The surface molecule that is complementary to the molecule at the end of AFM probe can be investigated via specificity of molecule-molecule interaction. Thus, this AFM cantilever chemistry, or chemical functionalization of AFM cantilever for the purpose of chemomechanical surface characterization, can be considered as an infinite source of applications important to understanding biological materials and material interactions.

This thesis is mainly focused on three parts: (1) AFM cantilever chemistry that introduces specific protocols in details such as adsorption method, gold chemistry, and silicon nitride cantilever modification; (2) validation of cantilever chemistry such as X-ray photoelectron spectroscopy (XPS), AFM blocking experiment, and fluorescence microscopy, through which various AFM cantilever chemistry is verified; and (3) application of cantilever chemistry, especially toward the potential of force spectroscopy and the imaging of biological material surfaces.

Thesis Supervisor: Professor Krystyn J. Van Vliet

Title: Lord Foundation Assistant Professor of Materials Science and Engineering

# Acknowledgements

First of all, I would like to express my sincere gratitude to my research advisor, Professor Krystyn J. Van Vliet for her dedication, sacrifice, support, and guidance. But for her endurance and understanding, I couldn't have finished my master's degree. She was willing to give me insights to address various kinds of problems and to listen to my hardships. Especially, she taught me how enthusiastic and diligent every professional should be. I am also grateful to the kind guidance of group members: Catherine A. Tweedie, M. Todd Thompson, Binu Oommen, Emilio Silva, Emily Walton, and Ellie Bonsaint. I thank Yemisi Afere who worked with me during summer 2005.

Outside the laboratory for mechanically coupled material systems, I thank God who has been always with me before even time began.

Finally, my love and this thesis are dedicated to my wife, Shinyoung, and my parents in Korea. I owe my whole life to my beloved.



# Contents

<b>1</b>	<b>Introduction</b>	<b>8</b>
1.1	Background	8
1.2	The Principle of Atomic Force Microscopy	9
<b>2</b>	<b>AFM Cantilever Functionalization Chemistry</b>	<b>14</b>
2.1	Adsorption Chemistry	15
2.1.1	Functionalization of Cantilevers	15
2.2	Gold Chemistry	17
2.3	Silicon Nitride Chemistry	21
2.3.1	Amine Derivitization	22
2.3.1.1	APTES Method	22
2.3.1.2	Ethanolamine Hydrochloride Method	24
2.3.2	Conjugation of Chemical Linkers	24
2.3.2.1	PEG-PDP Linker	24
2.3.2.2	PEG-biotin Linker	25
2.3.3	sATP Derivitization of Proteins	25
2.3.4	Disulfide Conjugation of Proteins	26
<b>3</b>	<b>Characterization of Modified Cantilevers</b>	<b>28</b>
3.1	Adsorption Reaction of BSA-biotin	28
3.2	Gold thiol chemistry	30
3.3	Si <sub>3</sub> N <sub>4</sub> chemistry	33
<b>4</b>	<b>Applications of Chemical Modification</b>	<b>39</b>
4.1	Resolution of Atomic Force Microscopy	39
4.1.1	Biotin or streptavidin-immobilized cantilevers	40
4.2	Intermolecular Interactions	42
4.3	Force Spectroscopy	43
<b>5</b>	<b>Conclusion</b>	<b>45</b>
5.1	Summary	45
5.2	Suggestions for future work	46
	<b>Bibliography</b>	<b>47</b>

# List of Figures

1-1	How AFM works . . . . .	10
2-1	Schematic of functionalization of AFM tips with streptavidin following BSA-biotin adsorption . . . . .	16
2-2	Streptavidin-immobilized gold surface via MUA coating . . . . .	19
2-3	Silicon dioxide-coated silicon nitride cantilever . . . . .	23
3-1	Validation of BSA-biotin adsorption protocol . . . . .	29
3-2	Validation of gold-thiol protocol . . . . .	31
3-3	Optimal concentration of MUA and streptavidin . . . . .	32
3-4	AFM blocking experiment . . . . .	35
3-5	anti-VEGFR2 conjugation on the fluorescence microscopy . . . . .	36
4-1	Fluorescence microscope images of biotinylated cell surface molecules . . . . .	40
4-2	Polymer-patterned surface . . . . .	41
4-3	Force spectroscopy data . . . . .	43

# List of Tables

3-1	Comparison with three chemical reactions .....	37
-----	--	----





# Chapter 1

## Introduction

### 1.1 Background

Since the invention of scanning probe microscopy [6, 7], a variety of new research areas have been opened. While scanning tunneling microscopy can map atomistic detail due to tunneling electron transfer between the conductive probe and conductive material surface, atomic force microscopy (AFM) can image the surface of insulators as well as conductors. In addition, one of the unique advantages of the AFM is the capability of this instrument to operate in liquid environments [8]. With all of these features, the AFM has stimulated the study of objects on a nanometer scale [9] and made it possible to provide a detailed image of diverse materials such as metals, ceramics, polymers, and biological structures. The fundamental principle of the AFM is the measurement of surface height and force interactions through either (1) displacement-enabled maintenance of a constant force between the cantilever and the sample or (2) force-enabled maintenance of a constant distance between the cantilever and the sample. In either case, the interaction forces between the molecules at the end of cantilever and molecules on the sample surface dictate image features.

This fundamental principle of the AFM has enabled the study of interactions between single molecules. At the same time, surface modification techniques using surface chemistry based upon self-assembled monolayers (SAMs) have been developed and are particularly important to AFM cantilevers that are made of silicon nitride or are gold-coated. Furthermore, molecular binding forces between a small number of various molecules have been measured directly by molecular force spectroscopy using the AFM [10-14].

Therefore, molecular recognition with the AFM has been facilitated by the chemical modification of the AFM cantilevers, together with the development of surface modification chemistry and delicate AFM force spectroscopy. Various ligands have been attached at the end of the cantilevered AFM probe, among which antibodies were preferred because these macromolecules exhibit complementary antigens and because this general antibody-antigen interaction has been thoroughly studied in the areas of biochemistry [12, 15]. While the primary research objective over the past decade has been to prove the versatility of the AFM in molecular recognition applications, the current challenge is to identify the location of specific molecules on a heterogeneous surface via immobilization of known molecules on the AFM cantilever. One of the most intriguing systems that displays a complex and heterogeneous surface is the living (or chemically fixed) cell surface [12, 16-19].

This thesis work concentrates on the surface modification chemistry that is an intermediate and critical step in the detection of molecules on complex biomaterial surfaces such as living mammalian cells.

## 1.2 The Principle of Atomic Force Microscopy

The atomic force microscope is a type of scanned-proximity probe microscope. All such scanning probe microscopes investigate a local property with a probe placed very close to the sample surface. The small probe – sample separation distance and the sharpness of the probe make it possible to measure features over nano- to microscale surface areas [20]. According to the interaction between the sample and the probe and the movement of the probe, there are two kinds of modes that were used in this thesis: constant contact mode and intermittent contact (or tapping) mode. When the basic operating principles of AFM are presented, the constant contact mode is usually discussed. Figure 1-1 schematically illustrates the principle of the AFM, specifically for constant contact mode imaging.

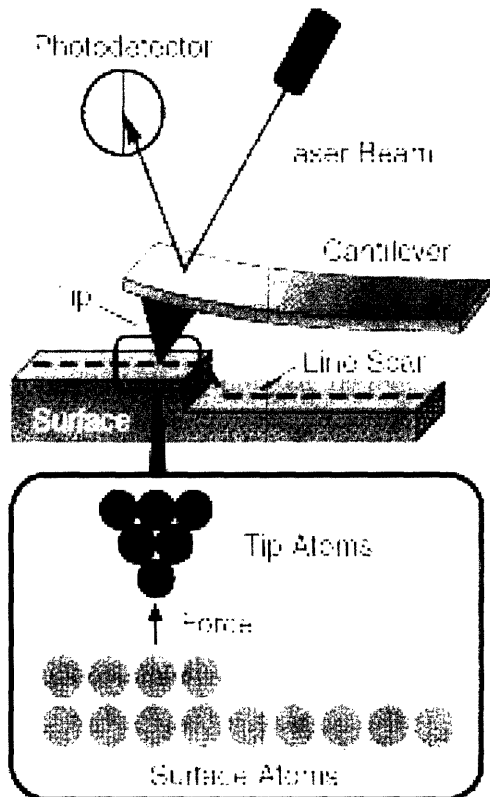


Figure 1-1: How AFM works [1] - The laser beam first emitted by the scanner and reflected by the photodetector is converted to a voltage signal. Three forces are involved in the interaction between tip atoms and surface atoms: van der Waals force, capillary force, and mechanical force of the cantilever.

Three forces are involved between the probe and the sample: repulsive van der Waals force, mechanical force of the cantilever, and capillary force of fluid between the cantilever and the sample surface. As the probe comes in contact with the sample surface, van der Waals forces become repulsive. Thus, van der Waals force plays a role in keeping the probe from attaching to the surface. When imaging in air, a layer of water condensation and other molecular contamination covers both the tip and sample, forming a meniscus that pulls the two surfaces together [21], which generates the capillary force. The mechanical force of the cantilever together with the capillary force acts as driving forces that attach the probe to the sample surface; in other words, these forces compensate for the repulsive van der Waals force. The probe – sample separation distance is determined by the balance between the van der Waals force and the summation of the capillary-mechanical forces. When imaging is conducted in aqueous environments, repulsive van der Waals force balances with the mechanical force of the cantilever and the capillary force is negligible.

While the force between the sample surface and the probe is controlled through

electronic feedback in constant contact mode, tapping mode instead controls the oscillation of a cantilever. This can be achieved either through magnetic alternating current (MAC) mode to oscillate the free end of a magnetically coated cantilever directly, or through acoustic alternating current mode (AAC) to oscillate the base of the cantilever or the sample stage. Soft or weakly bound samples may be damaged in contact mode by the direct exertion of force and considerable traction forces applied to the surface to maintain constant contact force. In contrast AAC mode operates in the intermittent contact regime by vibrating the cantilever or the piezoactuated sample stage, so relatively small forces ( $F < 0.5$  nN) are applied to the sample feature compared to contact mode imaging ( $F > 0.5$  nN). The cantilever base (in AAC mode) or cantilever free end (in MAC mode) is driven and oscillated by the alternating current (AC) or magnetic field, respectively. While the cantilever is moving along the surface of the sample, the amplitude of the oscillation changes due to the interaction forces between the tip and a certain surface feature. This change in the amplitude of the oscillation with respect to the user-requested driving amplitude of the cantilever is represented as an amplitude image; it is in fact an “error signal” image that represents imperfect control of the electronic feedback loop. The frequency of oscillation of the piezo or the cantilever is shifted with respect to the driving oscillation input signal (that is, an alternating current in AAC mode or a magnetic field in MAC mode) according to the mechanical or other properties of the sample surface (e.g., smooth, rigid or soft). This time lag between the input and output signal is presented as a phase image, and qualitatively represents the energy absorbed by the sample surface due the applied frequency and magnitude of contact [1, 22].

