EFFECT OF MEDIUM, BIOCONJUGATED AND BIOFUNCTIONALISED AFM TIP ON IMAGE AND ELASTICITY OF AUNP AND AUNP_IGA

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By

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LIST OF ABBREVIATIONS

AFM	Atomic Force Microscopy
APTES	3-Aminopropyltriethoxysilane
ASTM	American Society for Testing and Materials
AuNP	Gold Nanoparticle
AuNP_IgG	Gold Nanoparticle Conjugated Antibody
AVIS	Active Vibration Isolation System
CM	Contact Mode
FD	Force Distance
FS	Force Spectroscopy
HigG	Human IgG
HRP	Horseradish Peroxide
HRP-rb-IgG	HRP-labeled rabbit IgG
IgA	Goat Anti-Human
LFI	Lateral Flow Immunoassay
NCHR	Non-Contact High Resolution
NCM	Non Contact Mode
NNI	National Nanotechnology Initiative
NSOM	Near Field Scanning Optical Microscopy

OMCL	Olympus Micro Cantilever
PBS	Phosphate Buffer Saline
PEG	Polyethylene Glycol
PID	Proportional, Integral and Derivative controls
PLL	Poly-L-Lysine
PSPD	Position-Sensitive Photo Detector
PZT	Piezoelectric Transducer
RF	Resonance Frequency
rms	root mean square
SEM	Scanning Electron Microscopy
SPM	Scanning Probe Microscopy
STM	Scanning Tunnelling Microscopy
TEM	Transmission Electron Microscopy
XE-BIO	XE (Cross-talk Elimination)-BIO
XEI	Image processing program
XEP	Data Acquisition program

KESAN MEDIA, BIOKONJUGAT DAN BIOFUNGSIONAL AFM TIP KE ATAS IMEJ DAN KEANJALAN AUNP DAN AUNP_IGA

ABSTRAK

Protokol untuk menentukan daya interaksi dan keanjalan sampel, [nanopartikel emas (AuNP) dan konjugat AuNP kambing anti-manusia (AuNP_IgA)] di bentangkan. Kaedah melibatkan penyediaan sampel, pengukuran daya interaksi antara sampel dan tip dan juga pengukuran keanjalan menggunakan Mikroskop Daya Atom (AFM), dan pengimejan dalam persekitaran udara dan cecair dengan menggunakan tip pemfungsian. Daya interaksi diperolehi daripada daya tarikan keluar lenkung daya-jarak (FD) manakala keanjalan diperolehi daripada kekukuhan lengkung daya-jarak (FD) menggunakan kaedah Hertzian. Apabila lengkung FD yang diperoleh di udara, lapisan nipis air (lapisan bahan cemar cecair) terserap pada permukaan sampel menghasilkan meniskus atau daya kapilari. Sebaliknya, apabila di dalam cecair, daya kapilari dihapuskan dan daya-daya lain menjadi relevan. Antaranya adalah daya van der Waals dan daya elektrik dua lapisan. Daya bersih antara tip dan sampel adalah jumlah vektor daripada beberapa daya. Di udara, komponen dominan daya interaksi antara tip dan sampel adalah daripada daya kapilari manakala dalam cecair, komponen dominan adalah daya van der Waals. Daya interaksi di dalam julat 10 hingga 100 nN telah dilaporkan untuk pwngimejan di udara dan daya interaksi 5 hingga 104 nN yang diperolehi daripada kajian ini adalah terletak di dalam julat yang telah dilaporkan. Sementara itu, di dalam persekitaran cecair, daya interaksi yang diperolehi daripada kajian ini adalah dalam julat 0.1 hingga 40 nN dan ia terletak

dalam julat yang telah dilaporkan sebelum ini iaitu serendah 100 pN. Pengesanan daya adalah satu langkah penting ke arah membangunkan peranti analisis dan bioperubatan baru seperti biosensor.

