Investigation of biorecognition process using force spectroscopy, AFM with molecular functionalized tip

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UNIVERSITY

**INVESTIGATION OF BIORECOGNITION PROCESS
USING FORCE SPECTROSCOPY, AFM WITH
MOLECULAR FUNCTIONALIZED TIP**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2017

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

13 January 2017

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Date

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.....
SEONG-OH KIM

Abstract

Biorecognition process is the core of all biological interactions including interaction of cells, lipids, proteins and DNA. These Adhesion events in chemistry and biology are characterized by intermolecular interactions between particular chemical functionalities with noncovalent bonds, such as van der Waals, ionic or hydrophobic interactions. Furthermore, shape complementarities play a crucial role in the molecular recognition or biorecognition. Force spectroscopy measurement is one of the promising and versatile tool for the quantitative characterization of these binding forces and molecular interactions. On the basis of atomic force microscopy (AFM), force spectroscopy uses the tip functionalization as a means of evaluating binding specificity into interaction measurements. In practice, a chemically functionalized AFM tip is brought into contact with also chemically modified substrate with specific functionality and as the tip is retracted, the binding force between the two target molecular pair is measured.

In this dissertation, we investigated the biorecognition process with biomaterials using force spectroscopy, AFM. The determination of the binding interactions which can control the biological functions will be described, focusing particularly peptide-inorganic materials and peptide-cellular membrane components. Based on the previous force spectroscopy measurement, the investigation of biomolecular interaction with various experimental factors will be proposed. A force-distance curve, the main output of force spectroscopy measurement, contains a lot of information to determine the mechanical properties of samples. To extract dependable and reproducible information, there are several experimental parameters and statistical analysis which should be contemplated. Estimating the binding mechanism which governs molecular complexes and understanding the strength of biomolecular associations lead to a fundamental knowledge of how the specific peptide interacts to biomaterials and consequently elucidate the biorecognition system and applications.

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Abbreviations

AFM	Atomic Force Microscopy
AH	Amphipathic Helix
BSA	Bovine Serum Albumin
CCD	Charge-Coupled Device
CD	Circular Dichroism
CFM	Chemical Force Microscopy
ECSTM	Electrochemical Scanning Tunneling Microscopy
EFM	Electrostatic force microscopy
F-D Curve	Force – Distance curve
HCV	Hepatitis C Virus
HIS6	histidine 6
IOM	Inverted Optical Microscopy
MFM	Magnetic Force Microscopy
NAT	Nitrilotriacetate
NS5A	NonStructural Protein 5A
NMR	Nuclear Magnetic Resonance
PBS	Phosphate-Buffered Saline
PI(4,5)P ₂	Phosphatidylinositol 4,5-bisphosphate
PIs	Phosphoinositides
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PSPD	Position Sensitive Photo Detector
PSGL-1	P-Selectin Glycoprotein Ligand-1
QCM	Quartz Crystal Microbalance

QCM-D	Quartz Crystal Microbalance-Dissipation
RMS	Root Mean Square
SAMs	Self-Assembled Monolayers
SALB	Solvent-Assisted Lipid Bilayer
SBPs	Solid-Binding Peptides
ScFv	Single-chain Fv fragment
SCM	Scanning Capacitance Microscopy
SECM	Scanning Electrochemical Microscopy
SICM	Scanning Ion Conductance Microscopy
SLBs	Supported Lipid Bilayers
SPM	Scanning Probe Microscopy
SPR	Surface Plasmon Resonance
STM	Scanning Tunneling Microscopy
VE	Vascular Endothelial
3D	Three Dimensional

Chapter 1

Introduction

The Biomaterials or molecular interactions play a key role in fundamental biology and biochemistry such as cell adhesion, cellular signaling, immune system and cell architecture. The atomic force microscopy is the promising technique for high resolution sample surface imaging, but it also can measure the mechanical properties of the sample, force spectroscopy mode. Force spectroscopy mode is capable of resolving individual detachment events, as well as the overall force required to detach a tip and substrate interaction. This chapter introduces an overview of the biorecognition process and the strategy of how to investigate the biorecognition system using force spectroscopy with chemically functionalized AFM tip.

1.1. Problem of Statement

Molecular complexes system is commonly composed of huge number of molecules and collide together. In most cases, their connections are weak and nonspecific. However, one molecule bound to surface are complementary to its partner, that partner can interacts to surface, the interaction between two molecular partners has strong, long lifetime and specific binding affinity^{1,2}. Molecular recognition process refers to the specific binding event between two molecules pair such as receptor-ligand, protein-protein and protein-glycan³ (Figure 1.1). In the terminology, the macromolecule of an interaction is called a “*receptor*”, and a small molecule binding to its receptor partner produces a biological response is called a “*ligand*”. Through the specific interactions or binding forces mechanisms from the molecular complexes and how those forces affect to the biological and physical properties of the molecular interaction and their features allow the understanding of various biological functions such as cell adhesion, genome replication and signaling^{4,5}.

Biochemistry techniques have been employed to determine the thermodynamics and the kinetics of molecular complexes between pairs of biomolecules. From the viewpoint of thermodynamics, biorecognition process occurs due to the free energy gap between the receptor-ligand complex and unbound receptor-free ligand. To understand the biorecognition process, the factors which contribute to the free energies of the interactions have to be studied into the enthalpic and entropic components. For example, electrostatic interaction between negatively charged ligand and the positively charged receptor mainly affects the enthalpic term. To approach the investigation of binding affinity of biomolecular partners, many techniques are introduced such as surface plasmon resonance (SPR), nuclear magnetic resonance (NMR), optical or fluorescence spectroscopies and quartz

Figure 1.1. Schematic view of biorecognition processes.

When some specific sample or analyte interacts with the biological component or bio-receptor, response or signal can be detected by a transducer. The sensor responds only to a particular analyte or biomolecule of interest.

crystal microbalance (QCM)^{6,7}. However, these techniques with or without labelling, which can control an ensemble averaging, they cannot explain various factors of individual molecule and their interactions. Investigating the binding mechanisms that govern biological interactions and understanding the strength of bio-molecular interaction are challenging. Such bio-recognition processes are mainly governed by single or multiple non-covalent bonds that take place within desired the interacting molecular partners. Regarding performance sensitivity and selectivity, techniques using optical or magnetic tweezers are quite unique but, visible and ultraviolet radiation carry sufficient energy to disrupt nearly all types of bonds if directly absorbed and is therefore a limitation to many forms of optical imaging. Also, these techniques are not suitable to measure small force range (below 200 pN) and they require artificially attachment a micron-sized handle with which to apply a significant force without altering the behavior of the system under investigation. The invention of biorecognition detecting techniques, the exploration of the

single molecular interactions has become possible, providing a deeper insight of biological interactions. Among single molecule techniques, force spectroscopy, one of atomic force microscopy operation mode, is one of the most powerful tools to study biological complexes, allowing to detect intermolecular binding forces with high resolution and sensitivity in physiological conditions^{2,8-10}. Atomic force microscopy (AFM) is a very famous technique for obtaining the topography information of sample surface with sub-nanoscale resolution^{11,12}. It also can measure the mechanical properties of the sample with or without labelling or treatments. The force spectroscopy technique with high force sensitivity (piconewtons level), small contact region between tip and sample, displacement resolution (0.1 nm) is suitable for studying the molecular features of biorecognition system¹³. In particular, if one target molecule can attach to the AFM tip, the functionalized tip, and the substrate modified with target molecule partner, the biomolecular interaction such as receptor-ligand, protein-protein and antigen-antibody interaction can be measured in physiological condition¹⁴. When the functionalized tip contacts and retracts from the modified surface, the interaction between two molecular pairs is disconnected when the loading force overcomes a bonding threshold.

Although force spectroscopy can provide a variety of opportunities to determine the biorecognition processes at nanoscale level, still unknown, ambiguous and controversial results have been recorded in different experimental conditions. In this manner, a few issues related to experimental parameters or analysis procedures have to be considered to achieve the reliable and reproducible result.

- i. The investigation of biorecognition process requires that individual biomolecular pair is effectively involved in their interactions. The force-distance curve from force spectroscopy data shows sometimes very sharp or large or multiple binding events, instead of the desired specific binding. In order to obtain an accurate information of the binding event, a control of functionalization process of both AFM tip and the substrate surface is considered, such as the density of molecule attachment or incubation time.
- ii. Force spectroscopy results are influenced by the presence of nonspecific binding force among components of buffer, unexpected molecules and sometimes the desired molecular

pairs. Therefore, the establishment of criteria to distinguish between specific and nonspecific interaction is required at all data-set by determining false positive interaction.

- iii. The detailed and proper data interpretation with a number of force-distance curve results should be considered with statistical analysis. To establish the trustworthy quantitative information, performing many numbers of force curves and selection of dependable force curves are required for data analysis. During this process, nonspecific or strange shape of force curve has to be removed from data-set.

In this dissertation, we investigated the biorecognition process with biomaterials using force spectroscopy, AFM. The determination of the binding interactions which can control the biological functions will be described, particularly peptide-inorganic materials and peptide-cellular membrane components. Estimating the binding mechanism which governs molecular complexes and understanding the strength of biomolecular associations lead to a fundamental knowledge of how the specific peptide interacts to biomaterials and consequently elucidate the biorecognition system and applications.

1.2. Objective and Scope

The study of biological complexes with biorecognition processes including lipids, peptides and proteins, play a critical role in cellular life¹⁵. The biorecognition systems are mostly characterized by a collection of non-covalent bonds, hydrogen-bonding, ionic, and hydrophobic interactions⁸. In addition, shape complementarity appears to play an essential role in the process of biorecognition. One major challenge in the biorecognition system is the lack of biomimetic detection platforms to directly evaluate the binding affinity between biomolecular pairs, which is a critical point that can lead to molecular interaction. Also, optimization of experimental parameters for force spectroscopy measurement with the chemically functionalized tip is required to recognize the specific binding.

From this point of view, the main objective is to determine the peptide-inorganic materials and peptide-phospholipids interactions involved in the peptide attached AFM tip in physiological condition using mainly force spectroscopy technique. Currently, research

on biorecognition system using AFM is already studied widely by many researchers^{3,9,16-18}. However, still uncertain aspects remained, such as detailed binding mechanism, tip functionalization strategy, control of contact region and time between tip and surface and bulk data analysis. To approach these challenges in the context of solving remaining problems relating to biorecognition process at molecular interactions, a few experimental considerations are proposed in this dissertation.

First of all, the interaction between peptide and inorganic materials is investigated in physiological condition. The peptide adsorption on material surfaces have represented wide application across surface technology and biological science, expediting the development of surface modification^{2,19}. Using quantitative force mapping method, the binding affinity of engineered peptides and a variety of hydrophilic inorganic materials is determined regarding selectivity and sensitivity (Figure 1.2). Here, we combine the two different biorecognition technique, quartz crystal microbalance-dissipation (QCM-D) and AFM. QCM-D measurement are conducted to estimate the peptide binding affinity for hydrophilic oxide materials at the ensemble average level, and AFM are performed to evaluate the binding force of single peptide molecule. In accordance with the results and corresponding theoretical interpretation, the role of interfacial forces in peptide adsorption to inorganic materials can be elucidated at nanoscale level.

Figure 1.2. Force mapping images using force spectroscopy.

The engineered peptide and material interaction is visualized with different material combination. The AFM tip is functionalized material binding peptides and substrate consists of Gold (Au) / Aluminum oxide (Al_2O_3)

Secondly, based on peptide-inorganic materials interaction, the investigation of biomolecular complex binding have to be considered with various experimental factors. A force-distance curve, the main output of force spectroscopy measurement, contains a lot of information to determine the mechanical properties of samples^{20,21}. To extract dependable and reproducible information, there are several experimental parameters and statistical analysis which should be contemplated. Therefore, critical experimental parameters and conditions, such as the control of molecular density on AFM tip, contact time between molecular partner, the effect of ionic strength, the recognition of nonspecific and specific binding using positive or negative control measurement, are proposed to employ the evaluation of the interaction between a pair of biomolecular complex. These approaches can enable us to offer deep understanding of the force spectroscopy measurement on biorecognition process.

Thirdly, using optimized experimental parameters, we directly determine the interaction between the antiviral peptides and phosphoinositide receptors. Based on development of the AFM-based platform to measure the interaction strength between a synthetic peptide comprising the nonstructural 5A amphipathic helical peptides (NS5A AH) with basic amino acid pincer (BAAPP) domain and phosphoinositide molecules, we are going to study how structural features within the BAAPP domain influence phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) binding specificity and avidity.

1.3. Specific Aims

The aims of this dissertation are to mainly describe the use of force spectroscopy measurement for the evaluation of binding forces that control the biological interactions, focusing in particular on peptide-hydrophilic oxide solid substrate and peptide-cellular membrane component. All experiments have been conducted using optimized experimental parameters and proper strategy of AFM tip functionalization. By considering the other studies for determining the binding forces between biological complexes, we can then obtain the deeper understating of biorecognition process. The following specific aims will be achieved in this dissertation:

- i.** To investigate the binding affinity between solid binding peptides and inorganic solid substrates (Au, Al₂O₃, TiO₂ and SiO₂) using the combination of two experimental techniques (AFM and QCM-D).
- ii.** To determine the optimum experimental conditions (density of peptide on AFM tip, contact time, ionic strength, negative control treatment) for detecting the interaction between antiviral peptide and cell membrane component using force spectroscopy, AFM on physiological condition.
- iii.** To examine the biorecognition process of the synthetic peptides comprising the BAAPP domain and the panel of phosphoinositide receptors, PIP₂ receptors, including PI(4,5)P₂, PI(3,5)P₂, and PI(3,4)P₂ using force spectroscopy, AFM.

1.4. Dissertation Overview

The dissertation has been organized into the following chapter:

Chapter 1: “Introduction” presents the background information of the biorecognition system by force spectroscopy and outlines the board overview, objectives and specific aims of the Ph.D. research.

Chapter 2: “Literature Review” describes the recent advances in search of the biorecognition process on various biological complexes along with a detailed review of conventional and selective-sensitive force spectroscopy measurement approaches to evaluate the interactions of biomolecular pairs.

Chapter 3: “Experimental Methodology” records the chemical reagents, peptides, materials preparation, and details of the experimental techniques and their protocols used throughout this dissertation.

Chapter 4: “Correlating single-molecule and ensemble-average measurements of peptide adsorption onto different inorganic materials” discusses the specific interactions of a short peptide to four different inorganic material substrates. The adsorption kinetics and binding affinity of peptide attachment for the different substrates are determined using force spectroscopy measurement and quartz crystal microbalance-dissipation.

Chapter 5: “Quantitative Evaluation of Specific Binding Interactions between Phosphoinositide Molecules and an Amphipathic, α -Helical Peptide” introduces establishing a force spectroscopy platform to evaluate the interaction between antiviral peptides and phosphoinositide molecules, PI(4,5)P₂ using a variety of experimental parameters including contact time, density of peptide on AFM tip, ionic strength and inhibitor conditions.

Chapter 6: “Investigating How Structural Features within the NS5A AH BAAPP Domain Influence Phosphoinositide Binding Specificity and Avidity” presents the understanding how structural features of the BAAPP domain influence phosphoinositide binding specificity and avidity.

Chapter 7: “Conclusion and Future Outlook” introduces concluding remarks of the above chapters and makes recommendations for the future work on the basis of the outcomes in this dissertation.

1.5. Main Contribution of the dissertation

This dissertation led to several new findings and outcomes by:

- i.** Establishing the specific binding events between engineered 16 amino acid-long random coil peptides and inorganic solid substrates (gold, silicon oxide, titanium oxide and aluminum oxide) with selective and high binding affinity.
- ii.** Offering guidance for molecule attachment to AFM tip with gold chemistry, polyethylene glycol spacer and peptide modified with cysteine residue to N-terminus or C-terminus.
- iii.** Providing insights into biorecognition process of biomolecular partners using force spectroscopy measurement by controlling the experimental parameters such as density of peptide on AFM tip, contact time between tip and surface, ionic strength and positive or negative control of force-distance curve.
- iv.** Demonstrating the force spectroscopy measurement platform for quantitative profiling of the binding affinity and avidity between antiviral peptides and phosphoinositide evaluated by the rupture force required to break the bio-molecular interaction.

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Chapter 2

Literature Review

Molecular binding forces measurements using force spectroscopy have been studied recently by many other researchers. In this chapter, we introduce mainly three types of biorecognition processes using force spectroscopy measurement with the functionalized AFM tip. To achieve clear understanding of interaction, the force required to unbind a ligand (functionalized tip) from its specific binding site (substrate) is dissimilar to the force required to eliminate a non-specifically bound ligand. In other words, the specific binding force must be adopted to recognize bound molecules and determine their distribution on the sample surface. Biorecognition system determine the specificity of interactions between two molecules, the difference of binding force can be used to identify and make a force mapping image using the distribution of those functionalized tip and sample surface

2.1. Scanning Probe Microscopy

The invention of the scanning tunneling microscopy (STM) is significant work in the history of nano-science and nanotechnology^{1,2}. It is also beginning of scanning probe microscopy (SPM). Before the SPM technique, determining the surface features with nano-scale was extremely difficult and the most of cases, there were many imaging artifacts. However, after SPM invention, it has enabled researchers to gather and display the topographical information of many sorts of sample surface including biological materials with very nano-scale.

Among SPMs, the STM is the first invented. In order to perform imaging, the STM uses the quantum-mechanical phenomenon of tunneling current. Tunneling current, which is key parameter of STM, is occurred by the flow of electrons from the surface of one material to the surface of another. The magnitude of the tunneling current is appreciable. When the separation between the surfaces is on the order of a few nanometers, the tunneling current is highly increased. Using the tunneling current flows between the samples and the probes, STM can sample surface image. However, in initial STM model, it is almost impossible to achieve atomic scale images due to irrelevant vibrations. The extraneous vibrations made it difficult to maintain a small separation between the sample and the probe (**Figure 2.1**).

In 1981, Binnig and Rohrer carried out the first successful tunneling current experiment³. They used an innovative feedback loop system that controlled the separation between the sample surface and probe. It was a critical point of success. By using this set-up, they can image the topography information of material at the atomic scale¹, and their team finally succeeded to resolve the structure of silicon at the atomic scale using the STM⁴.

The basic principle of all SPMs have in common is based on the interaction between the sample surface and the STM probe with atomic-scale precision. A sharp tip scans across a sample surface by means of z-piezo, while a certain signal is recorded by the tip for every single image point. Since wavelength of the analytical signal information normally bigger

than the atomic force microscopy (AFM) tip size parameter (size of apex) and the distance between tip and sample surface. So, the resolution is no longer diffraction limited as given by the wavelength of the signal. Since the measured signal composes topography information of the surface below the probe, the term local tip technique can be found frequently. The most important perspective of this concept is to use the local signal for observing the distance between tip and sample surface. Thus, topographical information can be obtained in real situation. Moreover, The AFM system can be placed the AFM tips above a sample surface with nanometer lever or below, it can possible to the exciting potential of accessing local spectroscopic information even confined to spots as small as a single atom.