Alternatively, the AFM imaging modes can be categorized as constant force (or amplitude in tapping mode) mode and constant height mode. These two modes are related to whether the feedback control is on or off. When the feedback control is switched on, the positioning piezo that governs the movement of the cantilever base moves to keep the pre-

determined value in response to the change in the force (or amplitude) between the cantilever free end (probe) and the sample surface, resulting in a constant applied force (or amplitude) mode. If the feedback control is off or the electronic gains are significantly reduced (but nonzero to avoid the problems caused by thermal drift of the piezoactuator), the AFM is operated to keep the cantilever base or piezoactuator height fixed, which is called constant height mode. This mode is especially useful for a flat surface that requires imaging of nanoscale surface features [23].

The way in which the AFM maintains contact with and collects data from probe-sample interactions is to use the feedback loop control. A laser beam (Class II red) is reflected on the back of the cantilever free end, and this light is collected by a photodetector (quadrant photodiode) that converts the light signal to voltage. The sum of the voltages acquired on all four quadrants is the total laser signal voltage; vertical deflection of the cantilever is determined by the difference between the upper and lower halves of the photodiode, and lateral deflection is determined by the difference between the left and right halves of the photodiode. This voltage is compared with the setpoint that is also represented as a voltage value. The “deflection image” in contact mode or “amplitude image” in AC mode is based upon this difference between the setpoint and actual voltage value. The feedback loop orders the system to move the piezoactuator at the cantilever base up or down to keep the cantilever deflection (or amplitude) constant by adjusting the voltage applied to move the piezo. The topography or “height image” is acquired from the magnitude of this voltage [20]. In other words, topography image is composed of the piezoelectric voltage required to maintain the user-requested photodiode voltage (setpoint). The calibration constant that relates the photodiode voltage [V] to cantilever deflection [m] is determined by deflecting the cantilever against a rigid surface [24]. In such a case, the downward deflection of the cantilever base (which is known in [m] via prior calibration of the piezocrystal response) is equal to the

upward deflection of the cantilever free end. The slope of free end deflection (in [V]) vs. fixed end deflection (in [m]) should therefore be unity, and the conversion constant (in [m/V]) is thus determined. Because the relationship between the voltage and the cantilever deflection is set up by the calibration constant, the photodetector voltage can also be linked with force by a Newton's second force law,  $F = C x$  where  $C$  is a spring constant of the cantilever and  $x$  is the cantilever free end deflection. The spring constant is measured by considering the cantilever as a simple harmonic oscillator whose thermal energy is commensurate to the elastic energy of the cantilever. The resonance (vibrational) frequency of the cantilever is monitored, and this vibration receives thermal energy equal to elastic energy, that is,  $k_B T = C \langle x^2 \rangle$  where  $k_B$  is Boltzmann's constant,  $T$  is temperature, and  $\langle x^2 \rangle$  is a measured variance of the deflection, from which the spring constant,  $C$ , can be calculated [25].

## Chapter 2

# AFM Cantilever Functionalization Chemistry

The main purpose of modifying the cantilevered AFM probe is to effectively detect complementary molecules on the material surface of interest by optimizing the interaction between the molecules on the sample surface and those on the AFM probe. There are several factors that should be considered for this purpose. First of all, the molecules on the AFM probe should withstand mechanical interaction with sample surface. As discussed in Chapter 1, the fundamental principle of atomic force microscopy is to keep the force or the distance between the probe and the surface molecules constant, so it is natural that a shear stress is applied to the probe itself (or molecular modifiers added to the probe) while the probe is traveling along the surface of the sample. Second, the interaction between modifier molecules on the tip and the complementary molecules on the sample surface must be maximized to overcome the inherent signal-to-noise ratio in such experiments. For example, if a ligand-receptor interaction is to be detected by the AFM, the ligand (or receptor) on the AFM probe must physically contact and strongly interact with the receptor (or ligand) on the sample surface. The extent of this interaction is related to the number of modifier molecules, the extent to which modifier molecules aggregate, and the length and structural rigidity of chemical linkers that connect the ligand (or receptor) to the rigid AFM probe, as well as whether the modifier molecules at the end of probe remain active after the chemical reactions required to functionalize the probe with them. Another limiting factor can be the orientation of the terminal molecule extending from the AFM probe: Even though molecules may be active and have the ability to bind the complementary molecule on the sample surface, this

binding will not occur unless the binding pockets of the receptor encounters the ligand at the correct orientation, and at an optimal rate/duration of time.

Therefore, AFM probe functionalization through molecular chemistry is the starting point required to generate topographical images that faithfully reproduce the molecular detail of the actual sample surface.

## 2.1 Adsorption Chemistry

Adsorption is simply the preferential partitioning of a substance from the liquid (or gaseous) phase onto the surface of solid substrate [26], but now it is understood as an expanded concept that includes binding of molecules onto a solid surface. This adsorption is mediated by van der Waals forces or electrostatic interactions between adsorbate molecules and the atoms that compose the adsorbent surface. In general, it is caused by the combination of two forces. Thus, when adsorption between protein molecules and the solid surface is expected, these proteins are expected to expose the positive or negative amino acid side chains, so that these charged groups can bind to the oppositely charged adsorbent solid surface.

### 2.1.1 Functionalization of Cantilevers

It is important that the inorganic AFM probe surfaces are well-cleaned prior to chemical functionalization. Here, the  $\text{Si}_3\text{N}_4$  cantilevers were cleaned in acetone, ethanol, and dichloromethane and then further cleaned by UV plasma. This cleaning was followed by biotinylated bovine serum albumin (BSA) functionalization. After the functionalization, the BSA on the probe was treated with 1% glutaraldehyde for fixation, which means that amine groups on the BSA molecules and the silicon nitride cantilevers bind to each other via double-functionality of the aldehydes group of glutaraldehyde.



The AFM cantilever (that represents the adsorbent surface in this case) is made of  $\text{Si}_3\text{N}_4$ , and BSA is used as an adsorbate species. The main purpose of attaching the BSA is to introduce biotin molecules at the end of probe, so biotinylated BSA was purchased from Pierce Biotechnology. One BSA molecule is conjugated with eight (8) biotins by the manufacturer (on a molar basis) [2]. The adsorption and/or immobilization of BSA is maximized in the presence of monovalent cations,  $\text{Na}^+$  or  $\text{Cl}^-$  [27]. In addition, it is important that the adsorption of biotinylated BSA takes place at  $\text{pH} = 8.3$  or higher, because the basic conditions appear to facilitate BSA adsorption to the  $\text{Si}_3\text{N}_4$  cantilever [2]. The optimal buffer molarity and  $\text{pH}$  in the current experiments was found through iteration to be 0.4nmol for 5 cantilevers and 9, respectively. Before the adsorption, it is best that the cantilevers to be oxidized to form a  $\text{SiO}_x$  layer by placing them in a UV cleaner for 2 hrs at room temperature [28]. BSA proteins were fixed with 1% glutaraldehyde to prevent the BSA molecules from spontaneously desorbing from the surface. Some proteins are denatured by adsorption to a charged surface, but BSA is not [29].

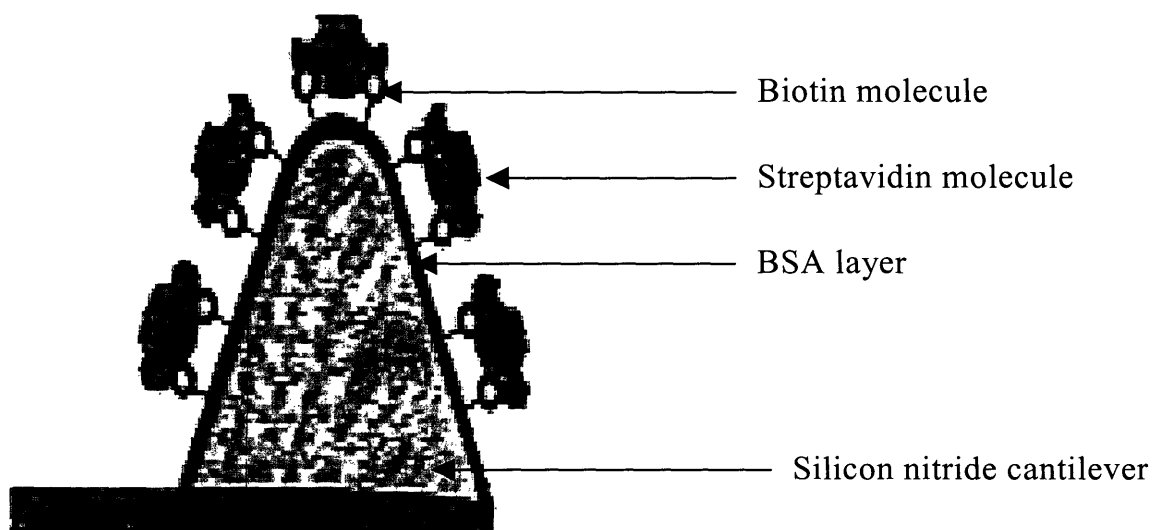


Figure 2-1: Schematic of functionalization of AFM tips with streptavidin following BSA-biotin adsorption [2] - BSA-biotin that forms black layer is adsorbed on silicon nitride cantilever followed by addition of streptavidin molecules. In this figure, two binding pockets to biotin are exposed to the outside, which provides another chance to add biotinylated molecules.

The advantage of this adsorption method is that it is relatively easy to execute this reaction, and it is possible to quantify the number of biotin from the fact that one BSA molecule has eight (8) biotin molecules. Biotin has a very high affinity to streptavidin ( $K_d = 10^{-15}$  M), which is the strongest non-covalent binding known [30]. Biotin and streptavidin are easy to conjugate with fluorophores and/or dyes such as fluorescein, which makes the characterization possible on the fluorescence optical microscopy (See Chapter 3). This adsorption reaction itself is stable. However, as far as the binding between the biotinylated BSAs and complementary molecules is concerned, this binding cannot be guaranteed, due to the fact that the linker that relates biotin to BSA via NHS biotinylation is short (0.3nm) [I calculated]. Thus, there may be insufficient space and mobility for the biotin molecules to bind to streptavidin attached to another rigid surface. In addition, if magnetic AC mode [22] of AFM is used, wherein the oscillation of the probe is generated by applying magnetic field directly against a magnetically coated probe, the vibration energy can dissociate the binding of adsorption because the adsorption is based upon relatively weak van der Waals forces or electrostatic interactions.

Adsorption can be used to attach avidin molecules to negatively charged mica surface to check whether biotin functionalized to the AFM probe is active. Avidins, like streptavidins, also bind to biotin very strongly. However, the charged groups of avidin are exposed to the outside of the molecule, while those of streptavidin are not. Thus, even though streptavidin arbitrarily aggregates and slightly adsorbs onto the surface via van der Waals interaction, streptavidin cannot be adsorbed to rigid surfaces. This will be more discussed further in Chapter 3.