EFFECT OF MEDIUM, BIOCONJUGATED AND BIOFUNCTIONALISED AFM TIP ON IMAGE AND ELASTICITY OF AUNP AND AUNP_IGA

ABSTRACT

A protocol for determining the adhesion force and elasticity of samples, [gold nanoparticle (AuNP) and AuNP conjugated goat anti-human (AuNP_IgA)] is presented. The method involved sample preparation, measurement of adhesion force between the samples and a tip as well as the measurement of elasticity using atomic force microscopy (AFM) in air and in liquid environment with bio functionalized tip. The adhesion force was computed from the pull-off forces of FD curve while the elasticity was computed from the stiffness of the FD curve using the Hertzian method. When the FD curve are required in air, a thin layer of water (liquid contaminant layer) adsorbed on the sample surface exerts a meniscus or capillary force. Instead, when working in a liquid, the capillary force is eliminated and other forces becomes relevant. The force present is the van der Waals force and electric double layer force. The net force between the tip and the samples is the vector sum of several forces. In air, the dominant component of the adhesion force between the tip and the sample is believed to be the capillary force while in liquid, the dominant components present is the van der Waals force. The adhesion force ranging from 10 to 100 nN have been reported for imaging in air and the adhesion force of 5 to 104 nN observed in our studies lies well within this range. Meanwhile, in liquid, the adhesion force obtained from this work is in the range of 0.1- 40 nN and it is lies within the range reported previously which is as

low as 100 pN. Detecting forces is an important step toward developing new analytical and biomedical devices such as biosensors.

CHAPTER 1

INTRODUCTION

1.1 Background

Atomic force microscopy (AFM) method is part of a scanning probe microscopy (SPM) technique used for surface characterization. It uses a sharp tip for scanning over a sample surface with the motion controlled precisely by a piezoelectric actuator to obtain information about the properties of the sample, including its topography image and surface interaction. AFM has been widely used in studying biomaterial characterization since it can be operated in air, vacuum, various gases and liquid environment. AFM visualises images by "feeling" the structure of a sample surface with a sharp probe rather than "looking" into the surface (Morris *et al.,* 2010).

In recent years, AFM has increasingly been used in nanotechnology especially for the visualization of biomolecules and force measurements. Originally AFM was used to image the topography of surfaces, but by modifying the tip it is possible to measure other quantities and also to perform various types of spectroscopy and analysis. AFM is more than a surface-imaging tool in that force measurements can be used to probe physical properties of molecules, such as molecular interaction, surface hydrophobicity, surface changes and mechanical properties.

The study of mechanical properties associated with biological molecules often refers as biomolecular mechanic/interaction. The elastic biomolecules is part of biomolecular mechanic which is a technologies that allow one to grab and stretch individual molecules and observed the interaction. Molecules which is elastic, viscous or viscoelastic properties are known to play a significant role in biological functions such as in the maintenance of cell and tissue mechanical integrity, sensing and/or relaying mechanical information.

The main advantage of AFM in biology as compared to other methods is that it usually does not require specific sample preparation and allows measuring in most of the physiological conditions biological objects are susceptible to. It is the most universal method in the sense that all the media including vacuum can be used for probing. The reason for choosing liquid media instead of air is not only because it is the natural physiological media for biological objects, but also due to the fact that all the interaction forces including unwanted ones are an order of magnitude smaller than in air allowing, for instance, to raise the resolution and to diminish image distortion. Although, it should be noted that measuring in liquids is much more complicated than imaging in air.

In nanobiotechnology, AFM is able to indicate the interaction of nanoparticles with biomolecules such as protein, DNA and others by measuring the force between the AFM tip and the sample surface via force spectroscopy (FS) measurement. Force spectroscopy or also known as force distance measurement is a method to measure interaction force occurring from the AFM tip to the surface of the samples. Force response is detected through beam deflection by a laser beam when a molecule of interest which is anchored at the end of the AFM tip to the surface of the sample is stretched (Ros *et al.,* 2004).

Force spectroscopy can be analogized as a fishing rod, to pick up molecules at a sample surface and then to stretch them. It sounds easy but it is not certain whether it is a single molecule or a mass of molecules attached to the tip. Hence, to overcome this limitation, functionalization of the AFM tip to specifically attach to a point of interest in a sample surface must be introduced. The choice of functionalization approach of nanoparticles-biomolecules to the cantilever tip is of importance in order to obtain information on the interaction of nanoparticles with biomolecules.

Gold nanoparticles (AuNP) have been found to be one of the useful tools in biomedical applications because of its unique properties of being inert and less toxic, small size and large surface area to volume ratio, high reactivity to living cells, stability in high temperatures, ease of detection and others (Tiwari *et al.,* 2011). It can be synthesized into different shapes and sizes due to its ability to react and agglomerate with other nanoparticles in the surroundings. Due to its nano size, it flows easily into various cells in the human body.