The SPM techniques use different type of probes, which based on the specific property. (**Table 2.1**) Normally STM uses a sharp conducting tip as the probe, but AFM can use a micro fabricated cantilever with a sharp tip as a force transducer. Magnetic, thermal, and biological properties of material can be investigated at the atomic scale by using proper the SPM probe. In case of a magnetic force microscopy (MFM), one of the SPM group, images magnetic forces using the magnetic material coated tip. In this manner, using biological material coated AFM tip, it can be made sensitive to bio-molecular forces.

Figure 2.1. Schematic view of simple principle of SPM.

Depicting the probe, piezoelectric element, and control system.

Table 2.1. SPM technique list and their measurement properties(Adopted and reprinted from Anariba et al., 2012)⁵

SPM Technique	Measuring interaction	Remarks
Chemical force microscopy ⁶ (CFM)	Van der Waals interactions	Complex tip preparation
Electrochemical scanning tunneling microscopy ⁷ (ECSTM)	Redox reactions	Used in electroplating and batteries
Electrostatic force microscopy ⁸ (EFM)	Electrostatic force	Sample can be immersed in solution
Magnetic force microscopy ⁹ (MFM)	Magnetic interactions	Used on magnetic samples
Scanning electrochemical Microscopy ¹⁰ (SECM)	Electrochemical activity	Poor image resolution
Scanning capacitance Microscopy ¹¹ (SCM)	Capacitance between probe and sample	Requires a conducting surface
Scanning ion conductance Microscopy ¹² (SICM)	Ion conductance just above sample	Feasible for imaging live cells in solution

2.2. Atomic Force Microscopy

Atomic force microscopy (AFM), invented in 1986 by Binnig, Quate and Gerber group¹³. The STM techniques mainly measure the tunneling current signal between their conducting tip and a conducting sample surface to image the information of sample surface and measure the electrical properties of the sample. However, the STM techniques have a main difficulty in that it cannot adopt a non-conducting material. The invention of the AFM figures out this problem and it can measure almost any sample, regardless of its electrical properties. Similar to other STM techniques, the AFM scans over the sample surface using a sharp tip and measures the force change between the sample and the AFM tip. Based on the distance between the AFM tip and surface, there are either repulsive or attractive force

which can be utilized to image the sample surface. **Figure 2.2** represents the basic configuration for most AFMs. The repulsive or attractive force between the sample surface and AFM tip can be detected by observing the AFM cantilever deflection signal. It can be quantified by the measurement of a beam which is reflected the cantilever reflective side and onto the Position Sensitive Photo Detector (PSPD), thus enabling the system to generate a map of the surface topography. The AFM can easily take a measurement of conductive, non-conductive, and even liquid samples without sample preparation.

Figure 2.2. Diagram of AFM system.

It shows traditional AFM principle. The cantilever deflection can be detected because of the attractive or repulsive force between the tip and the sample. As the cantilever deflects, the angle of the reflected laser beam changes, and the laser spot drops down a different part of the PSPD. Using this signal, the system (computer) makes topography of the sample surface.

2.2.1 Operation mode

Contact mode : In case of contact mode, the AFM tip makes soft but, real contact with the sample surface¹⁴. The imaging of sample surface is then conducted by utilizing the repulsive force that is exerted vertically between the sample and the AFM tip. In here, the repulsive force is very small level, on the order of piconewtons, the spring constant of the AFM cantilever is also appropriately small (contact mode cantilever, less than 0.1 N/m), thus allowing the AFM cantilever to react very sensitively. The cantilever deflects upward or downward depends on the sample surface structure, such as convex or concave area. The feedback loop uses the change of cantilever deflection signal to image topography of the sample surface. In order to generate the surface topography information, the z-scanner need to maintain the constant distance between the AFM tip and the sample by keeping the cantilever deflection. If the cantilever reaches a lower area, the z-scanner will move the cantilever down by that distance, or back up if the cantilever begins rising.

Figure 2.3. Relation between the force and the distance.

Black arrow indicates contact point between AFM tip and sample surface.

Non-Contact mode : As shown in **Figure 2.3**, in case of non-contact mode, the repulsive force between the AFM tip and the sample surface is tiny so that there are no sample surface damage or deformation during the imaging¹⁵. In contrast to non-contact AFM, contact mode AFM uses the “physical contact” between the tip and the sample surface and, sometimes the AFM tip damages the sample surface in contact mode AFM.

Figure 2.4 shows AFM cantilever movement which related to the imaging of sample surface both contact and non-contact mode AFM. In contact mode, it typically uses the real contact between the surface and the AFM tip using the attractive interaction. However, non-contact mode uses the repulsive force to image the sample surface so that there is no contact and no sample damage. In other words, the AFM cantilever just fluctuates very close to the sample surface, but no contact to sample surface. Furthermore the life time of the AFM tip is extended due to no physical contact during the imaging^{16,17}.

Figure 2.4. Conceptual illustration of contact mode and non-contact mode AFM.

AFM cantilever movement which related to the imaging of sample surface both contact and non-contact mode AFM.

2.2.2. Cantilever selection

The AFM cantilever senses deflection signals due to the interaction with the sample surface characteristics such as topographic information, physical robustness, electrical, magnetic and chemical properties. The AFM cantilever consists of the large piece of silicon chip, the cantilever placed at the end of the chip and the tip hanging at the edge of the cantilever (**Figure 2.5**). Depending on the material and application, the structures of the AFM cantilever is either triangular or rectangular shape. On the basis of geometry such as height, depth, width and thickness, there have a different spring constant. Typically, the upper side of the AFM cantilever has a metallic coating, e.g. gold or aluminum, to increase the reflectivity. The aluminum coating conducts its high reflectivity but, in biological sample, gold coating is better choice because of its chemical inertness.

The shape/geometry and the material of the AFM cantilever influences the properties that make the cantilever for contact or non-contact imaging modes. For contact mode, a soft cantilever with low spring constant (less than 0.1 N/m) is required to determine the small force between the sample surface and the tip. The hard or stiff cantilever can lead to pushing high forces to the surface so that the AFM tip or sample surface can be damaged easily. The cantilever for the contact mode has $\sim 1\ \mu\text{m}$ thickness to obtain a low spring constant because it makes a comparatively high deflection to a tiny force and it can provide a good quality surface imaging. In non-contact mode, compared to contact mode, the cantilever has a greater thickness (approximately $4\ \mu\text{m}$). It has a spring constant of more than 1 N/m which is very rigid, and a comparatively high resonant frequency. For non-contact mode, the AFM cantilever is oscillated at a high resonance frequency (approximately 200 ~ 300 Hz), and changing of the phase signal and amplitude can be occurred because of the interaction between the sample surface and the AFM tip. In liquid condition, or if the tip is positioned on a contaminated layer, it may often contact to the sample surface because of the surface tension of the AFM tip. When using the small value of spring constant cantilever, it happens more frequently. Due to the flexible feature of cantilever, it is difficult to bring it back to the original position. Therefore, to overcome the surface tension, the cantilever with properly big spring constant and sharp tip is required to reduce the surface tension.

Figure 2.5. The overall view from above and magnified view from the side of typical AFM cantilever.

Regarding the morphology of a sample surface, cantilever or imaging mode must be considered, such as when the tip curvature radius is bigger than the sample structure, the tip shape will influence the topography image (**Figure 2.6**). Therefore, a tip which sharper than the smallest sample structure should be selected in order to avoid these artifacts. However, the sharper tip has a shorter life time and are more expensive than general AFM tips. It is important to choose an AFM tip that corresponds to the sample structures.

Figure 2.6. AFM artifact - Tip convolution.

When the tip radius is bigger than the features of a sample, the tip shape will influence the resulting image. Therefore, a tip sharper than the smallest sample features should be selected in order to avoid these artifacts.

2.2.3. Force spectroscopy

The AFM is the best known for the sample surface imaging with high resolution, but it also can measure the mechanical properties such as Young's modulus and adhesion force of sample using force spectroscopy mode^{18,19}.

Cappella et al. introduced the theory and the application of force – distance curve (F-D curve) from AFM¹⁸. Force Spectroscopy is used in the investigation of a sample's mechanical properties. A force - distance plots show the cantilever's interaction with the sample changes as its distance from sample changes. In imaging modes, the tip scans over the sample surface to produce a three dimensional (3D) image of the surface. In force spectroscopy mode, the tip moves directly towards the sample until they meet together and then retracted again for measuring the interaction between the tip and sample. The first step of F-D curve measurement is the “approach” (**Figure 2.7, part 1**). The cantilever is positioned far from the sample surface where there are no forces affecting on the tip and no deflection changing. Next, the approach curve is displayed by movement of cantilever towards the sample surface and “contact” between them (**Figure 2.7, part 2**).

Figure 2.7. Schematic diagram of a typical force-distance curve.

It consists of mainly four processes; Approach, Contact, Adhesion or binding and Pull off. Using contact line (2), the Young's modulus (Elastic modulus) can be calculated and using adhesion phenomenon (3), the interaction between two biomolecules or materials can be determined at nanoscale level.

After the approach curve, the retract curve is obtained when pulling up the z scanner. During the retract curve, the “adhesion” force can be acquired due to interaction between tip and sample (**Figure 2.7, part 3**). The last step is the “pull-off” which the distance between tip and sample is so far that it reaches critical point, so there are no contact between sample and tip (**Figure 2.7, part 4**). During this approach-retract cycle, force spectroscopy mode is measure interaction between tip and sample on one point through Z scanner movement. Therefore, Z scanner will decline and incline, so tip will pull down on one point of sample (**Figure 2.7, red line**) and pull off (**Figure 2.7, blue line**). At this point, shown as **Figure 2.7**, there are changes on cantilever defection, also force value. Using F-D curve, force spectroscopy measurement, it can measure the strength of single hydrogen bond²⁰ or diffent type of covalent bond²¹. **Table 2.2** summarizes the types of forces-distance curve and illustrates the effect of each type of interaction on the appearance of the force-distance curve. Among these many type of interaction of forces, the adhesion force from retraction curve can utilize to detect the biorecognition event. To investigate the interaction between two partner molecules, they should be attached or immobilized onto the substrate and the tip. As described by many researchers, the detection of binding force and mechanical characterization of DNA²², proteins²³, polysaccharides²⁴, synthetic polymers (PEG)²⁵, supramolecular interaction²⁶ and antigen-antibody interaction²⁷ can be performed by force spectroscopy method. For studying the binding force between two molecules, the force required to break this interaction, the adhesion force or the rupture force, can be acquired under various conditions.

2.3. Tip functionalization

The main objective of AFM tip functionalization is to detect binding affinity effectively and properly by the interaction between the molecules on the AFM tip and on the target sample or substrate surface²⁸ (**Table 2.2**). Frisbie et al. established that methyl (-CH₃) or carboxyl (-COOH) groups attached tips can specifically bind with similar groups on the surface. Also they demonstrated that the spatial pattern of interaction mapping with functionalized tip and functional groups on the substrate²⁸. Wong et al. introduced the AFM tips covalently functionalized with carbon nanotubes. Using nanotube functionalized tip,

they could measure the specific ligand-receptor interactions particularly biotin-streptavidin interaction²⁹.

Figure 2.8. Schematic view of a representative force-distance curve with AFM functionalized tip.

The curve includes both the adhesion force (specific interaction) and the rupture distance between two molecules.

There are a few check points which must be considered for molecules attachment to AFM tip. First of all, the proper AFM cantilever has to be chosen. Typically, very soft AFM cantilever (spring constant < 0.1 N/m) and sharp AFM tip are used to detect the molecular binding forces. In order to obtain accurate result with quantitative measurement, the spring constant of AFM cantilever should be calibrated by thermal method³⁰. Second, the interaction strength between AFM tip and the attached molecule is bigger than the binding strength between the molecule (ligand) and the surface molecule (receptor). To prevent this phenomenon, covalent binding, such as gold-thiol interaction is used to AFM tip functionalization chemistry. Third, to investigate the specific interaction between two molecules, the linker or spacer molecule gives a chance that can provide in detecting specific binding events. When using non-functionalized tip or without spacer, the results

include many non-specific binding force and it is important to distinguish non-specific interaction and the targeted specific interaction. The spacer between AFM tip and molecule is typically flexible and it provides molecule mobility to easily access the binding of surface molecule. It can characterize the rupture distance due to entropic stretching of the spacer molecule which can recognize the targeted specific interactions from non-specific interaction (**Figure 2.8**).

Table 2.2. Types of interaction forces felt by the cantilever during the approach and retraction portion of the force curve

(Adopted and reprinted from Zlatanova et al., 2000)³¹

	Force	Description	Curve type
Approach curve	Van der Waals force (Attractive)	Creates jump to contact peak, meniscus forces must be eliminated by working in a low humidity environment or in liquid.
	Double layer electrostatic force (Repulsive)	Due to charging both sample and tip surfaces with like charges. (Adsorption of ions from solution and dissociation of functional groups on surfaces)
	Hydration force (Repulsive)	Comes from repellant interactions between hydrated ions bound to tip and sample surfaces, mostly at high salt concentration.
	Solvation force	When tip and sample are within a few molecular diameters from each other, oscillation between attraction and repulsion may be observed; caused by ordering of non-polar liquid molecules between the two liquid – solid interfaces; force follows density of solvent.
Retraction curve	Adhesion force	Due to van der Waals forces and indentation of sample by tip; Meniscus force must be eliminated.
	Hydrophobic force	Appear on imaging hydrophobic samples in water.

2.3.1. AFM tip functionalization chemistry

There are several methods to attach functional organic molecule to the AFM tip, the most common approach is formation of amino-self assembled monolayers (SAMs) with gold coated AFM tip³². The main strong point of this method is high binding affinity between thiol groups and gold. Binding strength between gold and thiol group can help withstand that AFM tip-molecule interaction is stronger than the tip attached molecule-molecule on substrate interaction. Also, gold coated AFM tip can be easily reused by washing all immobilized molecules.

The first step of AFM tip functionalization with this method is gold-thiol chemistry. To bind the thiolated molecules to gold coated AFM tip, alkanethiol molecule with 10 ~ 18 carbon atoms is generally used. This chain lengths can improve hydrogen bonding and stability of binding³³. After attached alkanethiol molecule, the desired ligand molecule can be immobilized directly to the AFM tip. However, in that case, many non-specific bindings occur, and to avoid it, spacer molecule is used. Besides distinguishing between non-specific and specific interaction, spacer with two different terminal group can control the density of the ligand molecules on the tip surface. For example, AFM tip with a spacer of 100% of molecule HS-R-X', where X' has a reactive chemical group can bind with the desired ligand, can produce 100% of the ligand. For the another type of AFM tip, it contains 50% of molecule HS-R-X' and 50% of molecule HS-R-X, where X has an inert terminal group, can only represent 50% of the ligand³⁴ (**Figure 2.9**).

Figure 2.9. Mixed spacers are bind on a gold coated tip.

(a) The ligand is fully attached to gold coated tip, (b) Due to spacer structure, ligand is attached to tip selectively.

2.4. Single-Molecule Adhesion Measurements using force spectroscopy with functionalized tip.

Molecular binding forces measurements using force spectroscopy have been studied recently by many other researchers. In this chapter, we introduce mainly three types of biorecognition processes using force spectroscopy measurement with the functionalized AFM tip (**Figure 2.10**). To achieve clear understanding of interaction, the force required to unbind a ligand (functionalized tip) from its specific binding site (substrate) is dissimilar to the force required to eliminate a non-specifically bound ligand³⁵. In other words, the specific binding force must be adopted to recognize bound molecules and determine their distribution on the sample surface. Biorecognition system determine the specificity of interactions between two molecules, the difference of binding force can be used to identify and make a force mapping image using the distribution of those functionalized tip and sample surface. The binding affinity of two molecular interactions can be identified by conducting the force–distance curve, force spectroscopy. In a general receptor-ligand interaction measurement, the receptor is attached to a sample surface and the ligand attached to the tip surface. When the ligand attached AFM tip contacts with the substrate containing receptor, the binding is occurred between them. The tip is then retracted from the surface, fitting with receptor, pulling the ligand out of its binding site. The deflection signal of the cantilever which can calculate to force by the receptor-ligand specific interaction is recorded. Typically, the maximum adhesive force from force-distance curve is known as the receptor-ligand unbinding force. In this chapter, various cases of single molecule adhesion force measurement using force spectroscopy is introduced such as interaction between receptor modified surface and chemically functionalized AFM tip.

Figure 2.10. Examples of single molecule force spectroscopy applications for biorecognition process.

(a) Peptide-Inorganic materials interactions, (b) Receptor-Ligand interactions, (c) peptide-peptide interactions.

Common force-distance curves measured for each type of molecular partners are described in **Figure 2.10**, to understand approach of the characteristic structures in each case and to allow comparisons. **Figure 2.11(a)** is show the no specific binding force which does not have any detectable binding event and no interaction. **Figure 2.11(b)** indicates nonspecific binding force between the tip and the substrate, likely without the connection of the molecules³⁶. **Figure 2.11(c)** explains that force-distance curve reflecting specific binding interactions between receptor modified surface and chemically functionalized AFM tip with flexible spacers³⁷⁻³⁹. In these curves, the rupture length which calculated from the nonlinear part of the retraction curve is expected to almost similar for that of the spacer under stretching. Particularly, in the presence of flexible spacer, specific interaction take place far away from the substrate, while the non-specific interaction still remain near the tip-sample surface^{19,40,41}. **Figure 2.11(d)** show the example of stretching of multiple binding event such as multivalent specific interactions, stretching of the molecules and a few case of nonspecific interactions. In this multi domain interaction case, if the last curve is starting and ending at zero deflection, it is commonly considered the specific interaction³⁸.

Figure 2.11. Examples of force-distance curve for a biomolecular complex.

(a) no binding event, (b) nonspecific binding event since the linear slope of the retract curve, (c) specific binding events with stretching (d) multiple binding events.

2.4.1. Binding of Peptide-Inorganic materials

The force spectroscopy is a useful technique to evaluate the specific interaction between biomolecules and inorganic materials. A variety of proteins or peptides have their own specific and selective binding affinity to target materials. Using force spectroscopy measurement, the adhesion forces associated with the connection of single biomolecules to various materials can be measured quantitatively.