## 2.2 Gold Chemistry

Gold-thiol binding is a well-known interaction because such reactions are straightforward to implement, chemical reagents are commercially available, and more importantly, the binding between gold and thiol groups is strong. There are many theories that explain the characteristics of the molecular level of this binding, but it is generally accepted that this is a covalent but strongly polarized bond because of the electronegativity of S atom that shifts the distribution of electrons after binding [31]. Because the gold-thiol binding has a feature of covalent bonding, it is stronger than adsorption-based binding which is the combination of weak van der Waals and electrostatic interactions. As AFM imaging exerts mechanical stress, it is required that modifier molecules adhere strongly to the AFM probe. In addition, we can easily purchase long molecules such as 11-mercaptoundecanoic acid (MUA) that has 11 carbons and is 16 nm in length. Such long, thiolated molecules increase the possibility for AFM probe modifiers to rotate sufficiently to detect surface molecules. Once this MUA monolayer is established, this layer prevents the aggregation and arbitrary adsorption of proteins to a gold surface. In this respect, this gold-thiol chemistry is a very good choice for functionalization of AFM probes. However, the process of gold coating (through, for example, sputtering) may structurally damage the AFM probe by coating and thus blunting the nm-scale probe radius. Further, the thin gold film may not withstand the mechanical stresses that the AFM probe undergoes during use without delaminating from the Cr-bond coated  $\text{Si}_3\text{N}_4$  cantilever.

The goal of gold chemistry within this thesis is to attach proteins to gold-coated AFM cantilevers without deactivating the protein. Streptavidin was used for the purpose of easy validation via fluorophore-conjugated biotin. The primary amines of the surface exposed lysine groups of streptavidin, which are involved in the amide bond formation via a nucleophilic displacement of N-hydroxysuccinimide (NHS) esters formed during N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) / NHS activation [5].

Instead of streptavidin, other proteins that have exposed lysine groups or amine conjugated molecules such as sulfo-NHS-biotin can also be used for this purpose.

$\text{Si}_3\text{N}_4$  cantilevers were first coated with a “bond coat” of chromium (20 nm thickness), and then coated with gold of 200 nm thickness using the sputterer in the Microsystems Technology Laboratories at MIT. The gold-coated cantilevers were first rinsed by piranha (70% of sulfuric acid and 30% of hydrogen peroxide) solution (or instead, acetone, ethanol, and dichloromethane subsequently), functionalized via MUA dissolved in ethanol (25mM); followed by the activation of carboxylic acid groups via the addition of EDC and NHS; and finally the conjugation of streptavidin (proteins or sulfo-NHS-biotin).

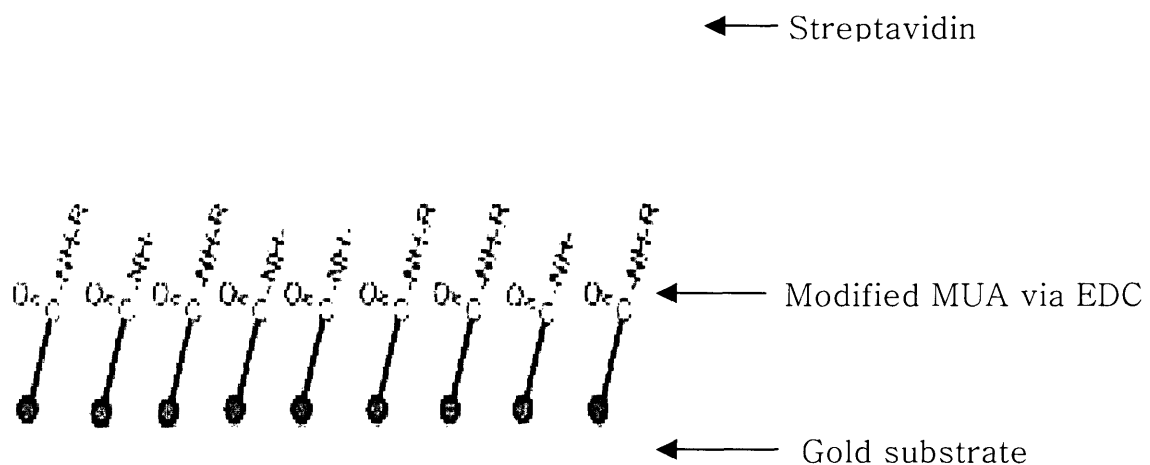


Figure 2-2: Streptavidin-immobilized gold surface via MUA coating [5]

This gold coating chemistry facilitates one of the most straightforward functionalization approaches possible. As Fig. 2-2 shows, the orientation of streptavidin (or proteins) on the AFM probe is arbitrary. There are 12 lysine groups on one streptavidin molecule, and all of these lysine groups can bind to activated carboxylic acid groups. If the size of streptavidin molecule is thought of as 5 nm (according to the molecular weight and the number of carbons), there can exist at the end of the probe about 50 active streptavidin

molecules that provide a variety of orientations. This estimate is calculated as follows: The geometry of the AFM probe is considered as the cone that has a round sphere at one end, and the diameter of this sphere is considered about 50 nm. When 1/8 of the surface area of the sphere probe is assumed to be exposed and active for the functionalization because of the curvature of the probe, the available surface area can be calculated as 981 nm<sup>2</sup>. The streptavidin molecule can be idealized as a circle that has a diameter of 5 nm, which will show that the cross-sectional area of one streptavidin molecule is 19.6 nm<sup>2</sup>. Thus, about 50 molecules of streptavidin can bind to the end of the AFM probe. The arbitrary orientations of streptavidin so added are an advantage and increase the chance to encounter biotin molecules on a sample surface, regardless of the movement of cantilever and the scanning rate. This is the main difference between this AFM probe functionalization and self assembled monolayers (SAMs) on flat surfaces that require uniform coverage and orientation. However, if the lysine groups in the binding pockets to biotin (or, more generally, binding sites to epitopes) are bound and become amide bonds, this may lessen the probability of streptavidin-biotin interaction. Once the functional group in the binding pocket is conjugated, the orientation of the actively binding portion of the molecule is towards the probe, not towards the sample surface. Another possibility is that if there is only one binding pocket for the epitope and the functional group in the pocket is bound, this binding pocket may be deactivated. All of these possibilities come from the fact that it is almost impossible microscopically to target a specific functional group using the bioconjugation techniques. Thus, the successful conjugation of a protein to the AFM probe surface through this approach is attained through statistical likelihood and therefore requires an excess of the molecule of interest to be conjugated to the probe surface.

Gold-coated cantilevers were cleaned with ethanol, acetone, and dichloromethane, followed by UV cleaning to attain highest level of purity. The cantilevers were soaked in

1mM MUA solution for 3 hrs or more and rinsed with ethanol by dipping in ethanol solution. Next step was activating carboxylic acid groups of MUA by adding 0.4M of EDC and 0.1M of NHS solution, leaving for 15mins; followed by adding 50uL of 0.1mg/mL of protein solution such as antibody or streptavidin, leaving for 2 hrs, and being rinsed with 1X PBS 5 times. Protein-conjugated tips should not be dried.

After the optimal protocol is established, this gold-thiol chemistry can be applied to attach antibody molecules (IgGs) to the probe because IgGs also contain prospective lysine groups exposed at the outside of this molecule. This attachment of IgG to gold surface via gold-thiol chemistry was attempted by Tlili et al.[32]. These authors activated the carboxylic acid group of MUA molecules on the gold surface with EDC and NHS, and then arbitrarily added these whole antibodies to react the activated carboxylic acid groups and lysine groups exposed to the outside of the antibody. Although the successful conjugation of the IgGs was not proven through an independent experiment, this reaction makes sense because there is no difference between this direct attack on lysine groups and the indirect attack via sATP-conjugated lysine group of the silicon nitride chemistry described in Section 2.3.

## 2.3 Silicon Nitride Chemistry

Although gold-thiol chemistry provides various advantages, gold-coated cantilevers are not easy to prepare, vulnerable to intrinsic damage caused by gold sputtering, and expensive to prepare and coat. Thus, it is important to consider alternative chemical reactions that do not require coating of the  $\text{Si}_3\text{N}_4$  probe. While the longest linker that can be commercially purchased for gold-thiol chemistry has 16 carbons (16-mercaptohexadecanoic acid) which is about 1.2 nm long, the longer linker commercially available for amine-derivitized surfaces is 7 nm. Such linkers can be attached to  $\text{Si}_3\text{N}_4$  cantilevers that are amine-derivitized. This is expected to improve the binding affinity between complementary molecules because this

longer linker will allow considerable movement of the terminal molecule while the probe is moving across the surface of sample.

## 2.3.1 Amine Derivitization

The main purpose of amine derivitization is to introduce amine groups to silicon nitride cantilevers. Primary amine groups are especially easily attacked by some functional groups such as carboxylic acids, aldehyde groups, and NHS esters. Thus, derivitization of amine groups on the sample can act as a starting point to bioconjugation chemistry.

### 2.3.1.1 3-(aminopropyl)triethoxysilane (APTES) Method

A silicon nitride surface in air has a several-nanometer-thick surface layer predominantly composed of silicon dioxide with surface silanol and silylamine groups [4]. A surface modification reagent used in this thesis is 3-(aminopropyl)triethoxysilane (APTES), which introduces amine groups on the silicon nitride surface. Many people use different methods [33], but two kinds of methods were employed herein: (1) chemical vapor deposition (CVD) in the gas phase and (2) aqueous phase adsorption of APTES dissolved in toluene. APTES is very sensitive to moisture, and will hydrolyze to produce ethanol and trisilanols when exposed to water molecules [34].

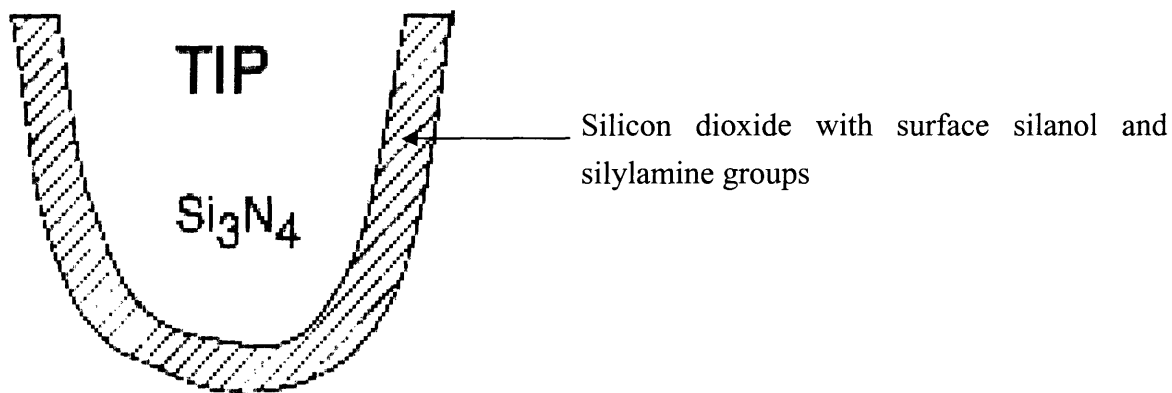


Figure 2-3: Silicon dioxide-coated silicon nitride cantilever [4]

Chemical vapor deposition (CVD) was used for amine derivitization. The silicon nitride cantilever was cleaned by acetone, ethanol, and dichloromethane, followed by ozone cleaning to introduce SiO<sub>x</sub> groups on the silicon nitride surface. Before putting the cantilever in a bell jar desiccator, the desiccator was evacuated with a slow but steady stream of Ar gas. APTES and N,N-diisopropylethylamine were prepared in the desiccator [22], and the cantilevers were inserted into the desiccator. After 2hrs of CVD reaction, the cantilevers were rinsed with dichloromethane or chloroform.