The AuNP surface and core properties can be manipulated for specific applications such as molecular recognition, chemical sensing, and imaging (Zeng *et al.,* 2011). The proper surface functionalization of nanoparticles will determines their interaction with the environment. These interactions normally affect the stability of the colloidal particles, and may yield to a controlled assembly or to the delivery of nanoparticles to a target, such as by appropriate functional molecules on the particle surface (Sperling & Parak, 2010). For example, the AuNP functionalized with antibody is used in the development of a immunoassay/sensing system and showed that AuNPs was efficient in preserving

the activity of the antibody and can serve as a sensing platform for many clinical immunoassays (Zeng *et al.,* 2011).

The conjugation of nanoparticles with biomolecules or also known as bioconjugation is a fascinating research with unique recognition interaction that provides a route into nanobiotechnology. Bioconjugation refers to a chemical process that combines two or more biomolecules together to create new molecules that are involved in the changing of biomaterial properties through covalent modification or immobilization (Schmid, 2004). Bioconjugation of nanoparticles combines novel material properties of nanoparticle compounds as well as the selective and specific interaction of biomolecules (Tessmer *et al.,* 2013). Biomolecules are commonly difficult to recognize directly due to their small size and invisible nature. In order to trace them and their biological activities, the probe or their target biomolecules are labelled by conjugating with a detection agent. The most recent nanoparticles used for conjugation are gold nanoparticles (AuNP) and magnetic nanoparticle labels (Kim Junkyu *et al.,* 2008).

Conjugation of nanoparticles to biomolecules generates hybrid materials that can be used to let the nanoparticles interact specifically with biological systems. Nanoparticle-biomolecule conjugates bring together the unique properties and functionality of both materials. Various biological targeting moieties such as antibodies have been conjugated to nanoparticle to adjust their biodistribution and to rise local therapeutic concentrations. If the aim is to use AuNP in biomedicine as diagnostic and therapeutic agents in cells or tissues, then, it is necessary to rightly choose the targeting component such as a monoclonal antibody (mAb), and the strategy to attach it on the surface of the particle (Cao-Milán & Liz-Marzán, 2014). Antibody conjugated gold nanoparticle probes allow for the study

and visualization of cellular and molecular processes. There are two methods that normally used for antibody conjugated gold nanoparticle such as non covalent (physical adsorption) and covalent conjugation. The covalent conjugation method provides more stable attachment between antibody and the gold nanoparticles compared to the non covalent method. The non covalent method depends on the affinity of certain functional groups on proteins such as amines and thiols to be attracted to the particle surface. Covalent modes are also used to bind functionalized groups to gold nanoparticle surface as well as to bind antibodies directly to the surface of nanoparticles by using a linker (Jazayeri *et al.,* 2016).

Physical and chemical interactions are used for attaching antibodies and other molecules to AuNP surface. Physical interaction between antibodies and AuNP depends on three phenomena: (a) ionic attraction between the negatively charged gold and the positively charged antibody; (b) hydrophobic attraction between the antibody and the gold surface; (c) dative binding between the gold conducting electrons and amino acid sulphur atoms of the antibody. Chemical interactions between antibodies and nanoparticle surface are achieved in the number of ways like (i) chemisorption via thiol derivatives; (ii) through the use of bifunctional linkers (iii), and through the use of adapter molecules like streptavidin and biotin (Jazayeri *et al.,* 2016).

For conjugation of antibodies to the AuNP, both covalent and non-covalent attachments are normally being used (Kumar *et al.,* 2008). The antibodies or other functionalized groups are non-specifically adsorbed onto gold nanoparticles while still keeping the nanoparticles negatively charged, providing stability in colloidal solution. In other words, the bio conjugation protocol uses non-covalent

attachment is based on a combination of electrostatic and hydrophobic interactions of the antibody and the AuNP surface (Rayavarapu *et al.,* 2007).

Non-covalent attachment is described as spontaneous absorption of antibodies onto the surface of citrate stabilized AuNP; there are several types of interactions that may occur in this process such as hydrophobic interactions, ionic interactions and others. Hydrophobic interactions are due to attraction between hydrophobic parts of the antibody and the metal surface that result in the formation of a non-covalent bond. Positively charged groups are abundant in antibodies such as positively charged amino acids and the N-terminal are present. Ionic interactions are formed between these groups and the negatively charged surface of the AuNP (Ljungblad, 2009).