Table 2.3 Peptide-inorganic materials binding force measurement

Biorecognition system	Functionalized tip	Modified Substrate	Mean value of binding force (pN)
Titanium binding peptide / gold (Au) and titanium oxide (TiO ₂) ⁴²	Titanium binding peptide	Gold and titanium oxide surface	Au : No specific interaction, TiO ₂ : > 100 pN
D-Ala-D-Ala peptide / stainless steel ⁴³	D-Ala-D-Ala peptide	Stainless steel	50 ~ 300
L-cystine crystals ⁴⁴	COOH, NH ₃ ⁺ and OH	L-cystine crystals	COOH : 3,140 NH ₃ ⁺ : 2,590 SH : 1,700
Amino acids residues with / silicon material ⁴⁵	Amino acids residues	silicon material	69 ~ 219
Gold binding peptide / hydrophilic oxide materials ⁴⁶	Gold binding peptide	Al ₂ O ₃ , TiO ₂ , SiO ₂ and gold substrate	Al ₂ O ₃ : < 100, TiO ₂ : 100 ~ 150, SiO ₂ : 250 ~ 550, Au : 200 ~ 600

Arai et al.⁴² proposed a noble method for studying the chemical composition analysis and force mapping images with solid inorganic materials and titanium binding peptide using force spectroscopy measurement. The bicompositional substrate surfaces of gold (Au) and titanium oxide (TiO₂) were recognized with titanium binding peptide functionalized AFM tips. The chemically modified surface obviously appeared as contrast in the binding force mapping images with nanoscale resolution. They employed the chemical imaging with three types of the peptide functionalized tip to optimize the experimental conditions. The type 1 tip was directly modified the titanium binding peptide without spacer. For the type 2 tip, the long and flexible spacer ((N-hydroxysuccinimide-PEG24 maleimide ester, PEG) was introduced between the tip and titanium binding peptide. Lastly, for the type 3 tip, the density of titanium binding peptide on the tip surface was reduced by mixing a methoxy-terminated PEG spacer. The final density of the peptide of

type 3 tip was estimated to be approximately 10% of the type 2 tip. In the case of the type 1 tip, it was difficult to recognize the Au and TiO₂ region from adhesion force mapping image. However, the type 2 and 3 tip case which modified spacer moieties between the tip and peptide, the measured binding forces and binding probabilities were increased on Au and TiO₂ surface (The averaged binding force > 100 piconewtons). The average value of rupture length which observed with the type 1 tip was approximately 2 nm, whereas that measured with the type 2 tip was 9 nm. This 9 nm of rupture length is quite similar for the theoretical length of the PEG spacer (9.4 nm) in the all-trans configuration. The results showed that the PEG spacer gives rise to a high degree of freedom in the structure of titanium binding peptide, resulting in a clear contrast for the binding affinity between peptide and Au / TiO₂ surface.

Landoulsi and Dupres⁴³ investigated the binding affinity between D-Ala-D-Ala peptide and a stainless steel substrate at single molecule level using force spectroscopy. The AFM tips were functionalized with D-Ala-D-Ala peptide and stainless steel substrate was modified iron oxide species to remain the stability. Depends on the logarithm of the loading rate, the binding force between peptide and inorganic surface increases linearly, as typically found for receptor–ligand interaction. From the all force-distance curves, the mean value of binding force was varying from 50 to 300 piconewtons, depending on the loading rate. All experiments were performed in NaHCO₃-enriched medium condition, this aqueous medium allows blocking of the electrostatic interactions. The results indicated that binding mechanism of D-Ala-D-Ala peptide and a stainless steel substrate could not be attributed to covalent bonds but rather due to a combination of hydrogen bonds and Van der Waals force.

Razvag et al.⁴⁵ used the single molecule force spectroscopy technique to probe the interaction of individual amino acids residues with inorganic materials. In this measurement, the individual negatively or positively charged, polar amino acid (glutamine, lysine, leucine, glutamate, or phenylalanine), aromatic and nonpolar was attached to AFM tips interacted with silicon substrate. They also studied the adsorption of the amino acid residues to the silicon substrate depends on buffer conditions such as pH and ionic strength.

The most probable force value from the statistical analysis of force-distance curves of leucine, lysine and phenylalanine were 68, 69 and 219 piconewtons, respectively. These results were mainly influenced by the role of hydrophobic interactions, so phenylalanine which has hydrophobic characteristic showed the strongest binding affinity to the silicon surface. To characterize the effect of pH and ionic strength on buffer solution, the electrostatic interaction, the force spectroscopy measurements were performed between lysine and silicon surface at different pH values. The mean value of binding force of pH 3.3, 7.0 and 11.6 were 72, 62 and 23 piconewtons, respectively. The results demonstrated that the hydrophobic forces and electrostatic interactions were crucial for the binding strength of amino acid residues and silicon surface.

Figure 2.12. Force mapping of peptide–material interactions.

(a, b) Au/Al₂O₃, (c, d) Au/TiO₂, and (e, f) Au/SiO₂. All images are 90 × 90 μm² in size and 32 x 32 pixels (Adopted and reprinted from Mochiduki et al., 2015⁴⁶).

Mochizuki et al.⁴⁶ quantitatively evaluated the interaction between gold binding peptide and inorganic materials by adhesion force mapping images. To estimate the binding affinity, the gold binding peptides were functionalized to gold coated AFM tip and Al₂O₃,

TiO₂, and SiO₂ with gold substrate were prepared by chemical vapor deposition method. The gold binding peptide has a +2 net charge under neutral buffer conditions and the isoelectric points of Al₂O₃, TiO₂, and SiO₂ are 9.1, 6.7 and 1.8, respectively. Considering the electrostatic interaction, the gold binding peptide is most attractive for SiO₂, negatively charged material, followed by TiO₂ and then Al₂O₃. As shown in **Figure 2.12**, all the height images presented a similar contrast (70 μ m), the adhesion force images exhibited obviously different contrasts depending on the binding affinity between peptide and inorganic materials.

2.4.2. Receptor-Ligand interactions

Since AFM, force spectroscopy was invented, a lot of receptor-ligand interaction studies have been measured at the single molecule level using functionalized tip. One of the most famous biorecognition system is the interaction between biotin-streptavidin or avidin (**Table 2.4**). These molecular partner has been studied as a model system due to their high binding affinity and thermodynamic data.

Table 2.4. Receptor-ligand binding force measurement

Biorecognition system	Functionalized tip	Modified Substrate	Mean value of binding force (pN)
Avidin / biotin ⁴⁷	Avidin	Biotin	160 $\pm$ 20
Streptavidin / biotin ⁴⁸	Biotin	Streptavidin	257 $\pm$ 25
Biotin / streptavidin ⁴⁹	Biotinylated BSA	Streptavidin	340 $\pm$ 120
Biotin / streptavidin ²⁹	Biotin	Streptavidin	200
Avidin / biotin ⁵⁰	Biotin	Avidin	173 $\pm$ 19
Streptavidin / biotin ⁵¹	Biotin	Streptavidin	454

Florin et al.⁴⁷ applied force spectroscopy to determine the interaction of avidin and biotin. The AFM tips were adsorbed the biotinylated bovine serum albumin (BSA), then incubated with avidin. The agarose beads functionalized with biotin, desthiobiotin or

iminobiotin were used for substrate. To allow a limited number of molecular partners to bind, the functionalized agarose beads were blocked by avidin first, and then force spectroscopy measurement was performed. The mean value of binding force is 160 ± 20 piconewtons for biotin, 125 ± 20 piconewtons for desthiobiotin and 85 ± 15 piconewtons for iminobiotin. Among those biomolecules, the biotin has the highest binding affinity. However, the desthiobiotin or iminobiotin cases, they performed the force spectroscopy measurement with approximately 200 data points, it is not enough to allow us to conclude that this is clear comparison. Also, to determine the quantitative evaluation of the binding forces, a detailed analysis of the time and force dependence of the unbinding process are required.

Moy et al.⁴⁸ applied an AFM to directly evaluate the specific binding forces between avidin-biotin complex and actin monomers in actin filaments. The avidin or streptavidin was attached to the tip with biotinylated BSA and biotin or derivatives are immobilized on substrate surface. The adhesion forces between receptors and their respective ligands are as follow; avidin-biotin is 160 ± 20 piconewtons, avidin-iminobiotin is 85 ± 10 piconewtons, streptavidin-biotin is 257 ± 25 piconewtons, avidin-desthiobiotin is 94 ± 10 piconewtons and streptavidin and iminobiotin is 135 ± 10 piconewtons. Based on the calculated binding force and energies, they have calculated 9.5 ± 1 angstroms of an effective rupture length for all biotin-avidin partners and around 1 ~ 3 angstroms for actin monomer interaction. They proposed a model for the correlation between binding forces and intermolecular potential.

Lee et al.⁴⁹ used an AFM to examine the interaction between streptavidin and biotin using Biotinylated BSA adsorbed on glass microspheres (stick to cantilevers) and streptavidin functionalized mica surface under physiological conditions. Also they directly compared the binding forces biotin-streptavidin system with blocked streptavidin-biotinylated surfaces. The average binding force between biotin and streptavidin was 340 ± 120 piconewtons, it is 3 ~ 8 times bigger than the nonspecific interaction (blocked streptavidin-biotinylated surfaces, 60 ± 20 piconewtons). The results indicated that the measured binding forces represent specific binding between the biotin and streptavidin.

Wong et al.²⁹ introduced new strategy for attachment of biomolecules to AFM tip using carbon nanotubes. The biotin group was covalently bound to carbon nanotube AFM tips and the streptavidin was linked to substrate surface using biotin group. They have used the carboxyl groups modified nanotubes as AFM tips to image functionalized samples on the basis of molecular interactions, and to measure the binding affinity between single receptor-ligand partners. In this method, the small tip curvature radius extremely enhances the lateral resolution compare with commercial silicon tips. The measured binding force between biotin-streptavidin was approximately 200 piconewtons, this value is quite similar for other researcher's results. However, due to the small effective radius of carbon nanotube tip, it enables to measure the force mapping of functional groups with true molecular resolution. The results indicated that nanotubes can be modified to AFM tip which is able to detect the biorecognition event at the molecular level.

2.4.3. Protein-Protein Interactions

In **table 2.5**, the intermolecular force spectroscopy measurement with high specific protein-protein interactions are presented with description of the functionalized AFM tips and the modified substrates. Depends on the experimental setups, the biorecognition system results were significantly diverse with respect to their organization and physiological condition. Various protein groups with mechanical functions contain multiple or individually folded domains with similar structures. Protein-protein interactions are accountable for cellular signaling pathway and establishing the structural stability of cells and tissues^{52,53}. Many researchers have been studied proteins interaction and engineering using AFM and it enabled the quantitative analysis of the unfolding and refolding of individual protein domains and their mechanical stability⁵⁴ (**Table 2.5**).

Table 2.5. Protein-protein binding force measurement

Biorecognition system	Functionalized tip	Modified Substrate	binding force (pN)
Cell adhesion proteoglycans ⁵⁵	Proteoglycans	Proteoglycans	400
P-selectin / P-selectin glycoprotein ligand-1 (PSGL-1) ⁵⁶	PSGL-1	P-selectin	165
Ab single-chain Fv (scFv) Fragment / fluorescein ⁵⁷	Fluorescein	Ab single-chain Fv fragment (scFv)	50 ± 4
Citrate synthase / <i>E. coli</i> chaperonin GroEL ⁵⁸	Citrate synthase	GroEL	420 ± 100
Insulin / insulin ⁵⁹	Insulin	Insulin	1300
Myelin basic protein / Lipid bilayers ⁶⁰	Myelin	Lipid bilayers	140 ± 60
Vascular endothelial (VE)–cadherins–Fc ³⁸	VE–cadherins–Fc	VE–cadherins–Fc	15 ~ 150
Nitrilotriacetate (NAT)/ histidine 6 (His ₆) ²⁶	His ₆	NAT	150 ~ 194
Phospholipid bilayers / recoverin ⁶¹	Recoverin	Phospholipid bilayer	48 ± 5
P-selectin / P-selectin glycoprotein ligand-1 (PSGL-1) ⁶²	PSGL-1	P-selectin	20
Cell adhesion proteoglycan ⁶³	Proteoglycans	Proteoglycans	-
Epimerase AlgE4 / mannuronan ⁶⁴	Mannuronan	Epimerase AlgE4	74 ~ 144
Pesticide / antibody ⁶⁵	BSA- mercaptopropanoic acid derivatives of atrazine or BSA- 2,4-dichlorophenoxyacetic acid	Protein A	511 ± 244 / 228.6 ± 163.1
Glucagon / anti-glucagon ⁶⁶	Glucagon	Anti-glucagon	256.4 ± 48.9
Myoglobin / aptamer or antibody ⁶⁷	Myoglobin aptamer or antibody	Myoglobin	Table 6 and 7

Dammer et al.⁵⁵ used force spectroscopy measurement to employ the investigation of binding strength between cell adhesion proteoglycans from a marine sponge under physiological conditions. The cell adhesion proteoglycans were covalently attached to organic monolayers on AFM tips and substrate surface. The average value of the adhesion force between two proteoglycans molecules was approximately 400 piconewtons. They also modified the substrate surface with Ca^{2+} and Mg^{2+} in various concentration to make different ionic conditions. At a 2 mM Ca^{2+} concentration, the average binding force was 40 ± 15 piconewtons but, at a 10 mM Ca^{2+} concentration, the average binding force was 125 piconewtons, ranging up to 400 piconewtons. They functionalized the surface with 2 mM Ca^{2+} and 10 mM Mg^{2+} concentration, the result was similar for 2 mM Ca^{2+} concentration case (40 ± 15 piconewtons). This result indicated that the binding affinity between cell adhesion proteoglycans is Ca^{2+} - selective and Mg^{2+} could not replace Ca^{2+} in this adhesion molecules interaction.

Yip et al.⁵⁹ introduced the forces measurement system with protein-protein and protein-receptor interactions which can explain the advancement of therapeutic proteins. They measured the binding forces between insulin-insulin receptor using covalently insulin attached AFM tips and insulin-modified mica surface. The both AFM tips and mica surface are functionalized by photochemical attachment of specific residues to these surfaces. The measured binding force was 1.34 nanonewtons with 100 nm/s of retraction rate calculated by analysis of numerous force-distance curves. The characteristics of interaction diverge noticeably for monomers and that dimerization binding forces which are influenced by binding partners such as free insulin and anti-insulin antibodies. They also measured the binding affinities at different buffer conditions (pH 3.5 and pH 7.5) with 3-APTES-functionalized AFM tips and similarly modified mica substrates, insulin-attached tip and a bare mica substrates. In PBS solution with pH 7.5 condition, the adhesion force was less than 0.1 nanonewtons but, in glycine buffer with pH 3.5 condition, the adhesion force was about 4.3 nanonewtons. It is mainly due to the strong electrostatic interactions between the positively charged insulin molecule and the negatively charged bare mica surface at pH 3.5 condition (below the isoelectric point of insulin ($pI = 6.4$)). Their results suggested an

experimental protocol for estimating the effects of antagonists on insulin-insulin dimer formation and investigating insulin-receptor interactions.

Fritz et al.⁵⁶ applied force spectroscopy to study the dynamic adhesion process between single complexes of P-selectin and P-selectin glycoprotein ligand-1. They investigated the adhesion forces, elasticity, and kinetics of the P-selectin and P-selectin glycoprotein ligand-1 interaction from force spectroscopy data. For functionalization strategy, the biotinylated P-selectin was attached to avidin-coated glass coverslips and biotinylated PSGL-1 was attached to avidin-coated AFM tips. The measured adhesion force was 165 piconewtons with chain-like elasticity with a molecular spring constant of 5.3 pN/nm. In this study, they applied a variety of loading rate (dissociation constant) from 0.02 sec⁻¹ under zero force up to 15 sec⁻¹ under external applied forces. The small logarithmic dependence of the binding interactions on pulling velocity Monte-Carlo simulation can explain the weak dependence of leukocyte rolling velocities on flow rates. The high molecular elasticity with the long length of the complex decreases the mechanical loading on the protein-protein binding. They explained that P-selectin and P-selectin glycoprotein ligand-1 interactions are a good evidence how the mechanical properties of single molecules control the characteristics of cell-cell adhesion. The biorecognition processes do not depend not only on buffer condition, but are also robustly influenced by applied loading forces and mechanical stability at the molecular level.

Mueller et al.⁶⁰ examined force spectroscopy to identify the interaction of mica and mixed dioleoylphosphatidylserine (20%) / egg phosphatidylcholine (80%) lipid bilayer surfaces with and without bovine myelin basic protein. Lipid vesicles containing dioleoylphosphatidylserine (20%) / egg phosphatidylcholine (80%) were adsorbed to substrate surface and further incubated with myelin basic protein and tips were coated with myelin basic protein. They mainly investigated the rupture distances of the last detachment peak from contact for myelin basic protein on bare mica and lipid bilayer modified mica. On bare mica surface or lipid bilayers had a short-range repulsive force (decay length 1.0 –1.3 nm) during the tip approaching and with the myelin basic protein, it always had an attractive force between tip and sample surface. During contact with the mica surface, the

myelin basic protein molecules showed elastic stretching behavior with rupture length of 0.5 ± 0.25 nm and 53 ± 19 nm of average contour length (**Figure 2.13**). However, when the myelin basic protein molecules contact with lipid bilayer containing dioleoylphosphatidylserine-egg phosphatidylcholine did not exhibited elastic stretching behavior. In the absence of lipid molecules, the myelin basic protein reacts like a common polymer chain in buffer condition. But, when the protein contact with lipid molecules, it seems more compact structure of the myelin basic protein. The results indicated that the protein has a different behaviors and conformational changes when in contact with lipid bilayer. It means that the lipid bilayer formation was firmly modified by the myelin basic protein attachment.

Figure 2.13. Statistical analysis of the distances of the last detachment peak for the myelin basic protein.

(a) Bare mica, (b) lipid bilayer. (Adopted and reprinted from Mueller et al., 1999⁶⁰)

Kaur et al.⁶⁵ introduced the single molecule force spectroscopy using functionalized tip to evaluate the binding affinity between pesticides and antibodies on a biosensor surface. The herbicide molecule mercaptopropionic acid derivatives of atrazine and 2,4-dichlorophenoxyacetic acid functionalized on a gold substrate, it was used to simulate an immuno-biosensor surface. The binding affinity between the functionalized antibodies and their partners was calculated by force-distance curve using a hapten-carrier protein immobilized AFM tip. They also measured the force measurement for BSA-mercaptopropionic acid derivatives of atrazine AND BSA- 2,4-dichlorophenoxyacetic acid attached AFM tips with their respective pairs only protein A-coated surfaces. The binding forces between the anti-atrazine antibody and BSA-atrazine-coated AFM tip exhibited a range distribution value of the adhesion force, varying from 200 to 940 piconewtons, the average value was 511 ± 244 piconewtons. This measured forces were smaller than the BSA-atrazine-coated cantilever was adopted to contact with only the protein A-coated surface (the average value was 31.5 ± 19.8 piconewtons). When they investigated the interaction between the BSA- 2,4-dichlorophenoxyacetic acid modified surface and AFM tips, the average value was calculated to be 228.6 ± 163.1 piconewtons, it was higher when BSA- 2,4-dichlorophenoxyacetic acid coated AFM tip interacted with protein A functionalized surface.