Another method related to the APTES method is using toluene solution that dissolves APTES. The silicon nitride cantilevers were cleaned by acetone, ethanol, and dichloromethane, followed by ozone cleaning. These cantilevers were dipped into an APTES solution in toluene (5 wt %) in a glove box to minimize humidity, followed by immersion in pure N,N-dimethylformamide. As a rinsing step, the cantilevers were cleaned with pure deionized water [35].

The most important step in such a procedure is to minimize moisture or humidity during this reaction. The CVD method requires only a couple of hours, so many people prefer this method due to time constraints. However, vacuum or dry Ar gas environments should be maintained between the step of filling gas or applying vacuum and that of putting APTES,



N,N-diisopropylethylamine, and cantilevers into a CVD chamber [28]. The solution method requires 24 hrs, the toluene solution should be anhydrous, and an additional instrument such as a glove box is required to minimize moisture. However, once these components are established, this solution approach is more reliable, as will be demonstrated in Chapter 3.

### 2.3.1.2 Ethanolamine Hydrochloride Method

While APTES provides three molecular linkages with  $\text{SiO}_x$ , ethanolamine provide one linkage. Thus, this bond is weaker than the one between the APTES and the silicon nitride surface. Ethanolamine hydrochloride was dissolved in dimethylsulfoxide (DMSO) (5.7 M) with molecular sieves to remove water without affecting other reagents. Cantilevers were cleaned with acetone, ethanol, and dichloromethane followed by overnight soaking in DMSO solution of ethanolamine hydrochloride.

## 2.3.2 Conjugation of Chemical Linkers

The length of the chemical linker is important. The optimal length of this linker is about 7 nm, which imparts sufficient molecular flexibility to allow the binding with the epitopes on a molecular sample surface during AFM imaging [36], while maintaining the bond strength via strong (covalent) bonding.

### 2.3.2.1 NHS-poly(ethylene glycol)-(pyridyldithio)propionate (PEG-PDP) Linker

This linker (provided by Molecular Imaging, Tempe, AZ, [37, 38]) has two functionalities: an NHS ester at one end and a disulfide bond at the other end. NHS ester can attack the primary

amine groups prepared by three methods mentioned above, and disulfide bonding enables sulfur exchange with another thiol group. The long PEG chain provides flexibility. After the binding of this linker, the proteins that have exposed thiol groups such as cysteine- or *N*-Succinimidyl-S-acetylthiopropionate (sATP)-derivitized proteins can switch to the disulfide bonds at the end of PEG-PDP linker.

After cleaning the amine-derivitized cantilevers with DMSO and dichloromethane, cantilevers were dried with N<sub>2</sub> gas. The PEG-PDP linker was dissolved in dichloromethane, and triethylamine was added as a proton scavenger. The cantilevers were immersed in this linker solution, followed by rinsing with dichloromethane. These cantilevers were dried with N<sub>2</sub> gas.

### 2.3.2.2 Poly(ethylene glycol)-biotin (PEG-biotin) Linker

While biotinylated BSA was adsorbed on silicon nitride cantilevers, as mentioned above, PEG-biotin provides a long linker of greater flexibility. The procedure of adding this linker is very similar to the above protocol utilizing the PEG-PDP linker. In other words, instead of the PEG-PDP linker solid, the PEG-biotin linker (provided by Molecular Imaging, [39]) is used.

### 2.3.3 *N*-Succinimidyl-S-acetylthiopropionate (sATP)

#### Derivitization of Proteins

Thiol groups are introduced to proteins in a protected form, allowing the modified molecule to be stored indefinitely and then later treated with hydroxylamine hydrochloride to expose the labile sulfhydryl group for the final conjugation reaction [40, 41]. The purpose of introducing this thiol group is to facilitate the attachment of proteins by elongating the

functional groups that can bind to the cantilevers. The PEG-PEG linker contains a disulfide bond (sulfur-sulfur), one sulfur of which can be substituted with a different thiol group to form another disulfide bond. Like the lysine amino acids (amine groups) of streptavidin that were conjugated with the carboxylic acid groups of MUA, this sATP also attacks the amine groups that are exposed to the outside of the molecules and plays a role in becoming a bridge for forming disulfide bond between the PDP-PEG linker and protein. When the molar ratio of sATP to protein is concerned, the number of lysine groups in protein is important. The larger number of sATP molecules will increase the probability of the completion of the reaction between the cantilever and the protein, but also has the potential to damage the intrinsic structure of the proteins and to denature them.

Two proteins were used: IgG and streptavidin. Derivatization of both proteins with sATP follows the same approach, except for the molar ratio of sATP to proteins according to the number of lysine groups in each protein. The number of lysine groups of IgG is about three times that of streptavidin. To conjugate streptavidin with sATP, a fivefold excess of sATP was used that came from the confirmation of the number of lysine groups in one streptavidin molecule. The IgG that was conjugated to the cantilever was anti-VEGFR2 (anti-Vascular Endothelial Growth Factor Receptor 2). This antibody contains 20 lysine groups, so a tenfold excess of sATP was used for this protein.

### 2.3.4 Disulfide Conjugation of Proteins

The proteins conjugated with sATP were prepared to exhibit protected thiol groups. To enable this stable thiol groups to attack disulfide bonding of PEG-PDP linker on the cantilever, hydroxylamine solution that exposes protected thiol groups was added to sATP-conjugated protein solution. Hydroxylamine solution was prepared by mixing hydroxylamine hydrochloride and ethylenediaminetetraacetic acid (EDTA) according to the protocol provided

by Pierce Biotechnology [42]. Biotin conjugated IgG that was also sATP-derivitized was provided by Molecular Imaging, Inc. The lysine groups of IgG were conjugated with NHS-biotin molecules that attack amine groups to form amide bonding. Hydroxylamine solution and this pre-derivitized IgG were added to the PEG-PDP functionalized cantilevers, all of which finally causes the attachment of biotin molecules to the AFM cantilevers for the purpose of detecting avidin or streptavidin molecules existing on a sample surface. If streptavidin is used instead of biotinylated IgG, this protocol can be terminated one step earlier to terminate the AFM probe with active streptavidin molecules.

Before and after the conjugation of sATP to proteins, proteins were purified with a desalting column. This column is a sort of chromatography where molecules are sieved according to the size and molecular weight. After collecting from the desalting column, every fraction was checked with a UV-VIS spectrophotometer (Cary). Proteins absorb at a wavelength of about 280 nm. The fraction that shows this peak contains protein molecules, and the concentration of the protein can be calculated by Beer's law. (Beer's law is the relationship between the absorbance and the concentration of molecules:  $A = ebC$  where  $A$  is an absorbance that can be measured by a spectrophotometer,  $e$  is an extinction coefficient,  $b$  is the length of a spectrophotometer cuvette, and  $C$  is the concentration of the solution. Every protein has its own extinction coefficient (e.g., the extinction coefficient of streptavidin is  $32 \text{ M}^{-1}\text{cm}^{-1}$  [43]).

# Chapter 3

## Characterization of Modified Cantilevers

### 3.1 Adsorption Reaction of BSA-biotin

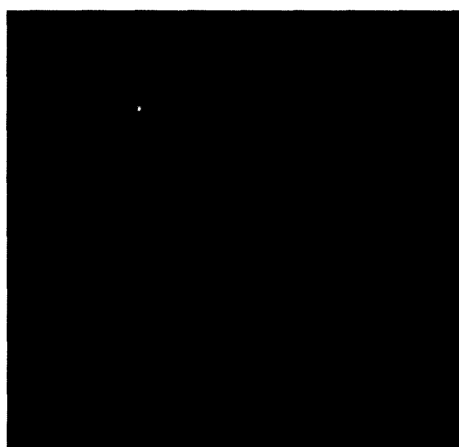
The adsorption protocol of Chapter 2 was optimized on bare  $\text{Si}_3\text{N}_4$  wafers prior to reaction on silicon nitride cantilevers. Fluorescence optical microscopy enables straightforward verification of the functionalization approach. Because the adsorbed molecule, BSA, contains biotin molecules, fluorophore-conjugated streptavidin can be used to check whether biotin is active, and the fluorophore-conjugated biotin can be used to check whether streptavidin is active.



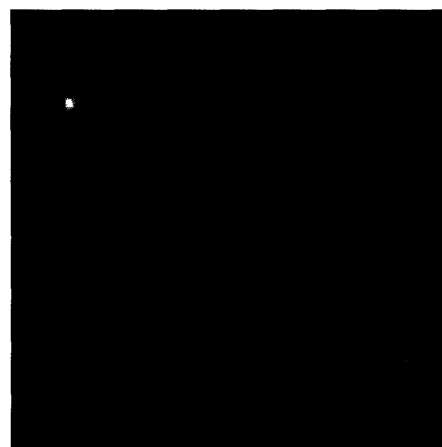
(a) Bare silicon nitride cantilever treated with streptavidin-fluorescein - no specific bindings, which acted as a control experiment

(b) BSA-biotin-adsorbed silicon nitride cantilever treated with streptavidin-fluorescein - specific bindings, which confirmed BSA-biotin molecules were active

(c) BSA-biotin-adsorbed silicon nitride cantilever, followed by streptavidin immobilization treated with biotin-fluorescein - specific bindings, which confirmed streptavidin attached to BSA-biotin molecules was active



(d) BSA-biotin-adsorbed silicon nitride cantilever, followed by streptavidin immobilization treated with streptavidin-fluorescein, which acted as a control experiment



(e) BSA-biotin-adsorbed silicon nitride cantilever, followed by streptavidin immobilization, streptavidin, and biotinylated molecules treated with biotin-fluorescein, which acted as a control experiment

Figure 3-1: Validation of BSA-biotin adsorption protocol

When this adsorption chemistry was first tried, the exact number of streptavidin and biotinylated molecules was calculated. However, there are a limited number of binding pockets to streptavidin and biotinylated molecules, which makes it more efficient to add an excess number of molecules, instead of adding the exact number. Even though molecules are left without binding to the binding pockets, unbound molecules are completely removed by rinsing with buffers. Because the binding affinity of streptavidin to biotinylated molecules (and vice versa) is very large (Chapter 2), the binding between two molecules is not destroyed after rinsing.

Figure 3-1 was obtained with the optimized protocol that was introduced in chapter 2. BSA-biotin was adsorbed followed by the addition of biotin-fluorescein, (b), and 90% of cantilever surface was covered with bright spots, from which above 90% of adsorbed BSA-biotin molecules are activated. Figure 3-1 (c) was obtained by adsorbing BSA-biotin, adding streptavidin and subsequently biotin-fluorescein to check if the streptavidin molecules bound to adsorbed BSA-biotin are active. About 50% of the surface of cantilever showed bright spots, which explains that the efficiency goes slightly down after binding pockets of streptavidin on the other side bound to BSA-biotin molecules. It is not possible to calculate the number of active streptavidin molecules, but it is secured that the cantilever tip should be active because of the fact that there exist about 50 streptavidin molecules at the end of probe tips whose diameter is only 50nm as explained in chapter 2.