Non-covalent attachment have several major weaknesses including the necessity of a high concentration of antibodies for the preparation of antibody-AuNP conjugates, random orientation of antibodies at the gold nanoparticle surface, and due to their electrostatic attraction they are making the biological response difficult to control; the binding is impressed by changes in pH, and ultimately because antibodies are non-covalently conjugated to nanoparticles (Jazayeri et al., 2016).

Covalent attachment are also used to bind functionalized groups to AuNP surface (Greg T. Hermanson, 2013). AuNP can be used for direct conjugation with thiol group-containing bio-molecules such as antibodies, and other biomolecules. Dative binding is a physical interaction that may occur between antibody and AuNP surface. It is the formation of a covalent bond between the AuNP and free sulfhydryl groups of the antibody (Kumar *et al.,* 2008). Covalent attachment used to bind antibodies directly to the surface of nanoparticles; acts as

a mediator linker, or can take place via adapter molecules like avidin and biotin for the formation of the complex.

Naturally, AFM can perform imaging in all environment including in air, liquid and gases. For bioconjugated samples, AFM imaging in air is able to clearly track and illustrate the performance of attaching the biomolecules to the nanoparticle surface for the ratification of the conjugation while in liquid, the AFM provides information with high spatial and temporal resolution related to the real time changes of shape or structure of bioconjugates (Tessmer *et al.,* 2013). Hence, AFM helps to understand the structural and mechanical properties of biomolecules.

In summary, AFM known as a powerful tool for the structural investigation of biomolecular conjugation to AuNP surface and analysis of bio conjugation-based nanostructures. Understanding the bio conjugation and the physical interactions of bio conjugation on the surface of gold nanoparticles, opens novel opportunities for their use in biomedicine.

1.2 Problem Statement

The conjugation of AuNP to antibodies can be used as a direct label for sensitive colometric detection of analytes in lateral flow immunoassay (LFI) tests (rapid test) so called test strips where the sample penetrates a membrane by capillary force. It proved to work well as detected by the LFI with good binding capability that can only be read by visual inspection. However, the strength of the binding is not known. Hence, force spectroscopy measurement can be a useful tool to ascertain the strength (force) of the conjugation. Detecting forces is an important step towards developing new analytical and biomedical devices such as biosensors.

To the best of our knowledge, this study is the first to be conducted on the determination of interaction between AuNP and conjugated antibodies and also the elasticity of these samples using AFM. Due to that, there are lack of information on physical interaction of biomolecules and nanoparticle. Besides, the protocols of microscopy profile for conjugation sample is not well documented.

1.3 Objectives of the Study

1. To develope and validate the protocols of microscopy profile for the gold nanoparticle conjugated with antibody.
2. To study the relation in adhesion force and Young's modulus (elasticity) of AuNP and conjugated AuNP_IgA for non-functionalized and functionalized AFM tip using force spectroscopy in ambient air and liquid environment.

1.4 Scope of the Study

In this study, the 40 nm spherical shape of AuNP was used since it is a noble metal (exhibits a high chemical stability), easily to functionalize and has extraordinary capability to conjugate with various biomolecules (agglomeration induces a shift of the maximum absorption towards higher wavelength that reflects a color changes when the biomolecules are present in the sample) as well as the spherical AuNP were less toxicity (depends on the type of cells used) compared to the nanorod AuNP (Murphy *et al*., 2008).

40 nm AuNP is used due to the facts that larger the size of particle will increased their stability while smaller the size will decreased their stability due to

the increasing active surface area (larger ratio of volume/area). Instead, smaller the size of AuNP will increased their sensitivity due to increasing active surface area. For 40 nm, larger the size will increased their vividity, therefore colour on dipstic assay is higher compared to 30 nm. 30 nm and 40 nm is not so different and almost identical in terms of size, however, 40 nm is well known to have a good stability, give vivid color and most used by the diagnostic company for the kit preparation.

Mica is commonly used as a substrate for high resolution AFM studies of biological samples because mica surface can be easily to cut to any sized and cleaves easily to obtain clean, flat and smooth as well as negatively charged surface (but it can be modified to make the surface positive). Sample binding is usually accomplished using electrostatic attraction between charges on the samples and charges on the mica surface. Therefore, the mica had been treated using different molecular weights of PLL in order to activate positively charged mica for enhancing the deposition of negatively charged AuNP and to make the sample immobilized on a surface after immersion in a phosphate buffer saline (PBS). In addition, mica is stable and inert to water, acid, alkalis and conventional solvent.