Lin et al.⁶⁶ determined the dynamic interaction of glucagon / anti-glucagon pairs with multiple binding events depends on z-direction pulling velocity and pH conditions. The adhesion forces were measured from force-distance curves between specific glucagon / anti-glucagon interaction system with multi-rupture events, which indicated single or multiple pull-off steps between glucagon and anti-glucagon. They measured the force spectroscopy with various pulling speed, varying from 16.7 to 166.7 nm / sec, to study the dynamic response of glucagon / anti-glucagon pairs. The results showed that the average value of the binding force was higher when the pulling speed was increased. Furthermore, they tested the binding affinity of glucagon molecules with different pH buffer conditions (varying from pH 4 to 10). The maximum binding force of glucagon / anti-glucagon pairs was 256.4 ± 48.9 piconewtons in acid buffer condition however, when the buffer condition was changed to basic or neutral, the interaction was decreased sharply. These results

explained that contact time or pulling speed and pH buffer condition affect the dynamic interaction of antigen–antibody.

The most recent research of protein-protein interaction using force spectroscopy is performed by Wang group⁶⁷. Wang et al. evaluated the medicine effects on the interaction of myoglobin and its aptamer or antibody. To study the effect of medicine molecules, the gold substrate was coated by myoglobin and it was incubated with promazine, amoxicillin, aspirin, and sodium penicillin, respectively. The mean value of binding force and the binding probability between myoglobin/aptamer were almost similar for each condition but, the parameters were changed for the interaction of myoglobin/antibody. The results showed that the binding force was changed and binding probability was increased for promazine and amoxicillin treatment. For aspirin treatment, only the binding force distribution was changed and for sodium penicillin, both the mean value of binding force and the binding probability were increased (**Table 2.6 and 2.7**). These results explained that the medicine treatments affected the binding sites between myoglobin-aptamer and myoglobin-antibody and conformational changes of myoglobin.

Table 2.6. The average binding force and binding probability of the myoglobin-Aptamer under the different medicine treatments

Medicine	Time interval	Binding probability (%)	Average binding force (pN)
Without medicine	-	23.05 ± 2.07	93.52 ± 4.07
Promazine	4 hours	23.24 ± 2.20	112.89 ± 7.16
Amoxicillin	4 hours	22.36 ± 2.35	115.91 ± 7.46
Aspirin	4 hours	22.89 ± 3.67	96.16 ± 8.75
Sodium penicillin	4 hours	22.94 ± 2.78	102.26 ± 5.74

Table 2.7. The average binding force and binding probability of the myoglobin- polyclonal Antibody under the different medicine treatments

Medicine	Time interval	Binding probability (%)	Average binding force (pN)
Without medicine	-	24.14 ± 1.28	118.59 ± 4.83
Promazine	4 hours	23.67 ± 2.80	165.72 ± 5.43
Amoxicillin	4 hours	22.83 ± 3.23	210.73 ± 3.68
Aspirin	4 hours	22.01 ± 3.49	181.32 ± 4.86
Sodium penicillin	4 hours	22.13 ± 1.83	121.01 ± 6.85

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Chapter 3

Experimental Methodology

In this chapter, we introduce the materials and their preparations that are utilized experimentally including the working principles, instrumentation, and experimental protocols. To produce the investigation of biorecognition processes, the engineered peptide and antiviral peptide are adopted and the binding affinity is studied with inorganic materials (SiO_2 , TiO_2 , Al_2O_3 and Au) and cellular membrane components. The force spectroscopy measurement with chemically functionalized AFM tip is used to monitor the interaction of biomolecular pair. Specially, this chapter mainly explains the principal of force spectroscopy, experimental protocol, data analysis and the strategy of molecules attachment to AFM tip.

3.1. Rationale for the selection of materials and experimental techniques

In this Experimental Methodology chapter, it consists of three main sections; (i) materials and their preparation (ii) experimental techniques and their protocols (iii) data interpretation. First, the materials used throughout this thesis are described and listed in detail, particularly peptide reagents (amino acid residue) and their synthesis, buffer solution, chemical reagents and lipid blot membrane. The peptides were synthesized by manufacturers using their own sequence. A point to be consider is the peptide have to be added Cysteine residue to the N-terminus or C-terminus to tip functionalization. The lipid blot membrane was utilized for phosphatidylinositol phosphate monolayer substrate to measure the interaction between peptide and lipid.

Secondly, the brief introduction of experimental techniques and their protocols which we used are presented with detail description. Atomic force microscopy with force spectroscopy mode was utilized to determine the binding affinity of biomolecules and materials with the functionalized tip. A quartz crystal microbalance with dissipation measurement was also adopted to measure the interaction of peptides and inorganic solid substrates. A combination of these two experimental approaches allowed us to compare the adsorption kinetics and interactions of biomaterials at nanoscale level.

Lastly, the final section in this chapter introduced the force-distance curve data analysis and interpretation. As a manner of force spectroscopy measurement, huge variability can be observed in force-distance curve data. To extract proper and reliable result, quantitative information, a statistic analysis of many number of force-distance curves have to be performed. This data analysis section explained how to recognize the nonspecific or specific interaction and proposed the optimum parameters of histogram and their fitting curve.

3.2. Materials

3.2.1. Peptide reagents

For chapter 4, High purity peptides (495%) were synthesized by Anaspec Corporation (San Jose, CA, USA). The sequence of the template peptide is H-Ala-Gly-Ser-Trp-Leu-Arg-Asp-Ile-Trp-Thr-Trp-Leu-Gln-Ser-Ala-Leu-NH₂. For AFM experiments, tip functionalization an additional Cys residue was added to the N-terminus or C-terminus. The as-supplied lyophilized peptides were solubilized in deionized water at a stock concentration of 2 mg mL⁻¹ peptide. The exact molar concentration of peptide in solution was determined by standard absorbance measurements at 280 nm and diluted accordingly before experiments.

For chapter 5, High purity AH peptide (>95%) was synthesized by Anaspec Corporation (San Jose, CA, USA). The sequence of the AH peptide is H-Ser-Gly-Ser-Trp-Leu-Arg-Asp-Val-Trp-Asp-Trp-Ile-Cys-Thr-Val-Leu-Thr-Asp-Phe-Lys-Thr-Trp-Leu-Gln-Ser-Lys-Leu-AspTyr-Lys-Asp-NH₂. The cysteine residue is highlighted because it is important for chemical attachment to the AFM tip.

For chapter 6, a molecular model of the synthetic peptide was first constructed using the DeepView/Swiss-PdbViewer software program with input from the nuclear magnetic resonance structure of the membrane anchor domain of the HCV NS5A protein 1R7C. Based on this structure, individual mutations of specific amino acid residues were made in order to create the wild-type AH peptide structure corresponding to the 1b genotype.

3.2.2. Chemical reagents

Ethanol (MerckMillipore, Billerica, MA) and dehydrated toluene (Fisher Scientific, Waltham, MA) were used to solution for AFM tip functionalization. To attached peptide to tip, 11-amino-1-undecanethiol hydrochloride (Sigma-Aldrich, St Louis, Mo), 2-aminoethanethiol (1 mM, Tokyo Chemical Industry, Tokyo, Japan) were used to make thiol bonding to gold coated AFM tip and N-hydroxysuccinimide–PEG24-maleimide ester (NHS–PEG–MAL) (Product No.10314, Quanta Biodesign, Plain City, OH) was used to long and flexible PEG chain spacer. For the aqueous buffer solution, Phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, CA) with 137 mM NaCl, 2.7 mM KCl salt concentrations and pH 7.2 was used as the running buffer. All aqueous solutions were prepared using Milli-Q-treated water (18.7 MO cm), (Millipore, Billerica, MA). For chapter 4, polyoxyethylene sorbitan monolaurate (Tween-20, Bio-RAD, Hercules, CA) was used to minimize the nonspecific interactions between the probe and the substrate.

3.2.3. Lipid blot microarray

For the purpose of studying the relative binding affinity of a peptide or protein for various types of phospholipids, the commercial nitrocellulose strips (2 x 6 cm) with hydrophobic membrane containing spots of equal amounts (100 picomoles) of 15 different phospholipids were purchased, and preparation was performed as recommended by the company (P-6001, Echelon Biosciences Inc., UT, USA), (**Figure 3.1**). These lipid blot membranes can be used to evaluate the specific interaction of lipid-protein or lipid-peptide, through a force spectroscopy with the functionalized tip. It is a convenient way to estimate if their peptide of interest interacts with one or more. The membrane has an oblique line cutting on the top left corner and Xylene Cyanol FF (blue color dye) is spotted in the bottom right blank corner to recognize the arrangement of the lipid blot membrane. Ponceau S staining (pink) was added to the each lipid spots to determine the location of lipids. All of the lipids have long chain (> diC16) with highly pure synthetic analogs.

Figure 3.1. Schematic representation of lipid blot membrane template.

3.3. Atomic force microscopy – Force spectroscopy

As we mentioned in chapter 2, the mechanical properties of the sample can be measured using force spectroscopy mode. The result from single force spectroscopy measurement is displayed as a simple x - y plot, so called force – distance curve (**Figure 3.2**). The z – scanner position or height position for cantilever is chosen as the x – axis, and the measured cantilever property, typically the vertical deflection of the cantilever, is set as the y – axis. The deflection can be measured by a laser beam deflection setup that carries an electrical signal which is proportional to the cantilever deflection. Using force – distance curve, we can collect the mechanical properties of the sample, such as elasticity which is calculated from the approach curve and the adhesion force which is obtained from retract curve. To explore the binding affinity between two materials, the adhesion force from retract curves are employed. When two materials have specific binding, the AFM tip is deflected down towards the substrate (sample) until the force is strong enough to rupture the interaction. This phenomenon can show a various adhesion of rupture events at each different case. The force spectroscopy for investing the binding affinity is measured at different positions repeatedly to make a force mapping image, or measured many times at the same position to provide statistical analysis of the binding force.

Generally, the representative force-distance curve of the specific interaction has a rupture length of around 13 nm, which is consistent with the PEG₂₄ spacer's length of ~9.4 nm along with the spatial proximity of the cysteine residue. The force required to break the specific bonding is around over the 120 pN, which is within the range of other protein-carbohydrate bonds. By contrast, when the tip is not functionalized with peptide or protein, there was no specific interaction between the two molecules. Force-distance curves with a maximum adhesion force between 0 – 1,200 pN and rupture length between 1 – 80 nm are selected for data analysis. The most probable adhesion forces and rupture lengths are determined by fitting a Gaussian curve to histogram plots of all rupture events within these criteria, and the statistical error is estimated by $\frac{2.35\sigma}{\sqrt{N}}$, where σ is the standard deviation and N is the number of rupture events in the set.

Figure 3.2. Example of force – distance curve from force spectroscopy result

Device setup – SPM system : The SPM system consists of five primary components: the AFM stage mounted on an inverted optical microscope, confocal and fluorescence microscopy, upright microscopy and acoustic enclosure (**Figure 3.3**).

Device setup – AFM, Force spectroscopy The Z scanner mounted on the AFM head allows the tip to maintain constant feedback conditions (force or distance) when moving over the sample surface. The Z scanner range is up to 25 μm and its resolution is 1nm. Alternatively, the minimum accessible Z-direction pixel resolution is determined by the voltage applied to the control unit and the Z scanner. Also, the noise level in Z axis can be acquired using zero-scan condition. It can be considered the vertical resolution for actual measurement. The X-Y scanner is a Flexure Hinge (leaf-spring type) guided system which fabricated with a solid aluminum block. It has a 100 x 100 μm scan range and Resolution of X-Y direction is 1.5 nm with closed loop 0.3 nm at open loop.

Figure 3.3. SPM experimental setup overview.

SPM System consists of six primary components: SICM, AFM, inverted optical microscopy, confocal microscopy, upright microscopy and acoustic enclosure.

Optical microscopy : We combine the SPM system with commercial inverted optical microscopy (IOM). It enables in-depth study of samples combining optical data and nanoscale image. This transmitted bright-field illumination mode is one of the most

commonly utilized to observe the sample in optical microscopy field. The sample which imaged with transmitted bright-field illumination mode is termed amplitude objects due to reducing of the amplitude or intensity of the illuminating wavefronts. Many sorts of samples, ranging from animal and plant tissues, animal and plant cells and living creatures can be in bright-field microscopy. Using the optical microscopy, the sample position can be fixed at one point, the AFM measurement also can measure the same region of bright-field image. In this system, the IOM consists of 4X, 10X, 20X, 40X, and 100X objective lens.

Confocal microscopy : Confocal fluorescence microscopy is a powerful technique for study biological sample especially cell. It is used to characterize specific biomaterials such as molecules that absorb light at specific energies for a certain time and simultaneously emit the light of a lower energy. It extends the value of traditional fluorescence-labeling techniques because its ability to remove background noise (out-of-focus) information from the image data certifies the acquisition of 3-dimensional intensity data sets that can be viewed as 3-dimensional images. We assembled confocal microscopy to left side of IOM equipped with a 60 $\times$ oil immersion objective (NA 1.49) and an Andor iXon + EMCCD camera (Andor Technology, Belfast, Northern Ireland). The acquired confocal fluorescence images can be acquired with 512 $\times$ 512 of pixels and 0.267 $\times$ 0.267 μm of size. It enables AFM imaging and confocal imaging at the same region simultaneously.

Upright microscopy : The digital charge-coupled device (CCD) camera (uEye – SE, Imaging Development System, Obersulm, Germany) is used to focus the beam onto the cantilever and to locate the cantilever to the interesting region on the sample surface that is to be measured. Effective high resolution digital camera is 1024 x 768 pixel 20 frame/sec. Digital magnification is up to 100 times.

Acoustic enclosure : To explore the nanoscale dimension, isolation from the external acoustic, vibration and light noise is essential. Typically, AFM technique measures sample properties at the nanometer scale, so all small factors can affect the result

accuracy. To facilitate such environment, we installed acoustic enclosure (Enhanced Acoustic Enclosures, Park Systems Corp, Suwon, Korea) with generate an environmentally sealed space for the SPM system. The dimension of the structure is $1,000 \times 1,000 \times 1,400$ mm (outer) and 646 kg of weight.

3.4. Tip functionalization

3.4.1. Cantilever selection

For force spectroscopy with molecular functionalized tip, two important properties of AFM cantilever are considered, one is the spring constant of the cantilever (k , N/m), the other is its resonant frequency (f , Hz). To investigate small forces on the soft biological material, such as protein interaction, very soft cantilever with low spring constant ($k < 0.1$ N/m) is required. Also, to minimize the thermal noise of cantilever due to Brownian motion, short shape (cantilever length < 100 μm) of the cantilever with low resonant frequency (bandwidth 0 – 60 kHz) is the most suitable¹.

In this study, we used soft commercial AFM cantilever with 0.03 N/m of spring constant and 10 kHz of resonant frequency (CGS-01, NT-MDT, Moscow, Russia). The maximum peak-to-peak noise level is 10 pN, therefore forces as small as 10 pN, which is at the smallest binding force for bio-molecular environment (20 – 240 pN) can be measured² (**Figure 3.4**).

Cantilever Length (μm)	Cantilever Width (μm)	Resonant Frequency (kHz)			Spring Constant (N/m)		
		Min	Typical	Max	Min	Typical	Max
350	30	4	9.8	17	0.003	0.03	0.13

Figure 3.4. Specification of AFM cantilever and schematic view.

3.4.2. Cantilever calibration

AFM cantilever calibration is quite an important issue for force spectroscopy measurement. Various different methods have been presented to calibrate the cantilever's spring constant, regarding speed and convenience, the thermal noise analysis is the most general method³. Measurement of the free fluctuations of the cantilever is the main factor of this method, so it is a passive analysis and it can be performed in air and liquid condition as well. Moreover, it is quite suitable for soft cantilever with low frequency because the free cantilever fluctuations are more critical⁴. The cantilever fluctuations come from the natural thermal environment of the cantilever, such as room temperature (**Figure 3.5**). The amplitude of this fluctuation is small, so the cantilever can be seen as a harmonic oscillator, and the equipartition theorem holds:

$$\frac{1}{2}k_c \langle q^2 \rangle = \frac{1}{2}k_B T, \quad k_c = \frac{k_B T}{\langle q^2 \rangle}, \quad [\text{Eq. 3.1}]$$

Where, k_c is the force constant of cantilever, $\langle q^2 \rangle$ is the root mean square (RMS) of amplitude, k_B is the Boltzmann constant, and T is temperature. In here, the determination of $\langle q^2 \rangle$ is required for spring constant calibration of cantilever. This method assumes that the cantilever is a simple harmonic oscillator. However, a correction factor should be required because cantilevers are not actual harmonic oscillators. If the length is longer than the width, and the cantilever is rectangular shape, the mean square amplitude at the end of the cantilever due to thermal energy excitation is as follows.

$$k_c = 0.817 \frac{k_B T}{\langle q^2 \rangle} \left[\frac{1 - \left(\frac{3D}{2L}\right) \tan \phi}{1 - \left(\frac{2D}{L}\right) \tan \phi} \cos \phi \right]^2, \text{ [Eq. 3.2.]}$$

Where, D is the height and L is length. These correction factors mainly contribute errors (uncertainty) the range of 10 – 20% when the spring constant calibration using the thermal noise analysis. Generally, it is acceptable range for variation in spring constant determination using the thermal noise method.

Figure 3.5. Power density measurement of the thermal noise of AFM cantilever.

3.4.3. Functionalization protocol

In this paper, commercial gold coated Si cantilevers (CSG-01, NT-MDT, Moscow, Russia) were used for all experiments. The reflective side of cantilever was coated with gold and the tips were coated with a 2 nm thick germanium adhesion layer first, followed by a 20 nm thick gold layer by thermal evaporation under vacuum with 1.0×10^{-4} *pa* of base pressure. The tip curvature radius is 6 nm and 35 nm, before and after gold coating, respectively. For the first step of the tip functionalization, the tips were cleaned by a UV – O3 cleaner (Novascan UV Ozone Cleaner, Novascan Technologies, Inc., USA) for 30 minutes. After the cleaning process, the tips were immersed in pure ethanol solution with 1 mM of 2-aminoethanethiol for 6 hours. After that, the tips were washed with pure ethanol and dried gently. The dried tips were immersed in anhydrous toluene solution with 1mM of N-hydroxysuccinimide–PEG24-maleimide ester for 6 hours. After immersion, the tips were washed with anhydrous toluene solution first, and rinsed with pure ethanol and dried gently. The tips were immersed in PBS solution with the desired peptide or protein for over the 6 hours or overnight. The peptide or protein functionalized tips were washed with clean PBS solution (**Figure 3.6**).