## 3.2 Gold thiol chemistry

As in the case of adsorption chemistry, fragments of gold-coated wafer were used instead of gold-coated cantilevers to confirm the gold-thiol chemistry protocol. As previously mentioned, the wafers and cantilevers were coated with gold at the same time, so the thickness of the gold coated on the wafers and cantilevers was 20 nm. The protocol that was provided in Chapter 2

was used, and the wafer pieces and cantilevers were imaged via fluorescence microscopy.



(a) Gold-MUA with biotin-fluorescein - gold surface was treated with MUA, and biotin-fluorescein was added as a control experiment.



(b) Gold-MUA with streptavidin followed by adding biotinylated molecule, and biotin-fluorescein - gold surface was treated with MUA, and streptavidin was conjugated. Biotinylated amino acid chain was added and blocked almost binding pockets to biotin. Adding biotin-fluorescein didn't show bright spots because of biotinylated molecules. This image was obtained as a control experiment.



(c) Gold-MUA with streptavidin followed by adding biotin-fluorescein - gold surface was treated with MUA, and streptavidin was attached. To check if conjugated streptavidin molecules are active, biotin-fluorescein was added. Bright spots were obtained, which showed positive confirmation.

Figure 3-2: Validation of gold-thiol protocol



According to the concentration of MUA and streptavidin added to the surface, the number of bright spots observed were different. The number of bright spots on the fluorescence microscopy was considered as that of active streptavidin molecules attached to the gold-coated wafer or cantilevers. Different concentrations of MUA (0.05, 0.1, and 1mg/mL) and streptavidin (0.2, 0.5, and 2mg/mL) were used to find the optimized experimental condition. As Fig. 3-3 shows, 1 mg/mL of MUA and 2 mg/mL of streptavidin resulted in fluorophore coverage over 85% of sample surface, as measured via fluorescence (reflected) optical microscopy.

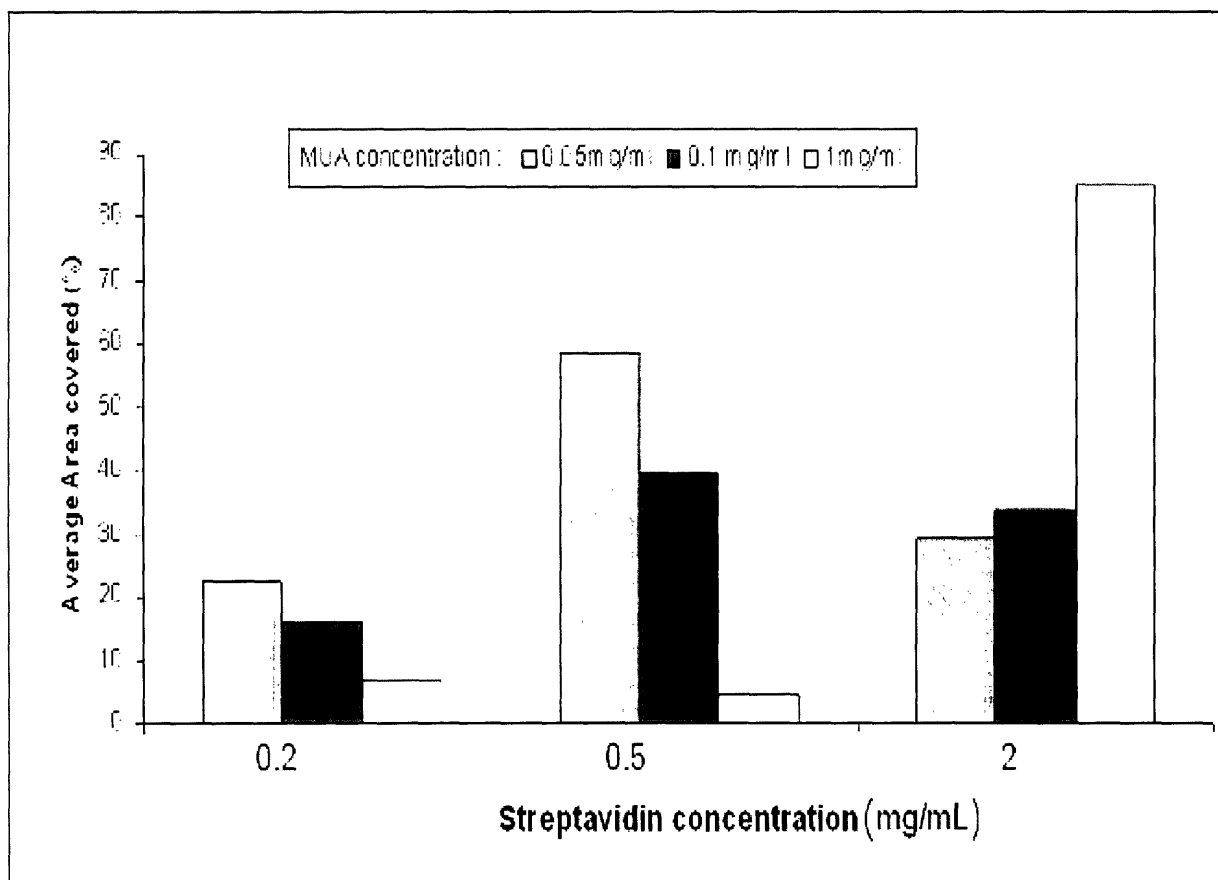


Figure 3-3: Optimal concentration of MUA and streptavidin

This protein immobilization technique can be efficiently applied to a variety of AFM imaging scenarios, but is useful only if the active sites or binding pockets of immobilized

proteins still remain after conjugation reactions. This will be discussed in Chapter 4.

### 3.3 Si<sub>3</sub>N<sub>4</sub> chemistry

Regardless of the approaches chosen for amine derivitization, such as chemical vapor deposition and APTES/toluene solution adsorption, it is crucial to confirm whether amine groups are introduced on the silicon nitride surface uniformly. Otherwise, subsequent steps following this amine derivitization could waste time and reagents.

One well-known technique with which surface molecules can be detected is X-ray photoelectron spectroscopy (XPS). The principle of the XPS is mainly based upon the photoelectric effect, where the surface electrons are ejected by the attack of photons. For each and every element, there will be a characteristic binding energy associated with each core atomic orbital, i.e., each element will give rise to a characteristic set of peaks in the photoelectron spectrum at kinetic energies determined by the photon energy and the respective binding energies. The presence of peaks at particular energies therefore indicates the presence of a specific element in the sample under study. Furthermore, the intensity of the peaks is related to the concentration of the element within the sampled region [44]. From XPS analysis of the amine derivitized surface, PEG-PDP linker-conjugated surface, and protein-terminated surface, the attachment of the various molecules in the functionalization protocol can be easily verified. Whether there are amine groups, PEG-PDP linkers, and proteins on the surface can be confirmed respectively from nitrogen peaks, sulfur peaks, and the increase in the percentage of carbon atoms that come from long protein chains of protein molecules.

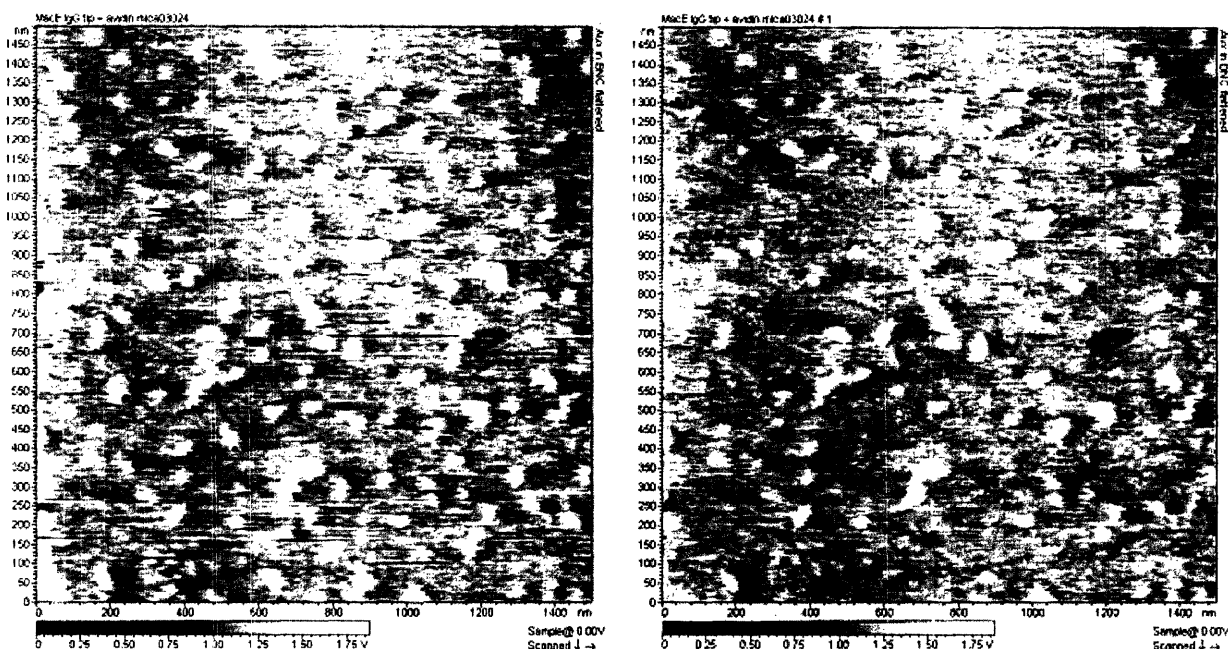
In the present experiments, when amine groups were first deposited, the atomic concentration percentage of nitrogen was 10%, and carbon that came from the APTES molecule increased to 30%. When PEG-PDP linker was added, the atomic concentration of sulfur was 1%; followed by the addition of protein that showed the increase of carbon atoms

to 60% due to the addition of polymer chains comprising long amino acids. Thus, the success of the amine derivitization reaction was verified through XPS.

Another method of confirmation is the contact angle analysis where the wettability of a surface is analyzed by measuring the surface tension of a solvent droplet at its interface with a homogenous surface. The size and size of droplets are determined according to the affinity (attraction or repulsion) between the surface molecules and droplet molecules [45]. Each functional group such as amine group has its own contact angle, so it is easy to find whether there exists a certain group on the sample surface. This contact angle method has not yet been tried to verify the above protocols.

A third method to confirm the viability of functionalized AFM probes includes the use of the AFM itself, specifically by assessing the specificity of binding forces between the supposedly functionalized probe and a complementary rigid sample surface. Whether biotin molecules were attached to the end of the cantilevers was checked by competitive binding or blocking experiments. Avidin molecules were adsorbed on the mica, and this surface was imaged by the PEG-biotin-functionalized cantilevers following the protocol provided in Chapter 2. Strong binding events were visible as dark spots detected by truncation of sinusoidal cantilever oscillations in the magnetic AC mode (See Chapter 2). After these bindings between biotin and avidin were detected as shown in Fig. 3-4 (a), streptavidin solution which bound to biotin molecules, thus deactivating the tip by shielding the biotin molecules at the end of probe was added to the sample plate, which is called probe-blocking. As shown in Fig. 3-4 (b), these binding events were not detected after addition of 0.1 M of streptavidin, demonstrating specificity of the biotin-avidin binding thus measured. Another blocking method can be to deactivate surface molecules, i.e., to block the binding of biotin to avidin molecules by adding BSA-biotin molecules that will compete for this same binding site on the surface-bound avidin molecules. This finding suggests the specificity of the binding

events (dark spots) as peculiar to the biotin-avidin interactions. The existence of biotin molecules at the end of probe was confirmed from the result.