Phosphate buffer saline (PBS) is used as a medium in liquid imaging because the pH value of PBS is set to be within the range of 7 to 7.6 as pH of most of biological materials fall between pH 7 to 7.6, but often set to be 7.4 as the pH of the blood nears 7.4. Furthermore, PBS has the osmolarity and ion concentration that matches those of the fluid inside the cells and cause isotonic and non-toxic to most cells. In other words, saline maintain osmotic balance between internal and external environment of cell while phosphate buffer maintain physiological pH.

Phosphate ion is known to all cells, so it is wise to use physiological buffer made of phosphate ion to keep the cell healthy. PBS helps to maintain the constant pH and osmolarity of the cells.

For force spectroscopy measurements, two different types (BLAC40TS and OMCLTR800PSA) of AFM tips with different dimension of spring constant, resonance frequency, shape, radius and length were used. The adhesion force can be determined if the deflection sensitivity and cantilever spring constant are stated (Azonano, 2011). The tip sharpness and spring constant plays an important role in force spectroscopy. The higher value of spring constant will contributed to higher value of adhesion force (Lee *et al.*, 1994). Large adhesion force can reduce imaging resolution, damage the sample and tip, and can create unwanted artifacts (Prater *et al.*, 1995). In addition, adhesion force between the tip and sample decrease with the tip radius. Sharper tip contributed to the lower adhesion force, high resolution topography image and more stable imaging.

The cantilever of BLAC40TS tip with high resonance frequency while being soft with a low spring constant was specially design for AFM measurement in liquid and allows the fast imaging of biological samples as well as short lever give a higher sensitivity with optical beam deflection sensors. Small spring constant makes a large deflection to a small force resulting in a high resolution topography image. Meanwhile, the triangular shape (V shape) lever of OMCL-TR800PSA was built to reduce torsional motion and prevent the cantilever to bend out of the position during sample imaging. However, for particle size analysis and determination of surface roughness, non-contact high resolution (NCHR) AFM tip was used for imaging in order to obtain high resolution topography images.

CHAPTER 2

LITERATURE REVIEW

2.1 AFM Tip Functionalization

AFM tip can be functionalized either by direct covalent attachment of ligand to the probe tip or by absorbing molecules on the probe. The AFM tip functionalization method using covalent attachment can improve binding affinity between complementary molecules because the longer linker allows considerable movement of the terminal molecules while the probe is moving across the sample. However it can be difficult to control the reproducibility of the silanization reaction because the silane reagent themselves are sensitive to contamination, easily crosslinks and can have a short shelf life. This can lead to wide variations in the surface density of the reactive functionalization groups, which are particularly undesirable when the experimental strategy calls for close control of this parameter in order to favour single-molecule interactions.

It is advantageous to attach the ligand molecules to the end of a flexible polyethylene glycol (PEG). The PEG linker's flexibility provides the ligand molecules the mobility to reorient its position as it approaches or comes in contact with the target so that it can bind easily to the receptor on a substrate. PEG is the most widely used crosslinker in force spectroscopy technique because it can be functionalized with a variety of useful end groups to permit the attachment of various molecular entities (Kienberger *et al.,* 2000). It has been demonstrated that elastic properties of the PEG obtained an independent and accurate measurement

of the number of interactions (Sulchek *et al.,* 2006). Functionalized tips are used to study the information of ligand-receptor pair interaction by force spectroscopy.

The AFM tips are normally functionalized in three steps: (1) aminofunctionalization, (2) crosslinker attachment, and (3) ligand-receptor coupling. Apart from these steps, adsorption or chemisorption can be very efficient methods for functionalization under certain circumstances (Ebner *et al.,* 2008). More importantly, the AFM tip must be cleaned prior to functionalization to remove any contamination during packaging and shipping. The AFM tip is normally made of silicon nitride (Si_3N_4) that easily produce asurface layer of silanol (SiOH) in ambient air conditions.