Figure 3.6. Detail procedure of the AFM tip functionalization.

3.5. Surface modification

3.5.1. Inorganic substrate

The gold material was patterned over Al_2O_3 , TiO_2 , and SiO_2 substrates to investigate distinct areas of surface chemistry using AFM peptide functionalized tip. First of all, 100 nm thickness layer of Al_2O_3 , or TiO_2 was formed on a clean Si(100) substrate by atomic layer deposition for the preparation of the substrates. For the SiO_2 substrate, thickness 100 nm layer of SiO_2 was deposited on a clean Si(100) substrate with tetraethyl orthosilicate (tetraethoxysilane) using the chemical vapor deposition method⁵. The photoresist masking in the oxide substrates was performed, and then they were coated with 20 nm thickness of chromium followed by coating with 100 nm thickness of gold layer using a thermal evaporation method. The photoresist masking has been removed from substrates and, the substrates were cleaned using sonication with toluene solution for 10 min.

3.6. Data analysis

In order to obtain a reliable result, hundreds number of force – distance curve should be taken for force spectroscopy measurement. In force spectroscopy for investigating binding affinity of two biomaterials, not all data show the binding force. No interaction or non-specific interaction between two biomaterials can be measured which are mixed with the specific binding force of interest. In order to recognize non-specific and specific interaction, statistical analysis such as histogram is required with each of binding data. Typically, the binding forces extracted from force-distance curves of specific interactions are represented as histogram; the binding forces being kept higher than 15 piconewtons based on machine noise level. The distribution of histogram is usually asymmetric shape with more or less spread and it contains single or multiple peaks. **(Figure 3.7)** To determine accurate statistic result, Scott's rule which relate with a theoretical study of synthetic data is used⁶.

$$h_N = 3.49\sigma N^{-\frac{1}{3}}, [\text{Eq. 3.3}]$$

Where, h_N is the optimized bin width, σ is the standard deviation of all data, N is the number of data points. This method can fit the distribution to a histogram of the forces measured with Gaussian or non-Gaussian distribution^{6,7}.

There are a few features in histogram graph such as single or multiple peak. In case of single peak, the most probable binding force can be evaluated at the highest peak point. In multiple binding events, the clearly distinct peaks can be observed in the histogram. Particularly, when the peaks in the histogram graph are equally arranged, the distance between two following peaks has been expected as the quantum for the binding force. However, multiple peak analysis in distribution requires a particular awareness due to different experimental configurations and conditions. In order to accurately analyze the statistical data, the effective number of interacting molecules must be investigated at each case. It means that when n number of receptor-ligand partners are pulled off at the same time, the effectively applied forces should be shared among them. In a manner of spatial organization of the biomolecules, to gather the reliable data set, the experiment have to be performed repeatedly with different parameters (Biomolecules density or the spacer length). Typically, the measured binding force can be estimated by the effective number, the strength, and the direction of the noncovalent bonds. Moreover, it is strongly influenced by experimental conditions, such as temperature, ionic strength and pH level. When the biorecognition system with similar biological functions are analyzed, many variances in the binding forces data have been observed, such as antigen/antibody pairs. However, a few cases of similar biomolecular complexes, such as biotin-avidin or biotin–streptavidin which are characterized by a strong binding affinity has similar interaction behaviors and binding force values. Only under the different experimental conditions, there are large variability in the measured binding force. These disparities have stimulated the binding process in terms of the biorecognition processes with their energy landscape. Force spectroscopy measurements performed at different experimental conditions, allow to reach a complete relaxation of the biomolecular complexes.

Figure 3.7. Example of binding force distributions of events with no interaction and specific interaction.

Blue graph: No interaction, red graph: Specific binding event between two molecules. The histograms were fitted to Gaussian distribution to recognize the most probable binding force values.

3.7. Quartz Crystal Microbalance with Dissipation Monitoring

The quartz crystal microbalance with dissipation monitoring (QCM-D) is originally proposed in 1959 by Sauerbray et al. who presented the relationship between the resonant frequency of the quartz crystal materials and the mass changing on the crystal surface⁸. It is a nanogram sensitive weighing balance that operates the piezoelectric characters of an AT cut-quartz crystal (cut with an angle of 35.25° to the z-axis). When the Quartz crystal, one of the most commonly used piezoelectric materials, exposed to an external electric field, it is mechanically deformed such as expansion or contraction. And, the quartz crystal recovers its original state or shape once the electric field is isolated. During these processes, due to the piezoelectric properties and cut orientation of the quartz crystal, an acoustic wave in the thickness shear mode of the crystal is produced by response of a quartz crystal material (**Figure 3.8**).

Figure 3.8. Schematic Representation of Piezoelectric Properties of Quartz Crystal.

(i) a quartz crystal, (ii) the induction of the piezoelectric effect by the application of an external force, (iii) reverse piezoelectric effect to induce oscillations for QCM measurements.

Following the QCM-D principle, the technique simultaneously measures changing of the resonance frequency (Δf) and the energy dissipation (ΔD) of the quartz crystal. The calculated values depend on both the quartz crystal itself and the molecules such as aqueous buffer, ions, lipid, peptide and protein which contact with the quartz crystal surface. When the target molecules adsorb to crystal surface, the resonance frequency changing is directly proportional to the mass adsorbed to the crystal surface. In a typical quartz crystal with two metal electrodes, it deforms its shape periodically and oscillates with the same frequency when an alternating potential is applied to the crystal surface. The resonance frequency of a quartz crystal material is generally depends on thickness of the crystals (5 MHz with $\sim 300 \mu\text{m}$ thickness), at this condition, the fluctuation signal shows a harmonic behavior with a maximized amplitude and minimal energy loss. The resonance frequency is changed or shifted when a uniform mass is adsorbed onto the crystal surface. This relationship between the resonance frequency shifting and the mass adsorption is proposed by Sauerbray as follows :

$$\Delta m = \frac{C}{n} \times \Delta f, [\text{Eq. 3.4}]$$

Where, Δm is the mass adsorption, Δf is resonance frequency shifting, C is the sensitivity constant and n is the harmonic number. Typically the sensitivity constant has

been determined as -17.7 ng/Hz.cm^2 for $300 \text{ }\mu\text{m}$ thickness of quartz crystals with 5 MHz , it can be expressed as⁹ :

$$C = \frac{t_q \times \rho_q}{f_o}, [\text{Eq. 3.5}]$$

Where t_q is the quartz crystal thickness, ρ_q is the density of quartz crystal, and f_o is the resonance frequency of quartz crystal. To validate the Sauerbray equation, the adsorbed mass have to be smaller than the quartz crystal mass. Also the adsorbed mass should be contact firmly and homogenously onto the quartz crystal surface.

As compared to normal QCM, QCM-D technique can detect the not only the shifting of resonance frequency but also the viscoelastic properties of the adsorbed mass. In case of QCM method, it is difficult to conduct the measurement in a liquid condition due to a variety of valuables from viscosity and elasticity of buffer solution. After QCM-D technique was developed, the researchers are able to perform the simultaneous measurements through monitoring the changes in resonance frequency and the energy dissipation between biomaterials such as between phosphoinositide receptors, supported lipid bilayer, synthetic peptides and proteins (**Figure 3.9**).

Rodahl et al. introduced the dissipation factor (D) as follows :

$$D = \frac{1}{\pi f \tau}, [\text{Eq. 3.6}]$$

Where, f is the frequency and τ is the decay time constant. If it is dimensionless, it is also described as follows¹⁰ :

$$D = \frac{1}{Q} = \frac{E_{dissipated}}{2\pi E_{stored}}, [\text{Eq. 3.7}]$$

Where Q is the quality factor, $E_{dissipated}$ is the energy loss per oscillation and E_{stored} is the total energy stored while the piezo is oscillating. Together with features of

the dissipation factor, the shifting of resonance frequency and the energy dissipation in liquid condition are redesigned as follows¹¹ :

$$\Delta f = -\frac{1}{2\pi t_q \rho_q} \sqrt{\pi f \rho_l \eta_l}, \quad \Delta D = -\frac{1}{t_q \rho_q} \sqrt{\frac{\rho_l \eta_l}{\pi f}}, \quad [\text{Eq. 3.8}]$$

Where ρ_l is the density of the buffer and η_l is the viscosity of the buffer.

In this thesis, QCM-D measurements were conducted using a Q-Sense E4 instrument (Q-sense AB, Gothenburg, Sweden) in order to investigate the binding affinity between peptides and the inorganic materials by tracking resonance frequency shift and the changes in energy dissipation of substrate surface as a function of time. During the QCM-D experiments, all measurements were conducted under continuous flow conditions, with the flow rate defined as 100 μ L/min and washing processes and 41.8 μ L/min as controlled by a Reglo Digital peristaltic pump (Ismatec, Glattbrugg, Switzerland). The temperature in the measurement cell was maintained at 25.0 ± 0.5 °C. The experimental data were collected at the 3rd ($n = 3$), 5th ($n = 5$), and 7th ($n = 7$) odd overtones using the QSoft (Q-Sense AB) software package, and the data were normalized according to the overtone number.

Figure 3.9. Measurement platform in order to measure the real-time binding kinetics.

The changes in resonance frequency and energy dissipation reflect the mass and viscoelastic properties of the bound peptide, respectively.

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Chapter 4

Correlating single-molecule and ensemble-average measurements of peptide adsorption onto different inorganic materials

The coating of solid-binding peptides (SBPs) on inorganic material surfaces holds significant potential for improved surface functionalization at nano-bio interfaces. In most related studies, the goal has been to engineer peptides with selective and high binding affinity to a target material. Herein, using a combination of experimental techniques, we investigated the adsorption of a 16 amino acid-long random coil peptide to various inorganic substrates – gold, silicon oxide, titanium oxide and aluminum oxide. Circular dichroism spectroscopy was employed to identify conformational changes associated with peptide attachment to inorganic nanoparticles. Quartz crystal microbalance-dissipation (QCM-D) experiments were performed in order to measure peptide binding affinity to inorganic solid supports, and atomic force microscopy (AFM) experiments were conducted in order to determine the adhesion force of a single peptide molecule. A positive trend was observed between the total mass uptake of attached peptide and the single-molecule adhesion force on each substrate. Peptide affinity to gold was appreciably greater than to the oxide substrates. Collectively, the results obtained in this study offer insight into the ways in which inorganic materials can differentially influence and modulate the adhesion of SBPs through triggered adsorption and conformational responses.

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4.1. Introduction

Solid-binding peptides (SBPs) are a versatile class of biomacromolecule which are widely employed as molecular building blocks to functionalize various types of inorganic and organic solid materials.^{1,2} One of the most intriguing aspects of SBPs is the possibility to utilize short amino acid sequences which selectively bind with high affinity to a target substrate. Indeed, SBPs are a useful tool for surface functionalization, offering a simple approach to improve the biocompatibility of a material surface or act as molecular ligands to facilitate the conjugation of other biomolecules. Among the scope of applications, SBPs enable the fine placement of biomolecules on solid surfaces,³⁻⁶ fabrication of self-assembled nanostructures,^{4,7} and destruction of virus*. For all these reasons, there is significant interest in understanding the factors which determine how SBPs selectively bind to target substrates.

The interaction between SBPs and material surfaces is governed by the interplay of various noncovalent intermolecular forces with different physical origins, including the double-layer electrostatic force, van der Waals force, hydration force, steric interactions, and the entropic force.⁸⁻¹¹ The binding affinity and selectivity of peptide attachment are difficult to predict on the basis of substrate properties and peptide characteristics (*e.g.*, amino acid composition, electrical charge, polarity) alone. Empirical investigations are necessary in order to directly address these questions, and typically focus on comparing the attachment of sequence-related peptides in a library to one target substrate or the attachment of one peptide to multiple substrates. In either case, a combination of different experimental techniques and theoretical analysis can help to establish correlations between material properties and peptide binding affinity.¹²⁻¹⁵ So far, a major emphasis in the field has been on engineering SBPs with optimal binding properties to a target material, and a wealth of knowledge remains to be learned about how material substrates differentially modulate the adsorption and conformational properties of SBPs.

The goal of the present study is to investigate the adsorption and conformational responses of one SBP candidate upon attachment to a series of inorganic substrates – gold,

silicon oxide, titanium oxide, and aluminum oxide. The SBP is a 16-amino acid amphipathic peptide (AGSWLRDIWTWLQSAL) engineered from a longer 27-amino-acid sequence which has demonstrated virucidal activity in solution¹⁶ and can be noncovalently immobilized onto hydrophilic solid supports for patterning applications¹⁷. The engineered peptide investigated here possesses one negatively and one positively charged residues (electrically neutral) as well as neutral hydrophilic and hydrophobic residues with an amphipathic disposition (**Fig. 4.1**).

Figure 4.1. Schematic of Experimental Design

Helix net wheel plot of the 16-amino acid long peptide is presented. By combining multiple experimental techniques, it is possible to investigate peptide adsorption kinetics and binding affinity to various inorganic substrates as well as corresponding changes in peptide secondary structure.

Systematic experiments are performed based on secondary structure characterization of peptides by circular dichroism (CD) spectroscopy, adsorption kinetics measurements of peptide binding affinity by quartz crystal microbalance-dissipation (QCM-D), and single-molecule force measurements of peptide attachment by atomic force microscopy (AFM). The combination of experimental techniques allows us to correlate changes in peptide secondary structure with attachment/detachment kinetics and peptide binding affinity.

4.2. Materials and Methods

4.2.1. Reagents

Phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, CA), ethanol (Merck Millipore, Billerica, MA), dehydrated toluene (Fisher Scientific, Waltham, MA), 11-amino-1-undecanethiol hydrochloride (Sigma-Aldrich, St. Louis, Mo), N-hydroxysuccinimide-PEG24 maleimide ester (NHS-PEG-MAL) (Product No. 10314, Quanta Biodesign, Plain City, OH) and polyoxyethylene sorbitan monolaurate (Tween-20, Bio-RAD, Hercules, CA) were used in the experiments. All aqueous solutions were prepared using Milli-Q-treated water (18.7 M Ω ·cm) (Millipore, Billerica, MA).

4.2.2. Peptides

High purity peptides (> 95%) were synthesized by Anaspec Corporation (San Jose, CA, USA). The sequence of the template peptide is H-Ala-Gly-Ser-Trp-Leu-Arg-Asp-Ile-Trp-Thr-Trp-Leu-Gln-Ser-Ala-Leu-NH₂. For AFM experiments, an additional Cys residue was added to the N-terminus or C-terminal. The as-supplied lyophilized peptides were solubilized in deionized water at a stock concentration of 2 mg/mL peptide. The exact molar concentration of peptide in solution was determined by standard absorbance measurements at 280 nm and diluted accordingly before experiment.

4.2.3. Circular Dichroism (CD) Spectroscopy

Far-UV circular dichroism spectra of peptide-nanoparticle mixtures were obtained by using an AVIV Model 420 spectrometer (AVIV Biomedical, Lakewood, NJ, USA) with a 1 mm path length cuvette (Hellma). Spectra data were collected with a step size of 0.5 nm and averaging time of 4 s. The peptide and nanoparticle concentrations were fixed at 100 μ M and 0.1 wt%, respectively, and the mixture was incubated in PBS solution [pH 7.2] for 30 min at ambient room temperature before experiment. All spectra were recorded at 25°C from 190 to 260 nm using a bandwidth of 1-nm and averaged over three scans. Baseline scans were obtained using the same parameters for buffer with nanoparticles only and subtracted from the respective data scans with peptides. The final corrected averaged spectra are expressed in mean residue molar ellipticity.

4.2.4. Quartz Crystal Microbalance-Dissipation (QCM-D)

QCM-D experiments were conducted with a Q-Sense E4 instrument (Q-Sense AB, Gothenburg, Sweden) in order to measure the binding affinity of peptides to the solid supports. The QCM-D technique monitors changes in the resonance frequency (Δf) and energy dissipation (ΔD) of an oscillating, piezoelectric quartz crystal sensor chip as functions of time, which reflect the acoustic mass and viscoelastic properties, respectively, of an adsorbate on the surface. The sensor chips had a fundamental frequency of 5 MHz and were obtained as-supplied with gold, silicon oxide, titanium oxide, or aluminum oxide coatings. Before experiment, the sensor chips were sequentially rinsed with water and ethanol, dried with nitrogen gas, and subjected to oxygen plasma treatment for 1 min with an Expanded Plasma Cleaner (model no. PDC-002, Harrick Plasma, Ithaca, NY). A baseline signal was recorded in PBS solution [pH 7.2] and then 7 μ M peptide in the same buffer was added. A washing step with PBS solution completed the procedure. In all steps, a peristaltic pump (Reglo Digital, Ismatec, Glattbrugg, Switzerland) was used to inject liquid samples into the measurement chamber under continuous flow conditions at a flow rate of 50 μ L/min. During the experiments, the temperature in the measurement cell was maintained at 25.0 ± 0.5 °C. The experimental data was collected at the 3rd ($n = 3$), 5th ($n =$

5), and 7th ($n = 7$) odd overtones using the QSoft (Q-Sense AB) software package, and the data was normalized according to the overtone number. Data processing was performed in the QTools (Q-Sense AB) and OriginPro 8.5 (OriginLab, Northampton, MA) software packages. All presented data was collected at the 5th overtone.

4.2.5. Probe Functionalization

In order to measure the peptide binding interaction, silicon AFM probes (CSG-01, NT-MDT, Moscow, Russia) with a spring constant around 0.03 N/m were employed. The probes were coated with 2-nm thick germanium adhesion layer, followed by a 20-nm thick gold layer by thermal evaporation in a vacuum (base pressure, 1.0×10^{-4} Pa). In order to attach the peptide to the probe tip, the gold-coated probes were first submerged in an ethanol solution with 10 mM 11-amino-1-undecanethiol hydrochloride for 2 hours and then washed with pure ethanol solution. The probes were next immersed in dehydrate toluene solution with N-hydroxysuccinimide-PEG24-Maleimide ester (1 mM) for 2 hours. After immersion, the probes were rinsed with pure toluene solution and allowed to air dry. The dried probes were incubated with 1 mg/mL peptide in deionized water. Before AFM experiments, the functionalized probes were rinsed with PBS buffer solution.