(a) Avidin-biotin binding in MAC mode - PEG-biotin-conjugated tip was used to image avidin-adsorbed mica surface. Black spots show specific binding between avidin and biotin.

(b) After blocking the functionalized tip - after the blocking the tip by adding streptavidin solution, block spots disappeared, which showed that black spots in (a) represented specific binding between avidin and biotin.

Figure 3-4: AFM blocking experiment

Antibody conjugation protocols can also be verified on the fluorescence microscopy. Anti-VEGFR2 antibody was attached to the cantilever by conjugation technique, and this functionalization chemistry was checked with a fluorescently labeled (FITC) secondary antibody that specifically binds to the primary IgG molecules. This result is shown in Fig. 3-5.

Note that XPS verification can be used for gold-thiol chemistry, as well, because MUA contains sulfur molecules and the proteins that were linked with MUA can be detected by an increase in the amount of carbon molecules detected. Conversely, if a biotin-adsorbed surface is imaged with the gold-MUA-streptavidin tip, the AFM blocking method can also be used for the confirmation of the existence and activity of streptavidin molecules at the end of probe. The reason that streptavidin-biotin system was preferred for these protocol

optimizations and verifications was because this system provides stable binding phenomena and is easy to confirm by the fluorescence microscopy images (biotin-fluorophore and streptavidin-fluorophore are commercially available) or AFM force spectroscopy blocking methods. Once protein binding protocols are confirmed in this system, other proteins such as various kinds of antibodies have the application potential according to protein structures. These applications are considered in Chapter 4.



(a) Chemical linker with secondary antibody-fluorescein - PEG-PDP linker was conjugated on top of amine-derivitized silicon nitride cantilever, followed by addition of fluorescein-conjugated secondary antibody. There was no specific binding. This image was obtained as a control experiment.

(b) chemical linker that was conjugated with anti-VEGFR2, followed by adding secondary antibody-fluorescein - PEG-PDP linker was conjugated on top of amine-derivitized silicon nitride cantilever, followed by the attachment of anti-VEGFR2. Fluorescein-conjugated secondary antibody was added and showed bright spots, which confirmed that attached anti-VEGFR2 was active.

Figure 3-5: anti-VEGFR2 conjugation on the fluorescence microscopy

The probe tip is located at the end of the triangular surface of cantilever, and about 1/8 of the length of triangle side is the length of probe tip. Thus, 1/8 of the high magnification image of the tip of the cantilever (~2  $\mu\text{m}$ ) was taken and analyzed. About 30 – 60% of this area was covered by bright spots, which does not necessarily mean that 30 – 60% of attached antibodies are active because the fluorophore-conjugated secondary antibodies bind to the conserved (and not the epitope) region of the primary antibody. It is reasonable that at least 30% of area was covered by anti-VEGFR2 molecules.

		Gold	Silicon nitride
Length of chemical linker	< 1 nm	1~2 nm	7~8 nm
Flexibility of linker	Not good	Intermediate	Good
Bond strength	Relatively weak	Strong	Strong
Chemical Reaction	Easy Straightforward	Intermediate	Sophisticated
# of active modifiers	Many modifiers survived (95% area covered)	Intermediate (50~90% area covered)	Intermediate (30~60% area covered)
Advantages	<ul style="list-style-type: none"> <li>• Easy reaction</li> <li>• Stable(streptavidin-biotin)</li> </ul>	<ul style="list-style-type: none"> <li>• Strong bonding</li> <li>• Relatively easy reaction</li> <li>• No non-specific adsorption</li> </ul>	<ul style="list-style-type: none"> <li>• Strong bonding</li> <li>• Good linker flexibility</li> <li>• Inexpensive preparation</li> </ul>
Disadvantages	<ul style="list-style-type: none"> <li>• Low linker flexibility</li> <li>• Weak bonding</li> </ul>	<ul style="list-style-type: none"> <li>• Expensive preparation</li> </ul>	<ul style="list-style-type: none"> <li>• Complex reaction</li> </ul>
Usage	Rigid surface	Rigid surface Biomaterials	Soft biomaterials

Table 3-1: Comparison among three chemical reactions

Table 3-1 summarizes the characteristics of the three kinds of chemical reactions considered in this thesis. Through many experiments that varied reagent molarities and reaction times, it is concluded that adsorption method is fit for rigid surface imaging especially due to the short length (< 1 nm) of the chemical linker. As mentioned in the beginning of Chapter 2, the flexibility of the linker and bond strength between modifier molecules and cantilever are crucial for accurate detection of soft biomolecules. The probability of success in terminating the cantilever probe tip with the molecule of interest using this chemistry is above 90%, which is a powerful advantage of this protocol. However, once the comparably sophisticated reaction procedures were established for both gold and silicon nitride protocols, these chemistries also resulted in repeatably obtaining at least 50% coverage of the available surface area with the molecule of interest.



# Chapter 4

## Applications of Chemical Modification

The methods to attach and verify attachment of certain molecules to the AFM cantilever were considered in Chapters 2 and 3. The focus of this thesis is the modification of a surface, in this case, the AFM cantilever. Each surface has its own energy that keeps the surface from being vulnerable to external, chemical or physical attacks. Thus, chemical modification and verification thereof for a stable inorganic surface is not an easy task. Chapter 4 includes discussion of why AFM probe functionalization is important, and explores applications in which this technique can be applied to through some practical examples.

### 4.1 Resolution of Atomic Force Microscopy

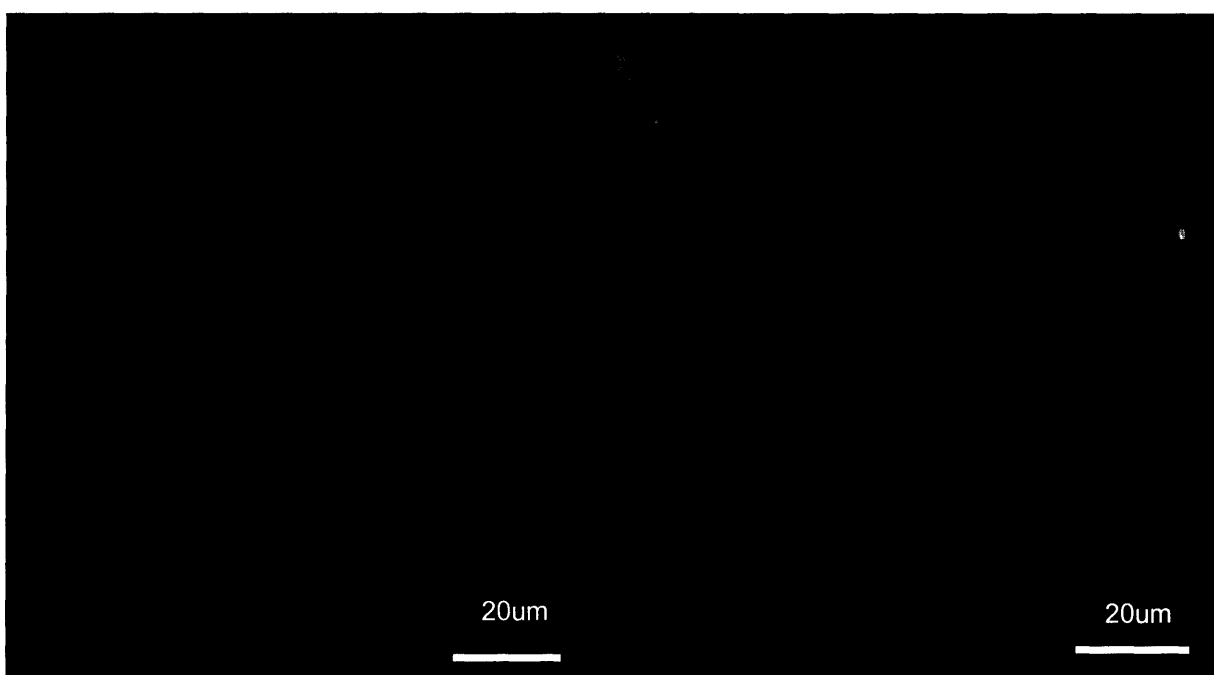
As emphasized in Chapter 3, streptavidin-fluorophore and biotin-fluorophore chemistries enable straightforward verification of surface functionalization through fluorescence microscopy. However, fluorescence microscopy exhibits significantly lower spatial resolution as compared to the AFM. The maximum resolution of the AFM is approximately 1 nm [8], but that of the fluorescence microscopy is at a micrometer scale. In other words, the fluorescence microscopy is useful and straightforward for larger imaging applications, but is limited by the intrinsic characteristic of light – spatial broadening of the emitted spectrum (i.e., point spread distribution function) and aggregation or overlapping among these spectra. Even though the size of one fluorophore molecule is very small (nano scale size), the light spots that can be observed optically are larger than the original size of fluorophore-bound molecules. In fact, the maximum resolution of the AFM is thought of as the minimum



resolution of the optical (fluorescence) microscopy.

### 4.1.1 Biotin or streptavidin-immobilized cantilevers

For example, to visualize the location and number of biotinylated surface molecules on fixed cells, the only information that can be provided by fluorescence optical imaging is whether such binding was specific or aspecific. It is impossible to determine the nanoscale distribution of such small molecules on the cells surface via fluorescence microscopy, as shown in Fig. 4-1.



(a) Non-specific binding after adding streptavidin-Texas Red to bare cell surface - Texas-red-conjugated streptavidin was added to cell surface that was not biotinylated. Non-specific binding was obtained. The brightness is less strong than (b).

(b) Specific binding after adding streptavidin-Texas Red to biotinylated cell surface - Cell surface molecules that have amine groups were specifically biotinylated by adding NHS-biotin, followed by the addition of Texas-red-conjugated streptavidin. This is 10 times brighter than (a), which confirms that brightness comes from specific binding. However, it is impossible to know the specific location of biotinylated surface molecules.

Figure 4-1: Fluorescence microscope images of biotinylated cell surface molecules

As previously mentioned, biotin-streptavidin system was used as a model system, mainly because of the ease of confirmation of binding and of the stability of the system. Furthermore, this system itself has many applications in biological functionalization and imaging.