The immersion of the tip in a substance made by mixing the sulphuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) which is known as piranha solution has been shown to be an effective method to remove the contamination (Lo *et al.,* 1999; Sirghi *et al.,* 2006). First, the tip is rinsed using ethanol and dried with nitrogen. Then, the tip is incubated in piranha solution with a ratio of H_2SO_4/H_2O_2, 70/30 (v/v) for 30 minutes. After that, the tip is washed using deionized water and ethanol and then left to dry under a low rate of nitrogen flow. Finally, the tip is baked at 100-150^{o}C for 30 to 60 minutes to ensure that there is no residue moisture.

In addition, a flat support is required for the immobilization of biomolecules. Mica, glass and silicon have shown to be effective supports for biomolecules. However, mica is most widely used due to its non-conducting mineral layer that can be easily cleaved using adhesive tape to produce a clean and flat surface

(Dupres *et al.,* 2007). Moreover, mica can be amino-functionalized for attachment of biomolecules using bi-functional crosslinkers (Ebner *et al.,* 2008)

Several important issues must be considered when functionalizing the tip and substrate with biomolecules. The forces which immobilize the molecules should be stronger than the intermolecular force being studied. Besides this, the attached biomolecules should have enough mobility so that they can freely interact with complementary molecules. In addition, the contribution of non-specific adhesion to the measured forces should be minimized (Hinterdorfer, 2002). In order to fulfill these requirements, several flexible spacer molecules have been introduced, including poly(ethylene glycol) and carboxymethylamylose. The biomolecules are covalently bound and able to move and orient freely, while non-specific adsorption is inhibited.

2.1.1 Amination by Silanization

AFM tips can be functionalized by direct covalent attachment of ligand to the tip of the AFM probe (Ebner *et al.,* 2007) by aminofunctionalization with aminosilanes (Chen *et al.,* 1997; Vinckier *et al.,* 1998; Ros *et al.,* 1998; Strunz & Oroszlan, 1999; Desmeules *et al.,* 2002; Jiang *et al.,* 2003; Almqvist *et al.,* 2004; Brogan *et al.,* 2004; Ratto *et al.,* 2004; Langry *et al.,* 2005; Wang *et al.,* 2005; Tsapikouni & Missirlis, 2010) or ethanolamine hydrochloride (HCl) (Hinterdorfer *et al.,* 1996; Riener *et al.,* 2003; Nevo *et al.,* 2003; Baumgartner *et al.,* 2003; Klein *et al.,* 2003; Stroh *et al.,* 2004; Ebner *et al.,* 2004; Ebner *et al.,* 2005; Bonanni *et al.,* 2005; Kienberger *et al.,* 2006; Kamruzzahan *et al.,* 2006; Ebner *et al.,* 2007; Puntheeranurak *et al.,* 2007; Lower, 2010; Wildling *et al.,* 2011).

The first step in functionalization is silanization of the tip surface with organosilane reagent. Silanization is the process of covering a surface with organofunctionalalkoxysilane molecules to form bonds across the interface according to their organic functions. This organofunctionalalkoxysilane represents the alkoxy group and a commonly methoxy ($-OCH_3$) and ethoxy ($-OCH_2CH_3$) group that are able to interact and undergo a chemical reaction with inorganic material for example a silica and glass. Moreover, the organosilane can act as a linker between the tip and functional molecules of interest, which is also known as silane coupling agent (Blanchette *et al.,* 2008). The silanization of the AFM tip using various organosilane reagents is summarized in Table 2.1.

There are various aminosilanes that have been used in the silanization of AFM tips such as 3-Aminopropyltriethoxysilane (APTES), Methyltriethoxysilane (MTES), Aminopropyltrimethoxysilane (APTMS), N'-(3-(trimethoxysilyl)-propyl)-diethylentriamin, and 3-aminopropyl-dimethylethoxysilane (APDMES). Silanes react with the silanol group (SiOH) on the surface of the tips which is covered by silicon.

In tip silanization, APTES are commonly used because it has three ethoxy groups that are able to produce Si-O-Si linkage to the tip which is capable of undergoing polymerization in the presence of water for covalent attachment. The silane group in APTES is highly reactive and silanizes the surface by forming covalent bonds with surface atoms. Polymerization of the silane existing at the tip due to the presence of water can be avoided by performing the tip silanization in the vapour phase. In the research investigated by Langry *et al.* (2005), the AFM tip was silanized with a vapour mixture of 1:10 molar of APTES/MTES that

provided a siloxane vapour concentration which produced the cantilever generally only one molecular interaction with the surface per measurement attempt.