4.2.6. Atomic Force Microscopy (AFM)

A commercial AFM system (NX-Bio, Park Systems, Suwon, South Korea) was employed for the force spectroscopy measurements. Before force measurement, the AFM probes were calibrated using the thermal method, which monitors thermal vibration of the cantilever. All force spectroscopy experiments were performed at room temperature in PBS buffer solution that contained 0.05 wt% Tween-20 in order to nullify hydrophobic interactions that may arise from surface defects or trace hydrophobic contaminants. The probe approach speed and maximum loading force were set at 2 $\mu\text{m/s}$ and 1 nN, respectively.

4.2.7. Evaluation of Single-Molecule Adhesion Force

Single-molecule adhesion force was evaluated by autocorrelation analysis of histograms of adhesion force observed for each system. With our conditions for preparation of AFM probes, the histograms exhibited quantized features (consisting of events of single and multiple bond attachments). Therefore, the single-molecule adhesion force corresponds to the periodicity in the autocorrelation lag.* Error bars were calculated from the standard deviation of the periodicity observed in the differentiated autocorrelation plots.

4.3. Results and discussion

4.3.1. Secondary Structure Characterization of Peptide in Solution

Using far-UV circular dichroism (CD) spectroscopy, the secondary structure of 100 μM peptide in PBS solution [pH 7.2] solution was first characterized in the presence and absence of 30-nm diameter inorganic silicon oxide, aluminum oxide, titanium oxide, or gold nanoparticles (**Fig. 4.2**).

Figure 4.2. CD spectra for peptide in presence and absence of inorganic nanoparticles.

All measurements were recorded in PBS solution [pH 7.2].

In the absence of nanoparticles, the free peptide in solution exhibited a predominately random coil character. A similar result was also obtained for peptide in the presence of aluminum oxide nanoparticles. By contrast, peptide in the presence of other nanoparticle types (silicon oxide, titanium oxide, gold) induced helical character, as denoted by peak minima in the regions of 208 and 222 nm, respectively. This finding supports that the peptide differentially attaches to various types of inorganic materials, motivating further investigation of the peptide-substrate interaction.

4.3.2. Peptide Binding Affinity to Solid Supports

In order to compare the binding affinity of the peptide to different substrates, quartz crystal microbalance-dissipation (QCM-D) experiments were performed. An initial baseline signal was recorded in aqueous buffer solution, followed by addition of 7 μM peptide at $t = 20$ min under continuous flow conditions. Peptide adsorption caused a significant decrease in the frequency and minor increase in the energy dissipation shifts (**Fig. 4.3A,B**). After reaching near-saturation values, a buffer washing step was performed and peptide desorption was monitored, as detected by a positive frequency shift and negligible change in the energy dissipation. In general, peptide affinity to gold was appreciably greater than to the oxide films on the basis of a larger frequency shift.

Figure 4.3. Representative QCM-D kinetic data for peptide adsorption onto solid supports. Changes in (A) frequency and (B) energy dissipation are presented as functions of time. Peptide was added at $t = 20$ min.

Depending on the substrate, there were quantitative differences in the frequency shift of the adsorption step (**Fig. 4.4A,B**). On gold, the frequency shift was around -30 Hz, while the shifts were around -24 Hz on aluminum oxide and titanium oxide. The smallest shift was around -20 Hz on silicon oxide. In all cases, the energy dissipation shifts were less than 1×10^{-6} . After buffer washing, the final frequency and energy dissipation shifts were also recorded and take into account the effects of peptide adsorption and subsequent desorption (**Fig. 4.4C,D**). The final shift was around -27 Hz on gold, and appreciably smaller on the oxide substrates, including -12 Hz on titanium oxide, -10 Hz on silicon oxide, and -7 Hz on aluminum oxide. Taken together, the QCM-D experiments indicate that peptide affinity to gold is the greatest among the tested cases. The results obtained with the oxide film substrates were more nuanced because there was not a general trend between the adsorption and desorption behaviors. The adsorption data support that the peptide has higher binding affinity to titanium oxide and aluminum oxide over silicon oxide. However, despite the observed adsorption behavior, peptide attachment to aluminum oxide was relatively weaker than to the other two substrates.

Figure 4.4. Comparison of QCM-D Measurement Responses.

Maximum changes in (A) frequency and (B) energy dissipation are reported for peptide adsorption on the different substrates. Final changes in (C) frequency and (D) energy dissipation after a washing step are also reported. All reported values are the average and standard deviation for $n = 4$ measurements.

4.3.4. Single-Molecule Adhesion Force Analysis

Adhesion force analyses at the single-molecule level were next performed in order to evaluate the interaction between the peptide and solid surfaces. Adhesion peaks in the force-distance curves were always observed after the stretch of PEG chain (around the separation distance of 10 nm) (**Fig. 4.5A**), indicating that the adhesion is due to the peptide-substrate interaction and not due to other non-specific interactions. As presented in **Fig. 4.5B**, the histogram of collected adhesion forces consists of several peaks due to the sum of the events for the rupture of single and multiple bonds. The autocorrelation of the histograms depicts step-like features and the differentiated curve clearly displays the periodic lag, which corresponds to the single-molecule adhesion force (**Fig. 4.5C**).

Figure 4.5. Single-Molecule AFM Force Spectroscopy.

(A) Representative force curves obtained with the P8C-SiO₂ system. Adhesion force is indicated with a double-sided arrow. (B) Representative histogram for adhesion forces observed in the P8C-SiO₂ system. (C) Autocorrelation-lag plot and its differentiation in order to determine the single-molecule adhesion force.

The evaluated single-molecule adhesion forces obtained in each system are all presented in **Figure 4.6**. Peptides with cysteine residues at the C- or N-terminus were both evaluated. The trends in the measured adhesion force were similar in both cases. Both types of peptide exhibited the strongest adhesion to gold among the tested substrates and interacted more weakly with the other solid supports. Comparing these results with those presented in Figure 4, there is a strong correlation between the affinities evaluated by the QCM-D and AFM measurements. Of note, bound peptides to aluminum oxide were sensitive to buffer washing and exhibited the most significant desorption behavior, as reflected in the final QCM-D measurement values (*cf.* Fig. 4C). Based on the CD measurement results, this finding is consistent with the lack of helix induction for bound peptides on aluminum oxide. By contrast, on the other three substrates, bound peptides underwent a conformational change that increased helical character in the peptide secondary structure.

Figure 4.6. Comparison of AFM Single-Molecule Adhesion Force Analysis on Different Substrates.

Evaluated single-molecule adhesion forces are presented for the peptide tethered to the NHS-PEG-MAL linker via the (A) C-terminus or (B) N-terminus.

4.4. Conclusion

In this work, we have investigated the adsorption of a short, random coil peptide to four inorganic material substrates. A combination of experimental approaches allowed us to compare the adsorption kinetics and binding affinity of peptide attachment to the different substrates as well as corresponding changes in the peptide secondary structure. A general trend in the total mass uptake and binding affinity was observed, with the greatest values obtained for bound peptides on gold. On each substrate, similar adhesion forces were determined for tethered peptides that were functionalized via the N- or C-terminus. In addition, peptide attachment led to helix induction on all substrates except aluminum oxide. In combination with the adsorption data, the substrate-specific conformational change suggests that helix induction is important for the stabilization of bound peptides.

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Chapter 5

Quantitative Evaluation of Specific Binding Interactions between Phosphoinositide Molecules and an Amphipathic, α -Helical Peptide

As the next step in developing force spectroscopy-based measurement approaches to study peptide interactions at interfaces, this chapter focuses on establishing an atomic force microscopy platform to evaluate the interaction between peptides and phosphoinositide molecules, which are a type of glycan that is important for numerous biological processes. Of interest in this study, I focus on the interaction between a synthetic peptide corresponding to the hepatitis C virus NS5A protein's N-terminal anchor and PI(4,5)P₂ molecules as this interaction is necessary for viral genome replication and blocking this interaction is therefore an attractive antiviral strategy. A chemical tethering strategy is established to functionalize AFM probe tips with the peptide, and different measurement parameters including the peptide coating density and the contact time of interaction are optimized. Based on the optimized settings, I then investigated the binding specificity of the platform along with the effect of ionic strength, revealing mechanistic insights into the factors governing the specific interaction. Lastly, I demonstrate that the platform is suitable for screening candidate inhibitors that block the interaction between peptide molecules and PI(4,5)P₂ molecules.

5.1. Introduction

Hepatitis C virus (HCV) is a significant cause of liver disease and affects over 150 million people worldwide¹. Although improved therapies have recently become available, much remains to be learned about the HCV life cycle, which could also be relevant for future strategies to combat cases of resistance². HCV is a positive, single-stranded, RNA virus that encodes a single ~3000 amino acid polyprotein which is processed by cellular and viral proteases into structural (components of the mature virus) and nonstructural (elements proposed to help replicate new virions) proteins³. Understanding HCV molecular virology has enabled the development of direct-acting antivirals against viral targets⁴. One of the most promising antiviral strategies involves targeting HCV nonstructural proteins involved in viral replication.

HCV genome replication occurs in close association with host intracellular membranes^{5,6}. HCV nonstructural proteins induce the formation of membranous web structures which host viral proteins and nascent RNA involved in membrane-associated RNA replication⁷. Each type of nonstructural protein plays a necessary role in the replicase complex, yet the specific function(s) of certain proteins is still somewhat nebulous. The NS5A protein is a key example⁸ and possesses a wide range of functions, including RNA binding activity⁹ and an N-terminal amphipathic helical (AH) segment which is involved in membrane association^{10,11}—a necessary step in viral genome replication. Significant effort has been placed on deciphering the mechanistic factors involved in NS5A AH membrane association as a potential antiviral strategy.

It was recently discovered that the NS5A AH specifically binds intracellular phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)¹². Immunofluorescence microscopy experiments demonstrated that NS5A protein and PI(4,5)P₂ lipid co-localize at HCV replication complexes, and that PI(4,5)P₂ binding induces a conformational change in the NS5A AH that is necessary for viral genome replication. A pair of positively charged lysine residues flanking a hydrophobic face within the NS5A AH was required for PI(4,5)P₂ binding and RNA replication, leading to the identification of the Basic Amino Acid

PI(4,5)P₂ Pincer (BAAPP) domain. A similar motif was found to be conserved across all HCV isolates, as well as amphipathic helices of many viral pathogens, including hepatitis A virus. While there are other known structural motifs that mediate PI(4,5)P₂ binding^{13,14}, the HCV NS5A BAAPP domain represents the first example of phosphoinositides mediating viral genome replication. Understanding the mechanistic details and consequences of the HCV NS5A BAAPP domain-PI(4,5)P₂ interaction can yield important new information about the viral life cycle and identify novel potential antiviral strategies for use against HCV and other viral pathogens.

To achieve this goal, a quantitative understanding of the HCV NS5A BAAPP domain-PI(4,5)P₂ interaction is essential yet precise determination of phosphoinositide binding affinities is difficult to achieve with conventional measurement approaches that rely on ensemble averaging. Such measurements provide estimates of binding affinity but have large errors¹⁵⁻¹⁷. As a result, systematic evaluation of protein-phosphoinositide interactions remains limited, especially within the scope of understanding how environmental conditions and amino acid mutations influence binding affinity^{18,19}. One promising approach involves direct measurement of the adhesion force underpinning individual phosphoinositide binding events at the single-molecule level, although to date such studies have only been studied using larger proteins. For example, Malkovskiy et al. measured the interaction between the PLC δ /1PH and PI(4,5)P₂ molecules by utilizing a polymerized liposome platform²⁰.

In this chapter, I report the chemical functionalization of an AFM probe tip with a synthetic peptide comprising the HCV NS5A BAAPP domain and demonstrate its high binding specificity to PI(4,5)P₂ molecules. Optimization of the measurement settings, including surface coating and AFM parameters, is achieved in order to achieve a large measurement response that is suitable for probing the influence of environmental conditions as well as evaluating pharmacological inhibition with a known small molecule inhibitor. These capabilities enable a quantitative measurement platform to identify key structural features in the NS5A BAAPP domain that will be of tremendous benefit to the study of hepatitis virology and aid development of new broad-spectrum antiviral strategies

5.2. Materials and Methods

5.2.1. Reagents

Milli-Q water (~18.7 MΩ·cm; Millipore, Billerica, MA), phosphate-buffered saline (PBS, Life Technologies Corporation, NY, USA), ethanol solution (Merck Millipore, MA, USA), dehydrated toluene (Fisher Scientific, GA, USA), 2-aminoethanethiol (Tokyo chemical industry, Tokyo, Japan), N-hydroxysuccinimide-PEG24 maleimide ester (NHS-PEG-MAL, 95%; Quanta Biodesign, OH, USA) and m-dPEG24-NHS (NHS-PEG-OMe, 98%; Quanta Biodesign, OH, USA) were used in this experiment. High purity AH peptide (>95%) was synthesized by Anaspec Corporation (San Jose, CA, USA). The sequence of the AH peptide is H-Ser-Gly-Ser-Trp-Leu-Arg-Asp-Val-Trp-Asp-Trp-Ile-**Cys**-Thr-Val-Leu-Thr-Asp-Phe-Lys-Thr-Trp-Leu-Gln-Ser-Lys-Leu-AspTyr-Lys-Asp-NH₂. The cysteine residue is highlighted because it is important for chemical attachment to the AFM tip.

5.2.2. Preparation of AFM tip functionalization.

Commercial rectangular shaped soft AFM cantilevers (CSG-01, NT-MDT, Moscow, Russia) with 0.03 N/m nominal spring constant were used to measure the binding interaction between peptide and Pls platform. Its AFM tips were coated with at first a 2.5 nm layer of titanium and then 35 nm thickness layer of gold (tip curvature radius is approximately 35 nm). For AFM tip functionalization, peptide attachment, the tips were washed with ethanol solution at three times and cleaned by UV Ozone cleaner (PSD UV Ozone cleaner, Novascan, USA). The cleaned tips were incubated in ethanol solution including 2-aminoethanethiol (10mM) for 6 hour. After incubation, the tips were washed with pure ethanol solution, dried with a very gentle stream of nitrogen gas, and immersed in anhydrous toluene solution containing N-hydroxysuccinimide-PEG24-Maleimide ester (1 mmol/L) for 6 hours. For the final step of AFM tip functionalization, the immersed tips were rinsed with anhydrous toluene and ethanol, dried with a very gentle stream of nitrogen gas again. After that, the rinsed tips were incubated in PBS buffer solution with the target

peptide (52 $\mu\text{M/L}$) for overnight. Before force spectroscopy, the peptide attached tips were washed by PBS buffer solution.

5.2.3. Preparation of phosphoinositides platform

To prepare the phosphoinositide platform, pre-spotted commercial PIs Strip membranes which nitrocellulose membrane-immobilized lipid (Echelon Biosciences, Salt Lake City, USA) were used. This PIs strip is serial dilutions of 15 different phospholipids include PI, PI3P, PI4P, PI5P, PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂ and PI(3,4,5)P₃ are spotted onto a hydrophobic nitrocellulose membrane. Before the measurement, PIs strip was immersed in PBS buffer solution for 1 hour and washed with same PBS buffer 3 times.

Figure 5.1. Chemical structure of phosphoinositides. (Reprinted from Tom D. Bunney et. Al. 2011)

5.2.4. Operation of AFM force spectroscopy

The force spectroscopy measurements were performed using a commercial AFM system (NX-Bio, Park Systems, Suwon, South Korea). For force mapping data-set acquisition, mapping resolution was 16×16 points with $10 \times 10 \mu\text{m}^2$ of scanning area,

256 force curves per one data-set. The speed of the AFM cantilever approaching and retraction were set at 1,000 nm/sec with the maximum loading force 3 nN. Before and after force spectroscopy, the spring constant of AFM cantilevers were calibrated using the thermal method which monitored thermal vibration of the AFM cantilever (approximately 0.0428 N/m). All force spectroscopy experiments were performed at room temperature in PBS buffer solution. For force curve analysis, calculation of the adhesion force and the rupture distance, Park Systems image analysis program (XEI v1.8.2), own analysis program (Python) and Origin Pro 9.1 (OriginLab Corp., Northampton, USA) were adopted. In order to acquire the reliable data, the adhesion force in the range 0-1,200 nN and rupture events with tip-sample separations in the range 1-80 nm were picked and analyzed.

5.3. Results and discussion

5.3.1. Platform Design

Because peptides like the one studied here are relatively small molecules with important secondary structure features, it is important to develop a chemical functionalization strategy that enables covalent attachment to the probe tip while avoiding denaturation that would hinder binding. To achieve this goal, I selected a tethering scheme that involves adding a heterobifunctional cross-linker to connect the peptide with the tip surface, as presented in **Figure 5.2**. First, the gold-coated tip was functionalized with 2-aminoethanethiol *via* gold-sulfur dative bonds²¹ and resulted in the presentation of free amine groups for further functionalization. The tip surface was then coated with N-hydroxysuccinimide-PEG24-maleimide ester, where PEG refers to the polyethylene glycol polymer and the amine group covalently attaches to the hydroxysuccinimide functional group. Afterwards, the free maleimide group on the other side of the PEG chain was incubated with the 27-amino acid long, synthetic peptide comprising the HCV NS5A BAAPP domain²² in order to promote covalent conjugation by formation of a stable thioether linkage between the PEG chain and peptide. In particular, the peptide contains a cysteine residue which has a free sulfhydryl group and this group irreversibly reacts with the maleimide group in phosphate-buffered saline (PBS) with pH 7.4 as it is known that

the reaction occurs spontaneously when the pH value of the solution is between 6.5 and 7.5 (see Ref. 23). In the simplest form, every PEG chain will be functionalized with a single peptide molecule. However, the coating density of peptide can be lowered by including a mixture of the bifunctional PEG cross-linker with a monofunctional PEG cross-linker which possesses the hydroxysuccinimide functional group but lacks the maleimide functional group. Controlling the coating density of the tethered peptide is further discussed below.

Figure 5.2. Chemical functionalization of AFM probe tip with peptide molecule based on utilizing a heterobifunctional PEG cross-linker.

5.3.2. Morphological Characterization of Phosphoinositide-Coated Lipid Blots

Before proceeding to evaluate the performance of functionalized tips, I first characterized the quality of the commercial nitrocellulose strips with hydrophobic membrane containing spots of phosphoinositide molecules (termed a “lipid blot”). Indeed, uniform surface properties are important for precise AFM measurements in order to determine binding affinities and minimize nonspecific interactions²⁴. Conventional AFM imaging with a non-functionalized tip was conducted in PBS solution, and operated in contact mode with a 0.8 nN loading force and 0.5 Hz scan rate. The results showed a flat surface with a 0.89 nm surface roughness, indicating a stable, flat and dense molecular layer over the nitrocellulose surface (**Figure 5.3**). The lipid blots were prepared following

a protocol which was recommended by the manufacturer. Using the protocol, reproducible AFM images were obtained, even after repetitive scans, with full coverage of PI molecules. The high concentration of uniformly covered molecules on the nitrocellulose strips is suitable for force spectroscopy measurements with the functionalized AFM tips.