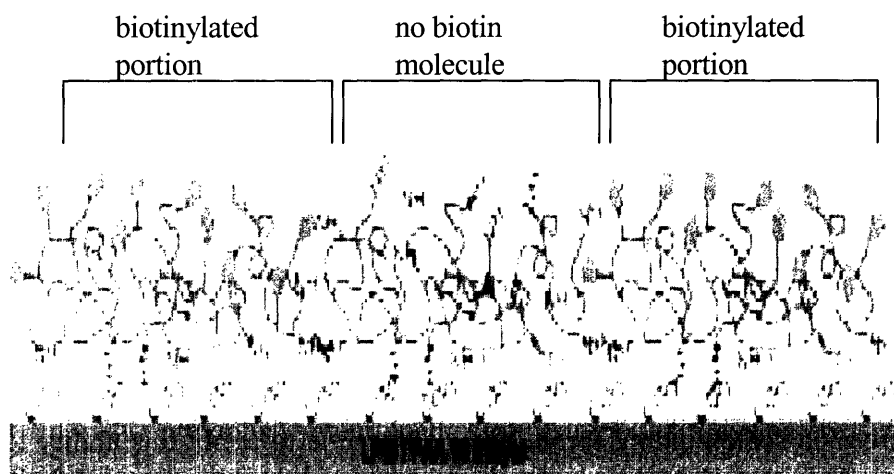


Figure 4-2: Polymer-patterned surface [3]

If polymers are patterned into features of nanoscale dimensions, the confirmation of the existence of certain molecules on the polymer surface is possible from the brightness of the surface after adding fluorophores, but whether the molecules are equally and uniformly patterned is not easy to confirm through optical microscopy. In this case, AFM imaging with a biotinylated cantilever will show the surface geometry from the characteristic shapes of AFM binding in the form of nanometer-scale spots that are due to the binding between complementary molecules even though these surface features, in reality, don't exist on the surface. The surface that contains biotin, as in Fig. 4-2, can be imaged with streptavidin-immobilized cantilevers using the protocol introduced in Chapter 2. In addition, the exact location of the biotinylated surface molecules (amine-containing or lysine-containing) on the fixed cell surface can be visualized by the streptavidin-immobilized cantilevers, which is

impossible using fluorescence microscopy. Various kinds of molecules can be biotinylated such as biotinylated DNA or antibodies, even gold nanoparticles through thiol-conjugated biotin, so the streptavidin-immobilized cantilevers can be used to detect these molecules via nanoscale imaging [46-48].

## 4.2 Intermolecular Interactions

Many results that are related to intermolecular interactions have derived from Florin's original result [49, 50]. This method was adapted by many people to measure molecular interactions such as between DNA single strands, carbohydrates, and antigen-antibody pairs [51-56]. However, most publications do not confirm nature of the molecules bound to the end of AFM probes. Instead, the main purpose of this early work was to show that the AFM is a powerful tool to detect and measure pico-scale binding force. Now is the time to apply this ability to measure intermolecular interactions as a tool to answer key scientific questions, not as a goal in and of itself. For example, after the confirmation of the existence of a certain molecule on the cantilever through the methods introduced in Chapter 3, whether complementary molecules to this truly exist, remain active, or how these complementary molecules move as a function of time in response to external stimuli can be elucidated. This application is especially exiting in biological systems such as live cell surfaces, which are attractive targets to which chemical functionalization techniques of the AFM cantilever can be applied.

## 4.3 Force Spectroscopy

One of the most powerful features of the AFM is the ease of the measurement of picoNewton-scale force, that is, molecular kinetics. This measurement is closely related to the loading rate of the piezoactuator that displaces the cantilever from the sample surface [57]. That is, the

force between molecules depends on the rate at which the AFM approaches or retracts from the sample. Once the relationship between the rupture force and loading rate is established for a system such as biotin-avidin binding, unknown molecular binding forces can be measured. Because various numbers of molecules are attached to the cantilever according to the curvature of the cantilever and the size of attached molecules [46], the rupture force can be doubled or tripled with respect to the number of binding events. However, it is expected that the magnitude of this binding event will be quantized, as determined by autocorrelation functions of distributions of such events for a given molecular pair. This is demonstrated in Fig. 4-3, where the probe has been functionalized with a biotinylated oligopeptide and the rupture forces were measured between this peptide and an endothelial cell surface. The peak rupture forces in the corresponding histogram indicate integer multiples of a minimal force of 0.3 nN, supporting the concept of quantization for a given molecular pair.

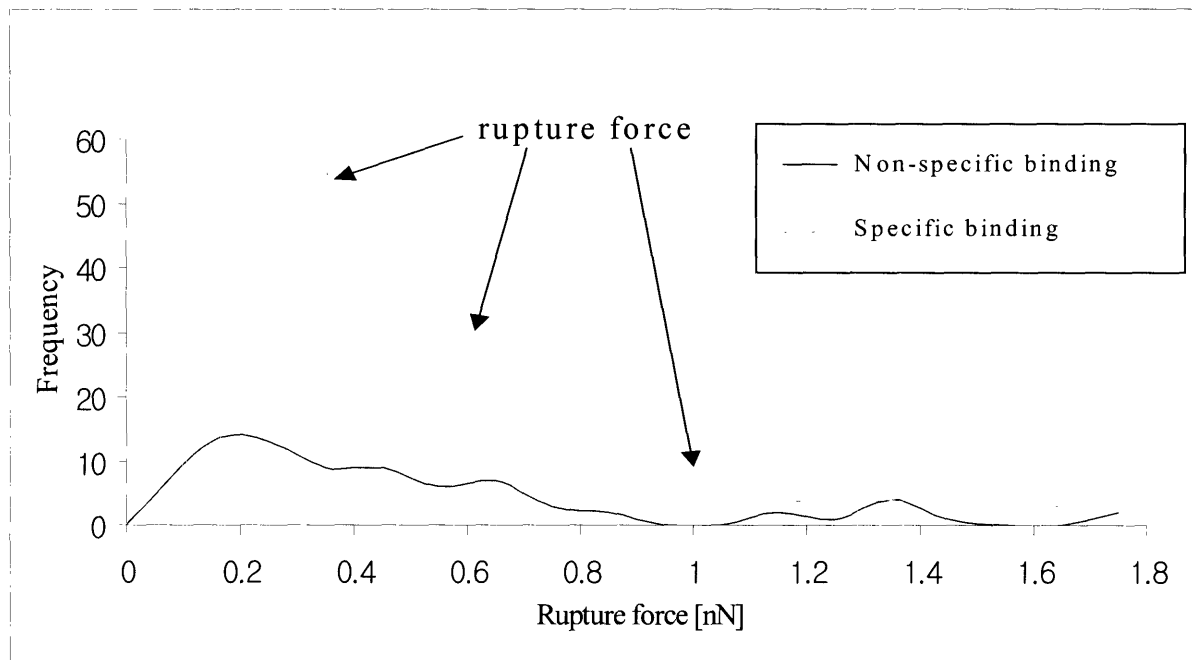


Figure 4-3: Force spectroscopy data: Biotinylated oligopeptide molecules were conjugated at the end of cantilever with which bovine capillary endothelial cells were imaged. Rupture forces were measured and quantized between putative receptors and oligopeptide at the probe. The rupture force between complementary molecules in this case is about 0.3 nN from the fact that the peaks at 0.3, 0.6, and 0.9 nN are integer multiples one another.

Rupture force, or the binding force required to separate two molecules, is thought to be a characteristic of a certain molecular pair. Various kinds of interactions such as electrostatic interactions between charged groups, hydrogen bonding, hydrophobic interactions, and van der Waals forces are involved in one binding pocket. The complicated combination of these forces macroscopically constitutes single binding, so each binding event has its own unique binding force, the average of which is considered an intrinsic property of the molecular pair for a given rate of loading.

A variety of chemical reactions besides those in Chapter 2 have been developed, so finding the optimized protocol for a specific use is now the most important step for a surface characterization. Most of the reactions are based upon gold-thiol interaction and silicon surface chemistry because these two systems provide stable and straightforward binding. Some simple examples except for the protocols introduce in Chapter 2 are the following: thiol-conjugated biotin can form SAM with gold-coated cantilever, and this tip can be used for detecting streptavidin molecules on a surface, and long covalent molecules such as PEG can extend chemical linkers that attach to gold surface via gold-thiol bonding.

# Chapter 5

## Conclusion

### 5.1 Summary

This thesis has improved methods for AFM cantilever modification, including significant efforts towards independent verification of functionalization steps. The contribution of this research is not to review previously achieved results, but to open up a new field by honing chemistry techniques towards a new method to measure and image molecular distributions on dynamic biological surfaces. In the introduction of this thesis, the history of developing the atomic force microscopy was recapitulated to demonstrate that chemical functionalization of the cantilever is a critical step toward new AFM applications. In particular, three kinds of chemistry protocols including physical adsorption method, gold-based chemical reactions, and silicon-based chemical reactions were explained in detail with respect to the characteristics of each reaction such as bond strength, bond length, and bond stability. The important step that naturally accompanies the chemistry protocols is the validation of these protocols. Thus, several verification methods such as x-ray photoelectron spectroscopy, contact angle method, and AFM blocking experiment were introduced. The ultimate goal of chemical modification of the AFM cantilever is to apply these techniques to various kinds of systems, and so practical examples were explored in Chapter 4. In summary, the major contribution of this thesis is to identify and optimize various kinds of surface reactions of the cantilevers for specific biomaterial and biological surface imaging applications.

## 5.2 Suggestions for future work

So far, the research of this thesis has been focused the characterization of surface molecules, and chemically modified cantilevers have been used for well-known system such as biotin-streptavidin for the purpose of confirmation of the cantilever chemistry.

Therefore, the next step is to explore biological systems such as living cell surfaces that exhibit versatile functionality and various surface molecules. In particular, surface molecules such as glycoproteins that are related to immunological responses and mediation of signals through cell membrane are of major interest. The powerful feature of the AFM is that the surface morphology can be tracked in real time and at molecular resolution, which can be fully applied to the movement of cytoskeletal filaments beneath the cell plasma membrane, as well as to the track of the reaction of cell surface molecules with a function of external stimuli. The proteins at the end of probe are active only if the cantilever is immersed in the solution. This is also compatible with the fact that adherent cells should be cultured and imaged in solution, e.g., buffer or media, and that the van der Waals force can balance with the spring force of the cantilever even in the solution, which makes the AFM an even more powerful for imaging molecular interactions in solution.

This work will require quantification of molecules at the end of probes, because the number of molecules on the cantilever is closely related to the magnitude and duration of binding force. In addition, such an extension of the functionalization chemistry will require careful consideration of how many surface molecules are aggregated, i.e., how many active binding pockets are exposed to the outside on the rigid sample surface as compared to a compliant cell surface. One thing that should also be kept in mind is that the bioconjugation technique for attaching proteins arbitrarily attacks the functional groups, so conjugation techniques that include more targeted protected/leaving groups should be studied further. To quantify the surface characterization, thermodynamic study using thermogravimetric analysis

(TGA) and differential scanning calorimetry (DSC) is required together with chemical analysis using Fourier transform infrared Spectroscopy (FT-IT). The thermodynamic study will include how tightly molecules are attached on the cantilever or the fraction of surface area covered by the molecules of interest on the cantilever and/or planar substrate of the same material, as a function of temperature. One example of this study can be the change in the adsorption bonding strength of BSA molecules to the cantilever surface before and after fixing (crosslinking) with glutaraldehyde. The thermodynamic characteristics of chemistry at the cantilevered probe tip is different from that of a flat surface because the chemical potential of a highly curved surface may differ substantially from that of a planar surface.