In the study by Jiang *et al.* (2003), each step of the protein modification on the tip was characterized using X-ray photoelectron spectroscopy (XPS). It was reported that the N_{1s} peak in the XPS spectrum did not appear on the bare silicon surface. However, upon APTES silanization, one peak N_{1s} appeared at ~400 ev.

APDMES is another chemical used for silanization. The presence of a single ethoxy group in APDMES caused a reaction between the functionalized tip to silica so that it was easier to control and resulted in an amino-functionalized monolayer (Smith & Chen, 2008). The effectiveness of the APDMES treatment for the generation of amine group on the tip revealed a homogenous and smooth topography image pointing to the formation of an amine monolayer. The results of the APDMES treatment was reported on a force graph obtained before and after tip silanization. Before the tip silanization, since the silicon nitride tip and substrate (glass) were negatively charged, they electrostatically repelled each other. The cantilever bent at a certain approaching curve recorded after the silanization, revealed the presence of an electrostatic attraction force between the tip and substrate caused by the generation of amine group covering the tip surface (Tsapikouni & Missirlis, 2010).

Aminopropyltrimethoxysilane (APTMS) has also been used in tip silanization (Brogan *et al.,* 2004). The AFM tip was modified with anti-ovalbumin IgG antibodies immobilized in either an oriented or a random manner. The oriented immobilization of whole IgG was accomplished through the use of Protein A, and random immobilization was carried out with glutaraldehyde.

After the silanization with APTMS, the tip was activated by incubation in glutaraldehyde solution which reacted with the amine groups. Glutaraldehyde was used as a binding agent for the attachment and immobilization of ligand to the tip. Glutaraldehyde coupling obtained a higher antibody surface coverage as well as antigen binding capacity relative to the Protein A-based immobilization. However, the Protein A-immobilized IgG had an antigen binding efficiency that was five times higher compared to the glutaraldehyde coupling, therefore representing a greater percentage of Protein A-immobilized antibodies which were oriented with their antigen binding sites exposed to the solution (Brogan *et al.,* 2004).

Other aminosilanes have rarely been used apart from APTES, APDMES and APTMS, for tip functionalization. APTES has been claimed to be the best aminosilane that provides a defined monolayer on a substrate (Moon *et al.,* 1997). Aminosilanization using APTES or APTMS in the gas phase or dry toluene is the best established and characterized method for amino-functionalization of AFM tips (Ebner *et al.,* 2008).

Table 2.1 Aminofunctionalization with aminosilanes.

Tip Binding	Aminosilanes	Reference
PEG-DNA PEG-H3 Antibody Antigen-Antibody GroEL-protein Biotin-PEG-NHS PEG-Concanavalin-A hSwi-Snf Aptamer-Protein Gold-Alkyl thiolate bond	3-Aminopropyltriethoxysilane (APTES)	Strunz & Oroszlan,(1999) C. M. Stroh *et al.,*(2004) Ros *et al.,*(1998) Vinckier *et al.,*(1998) Paris*et al.,*(2000) Ratto *et al.,*(2004) Wang *et al.,*(2005) Jiang *et al.,*(2003) Langry *et al.,*(2005)
PEG-Fibrinogen Antibody Bovine Serum Albumin (BSA) and Bovine Fibrinogen (BFB)	3-aminopropyl-dimethyl-ethoxysilane (APDMES)	Tsapikouni & Missirlis,(2010) Almqvist *et al.,*(2004) Chen *et al.,*(1997)
PEG-Concanavalin-A Gold-Alkyl thiolate bond	Methyltriethoxysilane (MTES)	Ratto *et al.,*(2004) Langry *et al.,*(2005)
Antibody	Aminopropyltrimethoxysilane (APTMS)	Brogan *et al.,*(2004)
Recoverin-DPCC bilayer	N'-(3-(trimethoxysilyl)-propyl)-diethylentriamin	Desmeules *et al.,*(2002)

2.1.2 Amination by Esterification

Amino-functionalization with ethanolamine hydrochloride solution is widely used in esterification reaction methods to form amine-terminated tip functionalization. Esterification describes the reaction between two chemicals (alcohol and carboxylic acid) to produce the final reaction result which is ester. The function of chloride is to activate the reaction and generate amino groups on AFM tips (Blanchette *et al.,* 2008). The esterification of silanol with alcohol leads the organic molecules to form a strong covalent bonds with surface covered by silanol.