Figure 5.3. AFM topographical image of phosphoinositide-coated lipid blot surfaces.

It shows even distribution at the correct height settings (~10 nm scale). Scale bar: 1 μ m

5.3.3. Evaluation of Measurement Operation

As a first attempt, I analyzed the interaction between the peptide-functionalized tip and a lipid blot of PI(4,5)P₂ molecules. A representative force-distance curve is presented in **Figure 5.4a** indicating a rupture length of around 15 nm, which is consistent with the PEG spacer's length of ~9.4 nm along with the position of the cysteine residue relative to the BAAPP domain (~2 nm) and some unfolding of the peptide's helical character during the unfolding (~3 nm)^{25,26}. The adhesion force is around -300 pN which is consistent with multiple binding interactions being broken simultaneously²⁷, as discussed before. By contrast, when the tip is not functionalized with peptide, there is no interaction between the tip and PI(4,5)P₂ molecules, indicating that the detected interaction is specific between the

peptide molecules and the phosphoinositides (**Figure 5.4b**). I next investigate various AFM measurement parameters in order to optimize the sensing performance.

Figure 5.4. Design of AFM platform.

Example of single force curve with specific interaction between target peptide and PIs. Black two-way arrow indicates the adhesion force, red two-way arrow shows the rupture length between two molecules (a), example of single force curve with no interaction between target peptide and PIs (b), schematic view of the peptide functionalized tip (c).

5.3.4. Effect of Peptide Coating Density

In order to compare the effect of peptide density bound to the AFM tip, a modified tip functionalization strategy using a mixture of spacers with NHS-PEG chains and methoxy-terminated PEG chains were tested. Then, the NHS-PEG spacers are able to bind to peptide but, in case of the methoxy-terminated PEG spacer, it cannot attach to target peptide due to the peptide-resistant group (OMe). In this measurement scheme, the density of peptide on the AFM tip was varied by incorporating 0 mol%, 20 mol%, 50 mol% and 100 mol% NHS-PEG-MAL spacer percentages. As shown in **Figure 5.5**, the statistical distribution of the adhesion force and the rupture length was calculated from

Figure 5.5. Statistical analysis of peptide coating density on AFM tip.

Peptide attachment to AFM tips with functionalized with (a) 0 mol%, (b) 20 mol%, (c) 50 mol%, and (d) 100 mol% Mal-PEG-NHS.

the force-distance curves. The histogram of the adhesion forces was measured from over 1,000 force-distance curves and the distribution of the rupture length was estimated for the same series of force-distance curves. In the case of 0 mol% NHS-PEG spacer, the peptides were not attached to the AFM tip so the average adhesion force and rupture force had the smallest values (43.47 pN and 6.06 nm, respectively) indicative of weak, nonspecific adhesion events. By contrast, for 20 mol% NHS-PEG spacer, the most probable rupture length was approximately 12 nm, which is close to the theoretical length of the PEG₂₄ spacer molecule (9.4 nm). It also compares well to the general rupture length found in other studies using PEG cross-linkers. This value provides a good agreement that supports specific binding events, based on the unfolding of the PEG spacer and following peptide interaction. The most probable adhesion force was 85.68 pN, and this value is higher than

0 mol% NHS-PEG spacer case but lower than 50 mol% and 100 mol% cases. Next, we studied the binding force between the peptide and PI(4,5)P₂ using 50 mol% and 100 mol% NHS-PEG spacer percentages. In both cases, the highest binding affinity was observed reaching around 175-180 pN, and the rupture length distribution could also be estimated with a dependable value around 13-15 nm. Such findings support that the PEG spacer conferred a high extent of spatial freedom to maintain the secondary structure features of the BAAPP domain peptide, resulting in strong binding interactions between NS5A AH and PI(4,5)P₂ molecules. Based on these results, it was identified that the measured adhesion force of the peptide- PI(4,5)P₂ interaction could be tuned based on adjusting the peptide coating density, implying that the interaction is multivalent which is further discussed in the next section. For all subsequent studies, the NHS-PEG spacer density was fixed at 100%.

5.3.5. Effect of Contact Time

It is well known that protein-glycan interactions are relatively weak in general, and depend on multivalency in order to create strong binding affinities^{28,29}. Hence, the measured adhesion force corresponds to the number of peptide-phosphonositide binding events and the average number of binding events is expected to relate to the dwell time of contact between the peptide and lipid blot. **Figure 5.6** shows the average adhesion forces, their corresponding rupture distances, and representative deflection curves of the AFM cantilevers as a function of contact time. In all cases, the rupture length was within the working range of the theoretical value, which supports that the specific interaction between the peptide and PI(4,5)P₂ molecules was being detected. In both the 0.1 and 1 sec contact time cases, the typical rupture lengths were similar at around 11.4 ± 1.6 and 14.8 ± 1.7 nm, respectively, and consistent with expected rupture lengths in the 10-15 nm range as described above. On the other hand, with a 3 sec contact time, the most probable adhesion force increased to 248.7 ± 20.3 pN, however, the typical rupture length was significantly larger at around 35.1 ± 2.9 nm and a large number of multiple rupture events were detected unlike the predominately single rupture events observed for shorter contact times. Therefore, to focus on the specific AH-PI(4,5)P₂ interaction, we selected a 1 sec contact

time for subsequent experiments due to the combination of a suitably large, most probable adhesion force and typical rupture lengths that are consistent with detecting single rupture events in this system.

Figure 5.6. Adhesion force as a function of contact time.

As a function of (a) 0.1 sec, (b) 1 sec, and (c) 3 sec contact times between peptides and PI membrane, the mean value of the adhesion force and the rupture length are presented with deflection of the AFM cantilever as a function of contact time (0.1, 1 and 3 sec). Over the 1,000 force-distance curves were performed at each measurement.

Hence, the contact time was fixed at 1 sec for subsequent experiments. Taking into account the short time scales of molecular interactions, the strong dependence of the adhesion force on contact time indicates that the kinetics of the peptide-phosphoinositide interaction are quite slow. Indeed, the time scales of structural changes of peptides are typically on the order of tens or hundreds of nanoseconds. In force spectroscopy measurements with the functionalized tip, due to the confinement of the peptide moieties between the AFM tip and the target receptors, there is low spatial freedom of the peptide binding configuration. It is one additional line of evidence that the NS5A AH peptide-PI(4,5)P₂ interaction is not simply caused by electrostatic forces, and that the structure of the NS5A AH is one of the essential factor in its specific interaction to PI(4,5)P₂ molecules. These observations are in line with previous biochemical results indicating that the AH peptide undergoes a conformational change in response to PI(4,5)P₂ binding.

5.3.6. Evaluation of Phosphoinositide Binding Specificity

Next, we evaluated the binding affinity of the NS5A AH peptide with a variety of phosphoinositides containing different degrees of phosphorylation at various ring locations, including PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂ and PI(3,4,5)P₃. The experimental conditions were fixed at 100 mol% of NHS-PEG-MAL and 1 sec of contact time. As presented in **Figure 5.7**, the NS5A AH demonstrated high specific binding affinity for PI(4,5)P₂, with the mean adhesion force around 183.49 pN. In the case of other PI bisphosphates (PI(3,4)P₂, PI(3,5)P₂), which have similar electrostatic character to the negatively charged PI(4,5)P₂, the peptide demonstrated significantly lower adhesion forces. The second-highest adhesion force was observed for the PI(3,4)P₂ molecule, which was around 44 pN, whereas the adhesion force to PI(3,5)P₂ was only 28 pN. By contrast, the adhesion force to PI(3,4,5)P₃ was 35 pN, the third-highest adhesion force among the tested cases. The other phosphoinositides, including PI, PI(3)P, PI(4)P, and PI(5)P, had no specific binding interaction with the peptide. Collectively, these findings indicate that electrostatic attraction is insufficient for high binding affinity, and that the phosphate group at the 4th position is particularly important for conferring strong binding affinity likely due to molecular-level, non-electrostatic interactions.

Figure 5.7. Quantitative profiling of NS5A-PIs membrane interactions.

The NS5A AH interacts with a variety of PIs membrane including PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂ and PI(3,4,5)P₃. The histograms for the measured adhesion forces in all measurement are presented (a), the mean value of adhesion force of each case is showing that histogram (b), the Gaussian non-linear fitting curves (c).

From a measurement perspective, it should also be noted that the AFM measurements agree well with previously reported results obtained using peptide monomers with the quartz crystal microbalance-dissipation (QCM-D) technique. One important distinction is that the AFM measurements further enable quantitative determination of the binding affinity between the peptide and PI(4,5)P₂ molecules with high precision due to stronger multivalent interactions enabling further investigation of environmental conditions.

5.3.7. Effect of Ionic Strength on Binding Affinity

While electrostatic attraction is insufficient to induce the high adhesion force observed for the peptide-PI(4,5)P₂ interaction, I hypothesized that the magnitude of the electrostatic force likely influences the magnitude of the adhesion force. To test this

Figure 5.8. Effect of ionic strength on BAAPP domain-PI(4,5)P₂ interaction.

The adhesion force histogram distribution is presented as a function of NaCl concentration (0, 10, 50, 150, and 250 mM).

hypothesis, AFM force spectroscopy measurements were performed in varying ionic strength conditions (**Figure 5.8**). Due to charge shielding, increasing ionic strength conditions decreased the surface charge of the peptide, especially in the region of the positively charged lysine residues in the BAAPP domains, as well as that resulting from the negatively charged phosphate groups of the PI(4,5)P₂ molecules. As a result, the magnitude of the attractive electrostatic force is expected to be weaker with increasing ionic strength³⁰. Experimentally, I observed that the magnitude of the PI(4,5)P₂ adhesion force decreased from 330.99 pN at 0 mM NaCl to 143.64 pN at 250 mM NaCl. This finding further reinforces the foregoing observation that electrostatic attraction is insufficient for governing the strong peptide-PI(4,5)P₂ interaction and highlights the importance of

additional molecular-level interactions that depend on the spatial configuration of phosphate groups on the PI molecules.

5.3.8. Pharmacological Disruption of the Peptide-PI(4,5)P₂ Interaction

Our foregoing observations establish that the AFM measurement platform is capable of detecting and quantifying PI(4,5)P₂ binding by the NS5A AH ligand in a highly specific manner. We next asked if the AH-PI(4,5)P₂ binding interaction could serve as a competitive probe to measure the association constant of phosphoinositide-binding small molecules that might be promising drug candidates. Indeed, as the force spectroscopic measurement is probing interactions between AH ligands and PI(4,5)P₂ receptor pairs, it would be expected that the occupancy of a fraction of receptor sites by a phosphoinositide-binding small molecule might influence the measurement response (**Figure 5.10**). Such capabilities would be useful for quantitative biochemical analysis of phosphoinositide-small molecule interactions as well as for evaluating potential antiviral drug candidates that interfere with the AH-PI(4,5)P₂ binding interaction.

To explore this possibility, we investigated how neomycin, an aminoglycoside antibiotic that is known to bind PI(4,5)P₂ receptors, affects the AH-PI(4,5)P₂ binding interaction. For these experiments, various concentrations of free neomycin (10 nM – 1 mM) were incubated with the phosphoinositide array for 1 hr to allow equilibration of free and bound neomycin prior to each AFM experiment, and then adhesion force measurements were conducted in the equilibrated solution conditions. A low concentration of neomycin (100 nM) did not affect the binding interaction and the recorded measurement values in this case were comparable to conventional AH-PI(4,5)P₂ binding (adhesion force of ~225 pN). On the other hand, higher concentrations of neomycin (1 – 10 μ M) exhibited dose-dependent inhibition of the AH-PI(4,5)P₂ binding interaction. Representative adhesion force maps (2 μ m $\times$ 2 μ m) obtained in the presence of different neomycin concentrations show the force magnitudes obtained at different pixel positions, indicating a transition from high adhesion forces corresponding to strong, specific binding forces at low neomycin concentrations to weak, nonspecific interactions at higher neomycin concentrations. With increasing neomycin concentration across this range, the most probable adhesion force decreased from

194.2 ± 11.1 to 64.5 ± 14.3 pN. Above this concentration range (≥ 100 μ M neomycin), the measured adhesion forces for AH-PI(4,5)P₂ binding reached a stable minimum value that was comparable to the magnitude of nonspecific adhesion events (~ 48 pN). Hence, competitive binding of neomycin to PI(4,5)P₂ receptors inhibited specific AH-PI(4,5)P₂ binding on account of neomycin molecules occupying PI(4,5)P₂ receptor sites. The sharp transition in the adhesion force data as a function of neomycin concentration suggests that the measurement approach may be probing single-molecule binding events.

Based on the adhesion force data, the probability of specific binding events was determined and ranged from 84% at 0.1 μ M neomycin to 20% at 1000 μ M neomycin. From these data, an efficacy curve of neomycin's inhibitory activity was constructed as a function of the drug concentration. The 0% and 100% efficacy levels were defined as the event probability corresponding to typical AH-PI(4,5)P₂ binding ($\sim 79\%$) and the event probability of no specific interaction (NHS-PEG₂₄-MAL without AH peptide) ($\sim 15\%$), respectively. Following this approach, the 50% efficacy concentration (EC₅₀) of neomycin was determined to be 1.96 μ M. As neomycin binds to PI(4,5)P₂ in a 1:1 stoichiometric ratio³¹, the EC₅₀ value corresponds to the bulk concentration of neomycin at which 50% of the PI(4,5)P₂ receptor sites are occupied. From this result, we can calculate the apparent association constant, K_a , which is equal to the reciprocal of the neomycin concentration that binds 50% of the PI(4,5)P₂ receptor sites. The calculated K_a value of 5.10×10^5 M⁻¹ agrees well with literature values obtained by conventional biochemical methods ($1 - 7 \times 10^5$ M⁻¹)^{60,61}. Of note, Živković *et al.* previously attempted using AFM force spectroscopy to measure the effect of 100 mM neomycin inhibitor on peptide-RNA binding interactions but still detected some specific peptide-RNA binding events despite the neomycin concentrations being well in excess (by a factor of 5×10^4) of the corresponding dissociation constant. From this viewpoint, our findings reveal for the first time that, by utilizing the AH-PI(4,5)P₂ binding interaction as a competitive probe, AFM force spectroscopy measurements can distinguish the extent of neomycin's inhibitory activity at five orders-of-magnitude lower drug concentrations than previously observed in other AFM force spectroscopy systems and provide quantitative readouts of equilibrium binding constants for phosphoinositide-small molecule interactions.

Figure 6. Evaluation of a small-molecule drug candidate that inhibits AH-PI(4,5)P₂ binding. (a) Schematic illustration of neomycin inhibitor binding to PI(4,5)P₂ molecules, thereby decreasing the number of available receptor sites. (b) Effect of neomycin concentration on the most probable adhesion force of the AH-PI(4,5)P₂ binding interaction. Dashed line represents most probable adhesion force for control experiment in the absence of neomycin. (c) Percentage of AH-PI(4,5)P₂ binding inhibition as a function of bulk neomycin concentration. The EC₅₀ value was computed from a 4PL fit.

5.4. Conclusion

In this chapter, I have developed an experimental approach based on atomic force microscopy that is able to measure the adhesion force associated with specific binding interactions between phosphoinositides and peptides. The experimental approach is based on covalently tethering the peptide to a functionalized, gold-coated AFM probe tip. The AFM measurement parameters were optimized with respect to the peptide coating density and contact time. Based on this optimized measurement format, I determined that the

experimental approach is suitable for detecting highly specific binding events while avoiding false positives generated from binding to other receptor molecules with similar molecular shapes. Utilizing these new measurement capabilities, I characterized the interaction between PI(4,5)P₂ molecules and a synthetic peptide corresponding to the NS5A AH BAAPP domain. It was determined that the peptide-PI(4,5)P₂ interaction is primarily related to non-electrostatic interactions and a precise quantitative readout of the binding affinity could be obtained by comparing multivalent interactions *versus* monovalent interactions that are evaluated in other measurement techniques, *e.g.*, QCM-D. As demonstrated by the successful pharmacological disruption of the peptide-PI(4,5)P₂ interaction, this assay demonstrates potential significance for drug screening applications and can also yield further insight into the molecular determinants of BAAPP domain interactions.

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Chapter 6

Investigating How Structural Features within the NS5A AH BAAPP Domain Influence Phosphoinositide Binding Specificity and Avidity

Based on successful development of the AFM-based platform to measure the interaction strength between a synthetic peptide comprising the NS5A AH BAAPP domain and phosphoinositide molecules, I extended this work to investigate how structural features within the BAAPP domain influence PI(4,5)P₂ binding specificity and avidity. The binding specificity was investigated by comparing peptide binding to a panel of PIP₂ receptors, including PI(4,5)P₂, PI(3,5)P₂, and PI(3,4)P₂. The binding avidity was quantitatively evaluated by measuring the most probable adhesion force based on >500 force-distance curves. Following this approach, a shortened, template peptide with enhanced binding avidity was first engineered in order to facilitate high-resolution deciphering peptide-PI(4,5)P₂ interactions. Then, a comprehensive series of mutations were systematically introduced into the BAAPP domain, including both the presence and composition of the pincer along with interior residues of the hydrophobic face and bulky residues surrounding the hydrophobic face. Interestingly, the wild-type BAAPP domain had the highest binding avidity to PI(4,5)P₂ receptors, further reinforcing that the PI(4,5)P₂ receptor is critical for hepatitis C virus genome replication and suggesting that this virally-encoded BAAPP domain has evolved to efficiently bind to the receptor as a means towards high-efficiency viral genome replication.