## Bibliography

- [1] [http://www.molec.com/apps\\_imagingmodes.html](http://www.molec.com/apps_imagingmodes.html).
- [2] A Chen, V.M., *Methods in cell biology*. **68**: p. 301-309.
- [3] H Kim, J.D., DJ Irvine, RE Cohen, and PT Hammond, *Large Area Two-Dimensional B Cell Arrays for Sensing and Cell-Sorting Application*. *Biomacromolecules*, 2004. **5**: p. 822-827.
- [4] Bliznyuk, V.T.a.V., *Adhesive and Friction Forces between Chemically Modified Silicon and Silicon Nitride Surfaces*. *Langmuir*, 1998. **14**: p. 446-455.
- [5] X Su, Y.W., R Robelek, and W Knoll, *Surface Plasmon Resonance Spectroscopy and Quartz Crystal Microbalance Study of Streptavidin Film Structure Effects on Biotinylated DNA Assembly and Target DNA Hybridization*. *Langmuir*, 2005. **21**: p. 348-353.
- [6] G Binnig, C.Q., and C Gerber, *Atomic Force Microscope*. *Physical Review Letters*, 1986. **56**: p. 930-933.



- [7] G Binnig, H.R., C Gerber, and E Weibel, *7 X 7 Reconstruction on Si (111) resolved in real space*. Physical Review Letters, 1983. **50**: p. 120-123.
- [8] Yang, J., *AFM as a High-Resolution Imaging Tool and a Molecular Bond Force Probe*. Cell Biochemistry and Biophysics, 2004. **41**: p. 435-448.
- [9] OH Willemsen, M.S., KO van der Werf, BG de Grooth, J Greve, P Hinterdorfer, HJ Gruber, H Schindler, Y van Kooyk, and CG Figdor, *Simultaneous Height and Adhesion Imaging of Antibody-Antigen Interactions by Atomic Force Microscopy*. Biophysical Journal, 1998. **75**: p. 2220-2228.
- [10] G Lee, D.K., and RJ Colton, *Sensing discrete streptavidin biotin interactions with atomic-force microscopy*. Langmuir, 1994. **10**: p. 354-357.
- [11] EL Florin, M.R., H Lehmann, M Ludwig, C Dornmair, VT Moy, and HE Gaub, *Sensing specific molecular-interactions with the atomic-force microscope*. Biosens. Bioelectron, 1995. **10**: p. 895-901.
- [12] R Ros, F.S., D Anselmetti, M Kubon, R Schafer, A Pluckthun, and L Tiefenauer, *Antigen binding forces of individually addressed single-chain Fv antibody molecules*. PNAS, 1998. **95**: p. 7402-7405.
- [13] U Dammer, M.H., D Anselmetti, P Wagner, M Dreier, W Huber, and HJ Guntherodt, *Specific antigen/antibody interactions measured by force microscopy*. Biophys. J. **70**: p. 2437-2441.
- [14] J Fritx, D.A., J Jarchow, and X Fernandez-Busquets, *Probing single biomolecules with atomic force microscopy*. J. Struct. Biol, 1997. **119**: p. 165-171.
- [15] P Hinterdorfer, W.B., HJ Gruber, K Schilcher, and H Schindler, *Detection and localization of individual antibody-antigen recognition events by atomic force microscopy*. PNAS. **93**: p. 3477-3481.
- [16] V Parpura, P.H., and E Henderson, *Three-dimensional imaging of living neurons and glia with the atomic force microscope*. J. Cell Sci, 1993. **104**: p. 427-432.
- [17] PG Haydon, E.H., and E Stanley, *The spatial distribution of individual calcium*

*channels in the release face of a presynaptic terminal.* Neuron. **13**: p. 1275-1280.

- [18] HG Hansma, a.J.H., *Biomolecular Imaging with the Atomic Force Microscope.* Ann. Rev. Biophys. Biomol. Struct, 1994. **23**: p. 115-139.
- [19] T Osada, A.I., A Ikai, *Mapping of the receptor-associated protein (RAP) binding proteins on living fibroblast cells using an atomic force microscope.* Ultramicroscopy, 2003. **97**: p. 353-357.
- [20] <http://stm2.nrl.navy.mil/how-afm/how-afm.html>.
- [21] AL Weisenhorn, P.H., TR Albrecht, and CF Quate, *Forces in atomic force microscopy in air and water.* Appl. Phys. Lett, 1989. **54**: p. 2651-2653.
- [22] C Stroh, H.W., R Bash, B Ashcroft, J Nelson, H Gruber, D Lohr, SM Lindsay, and P Hinterdorfer, *Single-molecule recognition imaging microscopy.* PNAS, 2004. **101**: p. 12503-12507.
- [23] <http://spm.phy.bris.ac.uk/techniques/AFM/>.
- [24] EP Wojcikiewicz, X.Z.a.V.M., *Force and Compliance Measurements on Living Cells Using Atomic Force Microscopy.* Biol. Proceed, 2004. **6**: p. 1-9.
- [25] JL Hutter, J.B., *Calibration of atomic-force microscope tips.* Rev Sci Instrum, 1993. **64**: p. 1868-1873.
- [26] <http://ias.vub.ac.be/General/Adsorption.html>.
- [27] Ayhan, H., *Model Protein BSA Adsorption and Covalent Coupling onto Methyl methacrylate Based Latex Particles with Different Surface Properties.* Journal of Bioactive and Compatible Polymers, 2002. **17**: p. 271-282.
- [28] M Ombelli, D.E.a.R.C., *Biomimetic Dextran Coatings On SiliconWafers: Thin Film Properties And Wetting.* Mat. Res. Soc. Symp. Proc. **734**.
- [29] JR Lu, T.S., BJ Howlin, RK Thomas, ZF Cui, J Penfold, *Protein adsorption at interfaces, Scientific highlights.*

- [30] A Holmberg, A.B., O Nord, M Lukacs, J Lundeberg, M Uhlen, *The biotin-streptavidin interaction can be reversibly broken using water at elevated temperatures*. Electrophoresis, 2005. **26**: p. 501-510.
- [31] J Reichert, R.O., D Beckmann, HB Weber, M Mayor, and Hv Lohneysen, *Driving Current through Single Organic Molecules*. PHYSICAL REVIEW LETTERS, 2002. **88**: p. 176804.
- [32] A Tlili, A.A., S Hleli, and MA Maaref, *Electrical Characterization of a Thiol SAM on Gold as a First Step for the Fabrication of Immunosensors based on a Quartz Crystal Microbalance*. Sensors, 2004. **4**: p. 105-114.
- [33] ET Vandenberg, L.B., B Liedberg, K Uvdal, R Erlandsson, H Elwing and I Lundstrom, *Structure of 3-aminopropyl triethoxy silane on silicon oxide*. Journal of Colloid and Interface Science, 1991. **147**(1): p. 103-118.
- [34] SAIM, S.I.A.R.f., <http://www.inchem.org/documents/sids/sids/919302.pdf>. 2003.
- [35] [http://repository.upenn.edu/cgi/viewcontent.cgi?article=1000&context=mse\\_papers](http://repository.upenn.edu/cgi/viewcontent.cgi?article=1000&context=mse_papers).
- [36] Molecular Imaging Company, T., AZ.
- [37] Christian K. Riener, C.M.S., Andreas Ebner, Christian Klampfl, Alex A Gall, Christoph Romanin, Yuri L. Lyubchenko, Peter Hinterdorfer, Hermann J. Gruber, *Simple test system for single molecule recognition force microscopy*. Analytica Chimica Acta, 2003. **479**: p. 59-75.
- [38] Peter Hinterdorfer, H.J.G., Ferry Kienberger, Gerald Kada, Christian Riener, Cordula Borken, Hansgeorg Shindler, *Surface attachment of ligands and receptors for molecular recognition force microscopy*. Colloids and Surfaces B: Biointerfaces, 2002. **23**: p. 115-123.
- [39] Karl Kaiser, M.M., Thomas Haselgrubler, Hansgeorg Shindler, and Hermann J. Gruber, *Basic Studies on Heterobifunctional Biotin-PEG Conjugates with a 3-(4-Pyridyldithio)propionyl Marker on the Second Terminus*. Bioconjugate Chem., 1997. **8**: p. 545-551.

- [40] protocol, P.B.
- [41] <http://www.piercenet.com/Products/Browse.cfm?fldID=02040619>.
- [42] <http://www.piercenet.com/files/0126as4.pdf>.
- [43] <http://www.prozyme.com/pdf/sa20.pdf>.
- [44] [http://www.chem.qmw.ac.uk/surfaces/scc/scat5\\_3.htm](http://www.chem.qmw.ac.uk/surfaces/scc/scat5_3.htm).
- [45] <http://www.uweb.engr.washington.edu/research/tutorials/contact.html>.
- [46] Jackson A.M., M.J.W., Stellacci F, *Spontaneous assembly of subnanometre-ordered domains in the ligand shell of monolayer-protected nanoparticles*. Nature Materials, 2004. **3**(5): p. 330-336.
- [47] Meiju Ji, P.H., Zuhong Lu, and Nongyue He, *Covalent Immobilization of DNA onto Functionalized Mica for Atomic Force Microscopy*. Journal of Nanoscience and Nanotechnology, 2004. **4**: p. 580-584.
- [48] Kobs, G., *In vitro Transcription from Biotinylated DNA Immobilized on Streptavidin MagneSphere?Paramagnetic Particles*. Promega Notes Magazine, 1996. **60**: p. 20.
- [49] VT Moy, E.F., HE Gaub, *Intermolecular forces and energies between ligands and receptors*. Science, 1994. **266**: p. 257.
- [50] EL Florin, V.M., HE Gaub, *Atomic Force Microscopy Experiments of Ligand Unbinding*. Science, 1994. **264**: p. 415.
- [51] GU Lee, L.C., RJ Colton, *Getting Physical with DNA*. Science, 1994. **266**: p. 771.
- [52] U Dammer, O.P., P Wagner, D Anselmetti, HJ Buntherodt, GN Misevic, *Binding strength between cell adhesion proteoglycans measured by atomic force microscopy*. Science, 1995. **267**: p. 1173.
- [53] Misevic, G., *Molecular self-recognition and adhesion via proteoglycan to proteoglycan interactions as a pathway to multicellularity: Atomic force microscopy*

*and color coded bead measurements in sponges*. Microsc Res Technol, 1999. 44: p. 304.

- [54] C Tromas, R.G., Topics in Current Chemistry, 2002. 218.
- [55] Y Harada, M.K., A Ishida, *Specific and Quantized Antigen-Antibody Interaction Measured by Atomic Force Microscopy*. Langmuir, 2000. 16: p. 708.
- [56] A Raab, W.H., D Badt, SJ Smith-Gill, SM Lindsay, H Schindler and P Hinterdorfer, *Antibody recognition imaging by force microscopy*. Nature biotechnology, 1999. 17: p. 902.
- [57] C Yuan, A.C., P Kolb and VT Moy, *Energy landscape of streptavidin-biotin complexes measured by atomic force microscopy*. Biochemistry, 2000. 39(10219-10223).



Room 14-0551  
77 Massachusetts Avenue  
Cambridge, MA 02139  
Ph: 617.253.5668 Fax: 617.253.1690  
Email: docs@mit.edu  
<http://libraries.mit.edu/docs>

## **DISCLAIMER OF QUALITY**

Due to the condition of the original material, there are unavoidable flaws in this reproduction. We have made every effort possible to provide you with the best copy available. If you are dissatisfied with this product and find it unusable, please contact Document Services as soon as possible.

Thank you.

**Some pages in the original document contain pictures or graphics that will not scan or reproduce well.**