In a study by Riener *et al.* (2003), dimethylsulfoxide (DMSO) was added as a non-volatile solvent and molecular sieve beads were added to bind moisture in the esterification method. Unfortunately, this ethanolamine did not work well in magnetically coated tips because it lost magnetism in hot ethanolamine/DMSO and became useless. Thus, the problem was solved by performing esterification at room temperature and just sufficient heat was used to dissolve all solids in DMSO (Fig. 2.1).

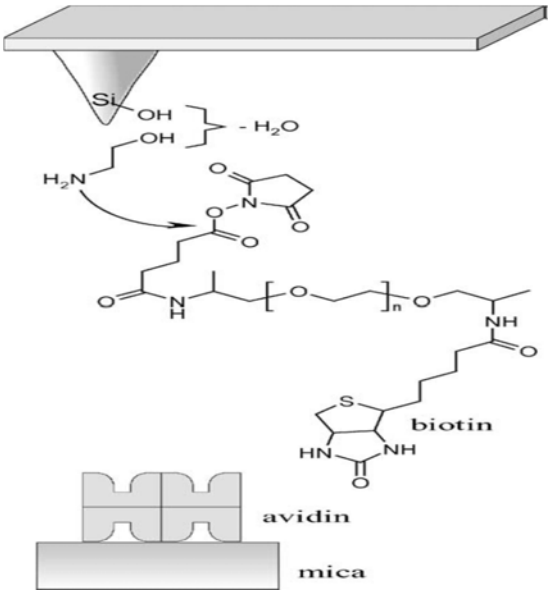

Figure 2.1 Amino groups are introduced on the AFM tip and biotin–PEG_{800}–NHS is attached via amide bond formation (Riener *et al.,* 2003).

Measurement of interaction forces of ligand-receptor pairs can be effectively done if the ligand and receptor were tightly bound and immobilized on the tip surfaces. Various researchers have investigated a heterobifunctional PEG as a tethering or linker of probe molecules to the tip. The standard procedure of tip-PEG-probe linking was conducted in three steps: (i) generation of amino groups or thiol functions on the tip surface, (ii) attachment with one end of the heterobifunctional PEG linker and (iii) the protein is coupled to the free PEG end. The method of tip functionalization for the tip-PEG-linking probe can be summarized in Table 2.2.

In another study investigated by Riener *et al.* (2003), a 6 nm PEG linker and a functional succinimide group at the other end were synthesized and coupled to the amine functionalized AFM tip. Avidin-biotin was chosen as the receptor-ligand pair. The approach of this research was to prepare AFM tips containing just one ora few PEG_{800} probe tentacles with a reach to the surface bound receptor in order

to probe single recognition sites with high lateral resolutions. PEG_{800} indicates the average molecular weight of the specific PEG at 800. By using ethanolamine/HCl/DMSO treatment at room temperature, a tip with the highest probability of specific binding was obtained. It also showed that the amino-functionalization and coupling of biotin-PEG-NHS with AFM tips, together with the immobilization of avidin on mica (Fig. 2.1), provided a convenient test system that is suitable for start-up experiments and methodical investigations of parameters. In addition, in a study by Bonanni *et al.* (2005), a 10nm long PEG that bore an aldehyde (ALD) at one end and NHS at the other end was used as a cross-linker (ALD-PEG-NHS). Azurin and Cytochrome C 551 were used as a ligand-receptor pair. Azurin was directly chemisorbed on a gold electrode whereas cytochrome c was linked to the AFM tip by heterobifunctional flexible cross-linker.

Figure 2.2 shows a schematic representation of a testing system for tip-antibody linking via disulphide bond formation. Esterification with ethanolamine hydrochloride in the first step proved to be equally suitable for attachment of PEG-biotin tentacles on AFM tip. Ethanolamine hydrochloride was used to generate amino groups on the AFM tip. The tip-bound PEG-biotin tentacle yielded a binding probability of 10-20% to a dense monolayer of avidin on mica. In the third step, the 2-pyridyl-S-S group on the free ends of the PEG chains reacted with biotinylated IgG to produce a stable disulphide bond (Kamruzzahan *et al.,* 2006). In comparison, a tip immersed in antibody that was prederivatized with S-acetylthiopropionyl (SATP) group by removing the acetyl group with hydroxylamine produced free thiol, which formed a disulfide bond with the PEG tether (Kienberger *et al.,* 2006).