6.1. Introduction

In the last main chapter, I developed an atomic force microscopy-based platform in order to investigate the interactions between the NS5A AH BAAPP domain and phosphoinositide receptors. This measurement platform enables quantitative profiling of the binding avidity^{1,2}—interpreted by the rupture force required to break the multiple peptide-phosphoinositide interactions present in the system (denoted herein as adhesion force)—between synthetic peptides comprising the BAAPP domain and phosphoinositide receptors³. It was possible to determine how ionic strength influences the binding avidity and a decrease in the adhesion force was identified with increasing ionic strength (50% decrease from 0 to 250 mM NaCl) as a proof-of-concept demonstration of how the measurement platform can probe subtle differences in rupture force. Aside from understanding the role of different environmental factors, such measurement capabilities also open the door to understanding how structural features of the BAAPP domain influence phosphoinositide binding specificity and avidity along with setting the framework to define the key structural features of the BAAPP domain. As first reported by Cho *et al.*, the BAAPP domain is comprised of a pair of basic amino acids that flank the hydrophobic face of the NS5A AH and similar amino acid sequences have been found by bioinformatic approaches in other viral proteins from hepatitis C virus, Japanese encephalitis virus, Dengue virus, Rhinovirus, and Enterovirus among other infectious diseases⁴. At the same time, a clear delineation of the BAAPP domain structural features is outstanding and the next step forward would be to scrutinize the importance of various structural features within the NS5A AH BAAPP domain, which is the only one that has been proven to be linked to biological function.

To date, the most detailed investigations into the binding interactions of the BAAPP domain have involved QCM-D measurements that measured the equilibrium amount of bound peptide to surface-adsorbed polymerized vesicles containing PIP₂ receptors^{5,6}. Approximately three-fold greater mass of the AH peptide was observed bound to PI(4,5)P₂ receptors versus PI(3,4)P₂ receptors, with appreciably less binding to all other PI receptors. The biological significance of these findings were corroborated by immunofluorescence

microscopy experiments that indicated the full-length NS5A protein colocalizes with cellular regions enriched in PI(4,5)P₂ receptors. Preliminary mutation studies have further shown that the two basic amino acid lysines of the HCV NS5A BAAPP domain are *necessary* for the PI(4,5)P₂ interaction, and that this interaction is necessary for *in vitro* replication of the hepatitis C virus genome. While HIV particle assembly has also been shown to depend on the interaction between a viral protein and PI(4,5)P₂ receptors in the plasma membrane⁷, this finding was the first demonstration that PI(4,5)P₂ receptors mediate viral genome replication⁴. Hence, there is a strong motivation for further characterizing the importance of other structural features within the BAAPP domain to mediate binding to PI(4,5)P₂ receptors. Within this context, building a detailed picture of the key structural features within the NS5A AH BAAPP domain will also aid discovery and validation of additional BAAPP domains in other viral and non-viral targets as a first step towards broader biochemical characterization.

As such, there is an outstanding need to decipher what structural features of the HCV NS5A BAAPP domain are *sufficient* for the BAAPP domain-PI(4,5)P₂ interaction. In this chapter, the goal is to systematically mutate different structural features of the NS5A AH BAAPP domain in order to determine the effects on the binding avidity and specificity of the NS5A AH to PI(4,5)P₂ lipid receptors. A shorter template peptide is first designed and validated with optimized PI(4,5)P₂ binding detection in order to facilitate tethering immobilization, facile mutagenesis, and higher resolution evaluation of mutant peptides. Then, mutagenesis of key elements is performed, including lysine deletion, pincer composition, hydrophobic face disruption with charged residues, and removal of bulky hydrophobic residues from the hydrophobic face. Both conservative and non-conservative mutation strategies are employed in order to evaluate the sensitivity of various amino acid changes. Using the atomic force microscopy-based platform, I investigate how these mutations influence the adhesion force between the peptides and PIP₂ receptors, identifying the key factors that modulate binding avidity and selectivity to PI(4,5)P₂ receptors. The findings demonstrate the utility of the force spectroscopy measurements with the atomic force microscope in order to probe fine details linking structural features of the NS5A AH BAAPP domain with its binding interactions. Indeed, as the first identified and validated

BAAPP domain, a detailed systematic investigation of the NS5A AH BAAPP domain is of tremendous benefit to the study of hepatitis virology and aid development of new broad-spectrum antiviral strategies⁸.

6.2. Materials and Methods

6.2.1. Reagents

Ethanol (MerckMillipore, Billerica, MA) and dehydrated toluene (Fisher Scientific, Waltham, MA) were used for AFM tip functionalization. To attach peptide to tip, 11-amino-1-undecanethiol hydrochloride (Sigma-Aldrich, St Louis, Mo) and 2-aminoethanethiol (1 mM, Tokyo Chemical Industry, Tokyo, Japan) were used to make thiol bonding to a gold-coated AFM tip and N-hydroxysuccinimide–PEG24-maleimide ester (NHS–PEG–MAL) (Product No.10314, Quanta Biodesign, Plain City, OH) was used as the long and flexible PEG chain spacer⁹. For the aqueous buffer solution, Phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, CA) with 137 mM NaCl, 2.7 mM KCl salt concentrations and pH 7.2 was used as the buffer solution. All aqueous solutions were prepared using Milli-Q-treated water (18.7 MO cm), (Millipore, Billerica, MA).

6.2.2. Peptide

In order to design mutations within the BAAPP domain, a molecular model of the synthetic peptide was first constructed using the DeepView/Swiss-PdbViewer software program with input from the nuclear magnetic resonance structure of the membrane anchor domain of the HCV NS5A protein 1R7C (Ref. 10) that is available at www.rcsb.org/pdb/explore.do?structureId=1r7c. Based on this structure, individual mutations of specific amino acid residues were made in order to create the wild-type AH peptide structure corresponding to the 1b genotype. As presented below, the surface features of the molecular model are visually represented by their electrostatic potential. Positive and negative electrostatic potentials correspond to the blue and red colors, respectively. The mutations introduced into the BAAPP domain were first evaluated using

this model in order to design a truncated peptide and then identify key amino acids that are likely involved in the PI binding interactions. Based on the mutation designs, high-quality peptides (>95%) were synthesized by Anaspec Corporation (San Jose, CA, USA) and supplied in lyophilized form.

6.2.3. Preparation of AFM tip functionalization.

Commercial rectangular shape and trapezium shape of cross section AFM cantilevers (CSG-01, NT-MDT, Moscow, Russia) with 0.0426 N/m of nominal spring constant were used to measure the binding interaction between peptide and PIs platform. The AFM tips were coated with at first a 2.5 nm layer of titanium and then 35 nm thickness layer of gold. Before and after gold coating, the tip curvature radius is approximately 6 nm and 30 nm, respectively. Before AFM tip functionalization, the tips were washed with pure ethanol solution at three times and cleaned by UV Ozone cleaner (PSD UV Ozone cleaner, Novascan, USA) for 15 min. The cleaned tips were incubated in ethanol solution including 2-aminoethanethiol (10mM) for 6 hour. After incubation, the tips were washed with pure ethanol solution, and dried with gentle stream of nitrogen gas. Then, the tips were immersed in anhydrous toluene solution containing N-hydroxysuccinimide-PEG24-Maleimide ester (1 mmol/L) for 6 hours. For the final step, the immersed tips were rinsed with anhydrous toluene and ethanol, dried them. After that, the rinsed tips were incubated in PBS buffer solution with the target peptide (52 $\mu\text{m/L}$) for overnight. Before force spectroscopy, the peptide attached tips were washed by PBS buffer solution.

6.2.4. Preparation of phosphoinositide platform

In order to determine the binding forces of peptides for phosphoinositides, the commercial nitrocellulose strips with hydrophobic membrane containing spots of equal amounts (100 picomoles) of 15 different phospholipids (P-6001, Echelon Biosciences Inc., UT, USA) were obtained, and preparation was performed as recommended by the company protocol. The lipid blot membranes can be used to evaluate the specific interactions between lipid-protein or lipid-peptide pair^{11,12} through a force spectroscopy measurement

setup with a functionalized tip. All of the lipids have long hydrocarbon chains ($> \text{diC16}$) with highly pure synthetic analogs.

6.2.5. Operation of AFM force spectroscopy

The force spectroscopy measurements were performed using a commercial AFM system equipped with a customized fluidic chamber (NX-Bio, Park Systems, Suwon, South Korea). Force-distance curves were recorded at different positions with 16×16 points, $10 \times 10 \mu\text{m}^2$ of scanning area. All measurements were performed in aqueous PBS buffer solution at room temperature. The speed of the AFM cantilever approaching and retraction were set at 1,200 nm/sec with the maximum loading force 3 nN. Before and after force spectroscopy, the spring constant of AFM cantilevers were calibrated using the thermal method which monitored thermal vibration of the AFM cantilever (approximately 0.0426 N/m). For the analysis of the adhesion force and the rupture distance, Park Systems image analysis program (XEI v1.8.2), own analysis program (Python) and Origin Pro 9.1 (OriginLab Corp., Northampton, USA) were adopted. In order to acquire the reliable data, the adhesion force in the range 0-1,200 nN and rupture events with tip-sample separations in the range 5-60 nm were picked and analyzed.

6.3. Results and Discussion

The wild-type AH peptide (WT AH) is 27 amino acids long and possesses a cysteine linker at the 13th amino acid. As explained in the previous chapter, the cysteine amino acid is the linker residue that is connected to the tethering chain. In order to generate a library of mutant peptides, I first explored designing a modified template peptide that is shorter (less expensive to synthesize) and shifts the cysteine residue farther away from the BAAPP domain residues (more structural flexibility). As presented in **Figure 6.1.**, I first designed two truncated peptides that from which either 6 ($\Delta 1-6$) or 9 ($\Delta 1-9$) amino acids are removed from the N-terminus of WT AH. In these model drawings, I present the nonpolar and polar faces of each designed peptide as well as the polar-nonpolar interface. Based on the AH ($\Delta 1-6$) design, I proceeded to design a modified peptide in which the

cysteine was replaced with a serine residue (a conservative mutation because both residues are polar) and an additional cysteine was placed on the N-terminus; this peptide was called P4 and served as the candidate template for future mutations. The mutation positions remain defined based on the WT AH.

Figure 6.2. Molecular models and sequences of wild-type AH peptide with BAAPP domain as well as truncated sequences and engineered sequence (P4). Note that the two lysine residues (blue) remain configured as a pincer in all designs.

The recognition of a single interaction between the peptide-functionalized AFM tip and PI(4,5)₂ was observed in the force-distance curve (**Figure 6.3**). When the AFM cantilever is positioned far from the substrate, there are no interactions between the AFM tip and sample surface, and hence no cantilever deflection as denoted by the zero baseline. When the AFM cantilever moves toward the sample surface and comes into attractive contact with the substrate, the cantilever is bent upwards and the approach curve displays a linear slope. After contact, the retract curve can be obtained when the z scanner is pulled up. Upon further retraction, the binding force can be observed due to the interaction between AFM tip and substrate until connection is broken. At this point, the cantilever jumps off back to the previous position. During a force-distance cycle, the force spectroscopy measurements yield the binding avidity between molecules on the tip and the sample surface using AFM cantilever deflection signal. As more than one peptide molecule is involved in the binding interaction, I refer to the affinity as avidity. When a specific

binding event occurs between the biomolecular pairs, the force-distance curve is displayed as seen in the red and black curves of Figure 6.2. Besides the adhesion force, other characteristic features, including the rupture distance, can be determined from the specific interaction. The 13 nm of rupture length reflects the length of peptide and PEG₂₄ spacer. As presented in Figure 6.2., binding of both the wild-type AH and P4 peptides can be detected in this measurement configuration.

Figure 6.3. Representative force-distance curves with peptide-functionalized AFM tips.

Single interactions of WT AH (black line), P4 (red line) and no binding event (blue line) with PI(4,5)P₂ are presented and show an approximately 13 nm rupture length.

At first, I studied binding affinities between WT NS5A AH, P4 and shorten sequence of WT AH peptide and PIs molecules, such as PI(3,4)P₂, PI(3,5)P₂ and PI(4,5)P₂ (Figure 6.4). In the presence of a specific binding event, a clear binding force is observed from which the rupture distance and magnitude of the rupture force can be determined. More than 500 force-distance curves were recorded at different positions across the surface and

Figure 6.4. Adhesion force histogram of WT AH along with shortened and engineered sequences.

Each data set consists of more than 500 force-distance curves.

each set was analyzed in order to calculate the most probable adhesion force. The statistical analysis showed the most probable adhesion force in each case at the piconewton-level. In all cases, the WT AH, AH $\Delta(1-6)$, AH $\Delta(1-9)$, and P4 exhibited specific interactions with PI(4,5)P₂ receptors, with most probable adhesion forces of 217, 278, 309, and 329 pN, respectively. By contrast, no specific interactions were detected between any of the peptides and PI(3,4)P₂ or PI(3,5)P₂ receptors. Taken together, the data support that peptide shortening was possible without comprising PI(4,5)P₂ binding avidity or specificity. Furthermore, the P4 peptide, on which the cysteine residue was moved to the N-terminus for more flexible tethering, demonstrated a greater than 50% increase in the most probable adhesion force (112 pN increase) while the background level increased by less than 20 pN. As a result, the resolution between nonspecific and specific binding interactions was improved and I decided to use the P4 peptide for incorporating mutation designs.

I next proceeded to investigate the effect of removing one or both lysine residues from the BAAPP domain. The lysine residues are positively charged and are known to be critical for recognition of negatively charged PI(4,5)P₂ receptors. As presented in Figure 6.5., the removal of one lysine residue significantly decreases the positive charge character of the peptide, and removal of both lysines fully abrogated the positive charge character except for the amine group on the N-terminus.

Figure 6.5. Molecular models and sequences of P4 mutations in which one or both lysine residues are removed.

As expected, all three peptides did not exhibit binding to the PIP₂ receptors independent of the phosphate group positioning (Figure 6.6). Hence, despite the greater adhesion force of the P4 peptide for PI(4,5)P₂ receptors, the peptide maintained identical structural requirements for binding avidity as is known for the BAAPP domain in the WT AH. These findings reinforce that positively charged amino acid residues are necessary in these positions. However, it is unclear if lysine residues are the only amino acid candidate that is able to support PI(4,5)P₂ binding. A previous study showed that WT AH with K20A and K26A mutations reverted back to the wild-type, indicating that lysine residues are preferred in these positions. Indeed, the correlation between this finding and the avidity of PI(4,5)P₂ binding is unknown. Arginine residues are another positively

Figure 6.6. Adhesion force histogram of P4 sequences in which one or both lysine residues are removed.

Each data set consists of more than 500 force-distance curves.

charged amino acid candidate that are common in phosphoinositide-binding domains. In the context of membrane binding, it should also be noted that arginine amino acids maintain their positive charge on account of delocalization through π -bonding of the guanidinium ion, whereas the side chain of lysine residues can become neutral within proteins or upon membrane association^{13,14}.

As presented in Figure 6.7., replacement of one or both lysine residues with arginine residues maintains the pincer configuration comprising two positively charged residues. Of note, the side chain in the arginine residue is appreciably longer than the side chain in the lysine residue. This greater length might provide greater conformational flexibility to bind to different PIP₂ receptors or result in a mismatch between the mutant

Figure 6.7. Molecular models and sequences of P4 mutations in which one or both lysine residues are substituted with arginine residues.

Figure 6.8. Adhesion force histogram of P4 sequences in which one or both lysine residues are substituted with arginine residues.

Each data set consists of more than 500 force-distance curves

BAAPP domains and PI(4,5)P₂ receptors. As seen in Figure 6.8, the binding specificity for PI(4,5)P₂ receptors was maintained and the avidity was graded across the mutant peptides. In comparison to the most probable adhesion force of 329 pN for P4 peptide binding to PI(4,5)P₂ receptors, the adhesion force for P4 K20R decreased to 240 pN. By contrast, the adhesion force for P4 K26R was 194 pN and the double mutant P4 K20R K26R had an adhesion force of 86 pN. No specific binding interaction to PI(3,4)P₂ or PI(3,5)P₂ receptors was detected. Collectively, the data support that KR substitutions are permissible although there is a measureable decrease in the binding avidity. The 20th position in the BAAPP domain appears to be the most permissible to the substitution, although it is clear that the lysine pincer is still preferred. As discussed above, lysine residues can become neutral when contacting lipid membranes. Taken into consideration alongside the foregoing observation that non-electrostatic factors are also important in dictating the PI(4,5)P₂ binding specificity as discussed in the previous chapter, it is likely that the molecular fit of the wild-type BAAPP domain with PI(4,5)P₂ receptors is optimal from an energetic perspective.

Aside from the lysine residues, the BAAPP domain also consists of a hydrophobic face and the central residue is a leucine, which contains a hydrophobic isobutyl side chain. To investigate the importance of this residue on PI(4,5)P₂ binding avidity, I designed a series of mutations in which the amino acid was substituted with amino acids containing negatively charged, positively charged, polar, or smaller hydrophobic side chains. Figure 6.9 shows the molecular surface models of the various mutations. The L23D mutation increased the negative surface charge properties of the peptide, while the L23K mutation did the opposite. By contrast, the L23T and L23A mutations had more moderate effects on the electrostatic potential of the model, essentially preserving the wild-type form.

Figure 6.9. Molecular models and sequences of P4 mutations in which the hydrophobic face is disrupted with amino acids of varying properties.

Figure 6.10 presents the adhesion force results obtained for the P4 peptide mutations of the hydrophobic face. The L23D mutation had the greatest effect with the adhesion force decreasing to PI(4,5)P₂ receptors decreasing to 126 pN. The L23K mutation was similarly disruptive and the adhesion force decreased to around 159 pN. By contrast, the L23T mutation was more moderate and the average adhesion force decreased to around 186 pN. The L23A mutation was the least-disruptive and the average adhesion force was still 251 pN. These findings demonstrate that amino acid substitutions in the hydrophobic face can subtly change the binding avidity although the specificity remains the same even with the addition of a charged amino acid. Again, no specific binding interaction to PI(3,4)P₂ or PI(3,5)P₂ receptors was detected. Hence, additional features other than the pincer residues can also strongly influence the binding avidity based on the adhesion force measurements.

Figure 6.10. Adhesion force histogram of P4 sequences in which one or both lysine residues are substituted with arginine residues.

Each data set consists of more than 500 force-distance curves.

Along this line, structural analysis of the NS5A AH BAAPP domain indicates that bulk hydrophobic amino acids flank both sides of the hydrophobic face. There is a phenylalanine residue at the 19th position and a tryptophan residue at the 22nd position. Such residues are known to be important for membrane association of amino acid sequences. When either or both residues are substituted with less sterically hindered alanine residues, the basic structural configuration remains the same but the molecular density of the BAAPP domain decreases, as presented in Figure 6.11.

Figure 6.11. Molecular models and sequences of P4 mutations in which bulky residues at the edge of the hydrophobic face with smaller residues.

Interestingly, the AFM measurements indicate that all three mutant peptides displayed significantly reduced binding avidity to PI(4,5)P₂ receptors, with adhesion forces in the range of 111 to 130 pN (Figure 6.12). The replacement of the tryptophan residue with an alanine residue was particularly compromising, which is consistent with the notion that the BAAPP domain's binds to membrane-associated PI(4,5)P₂ receptors. At the same time, these mutations also caused a loss in binding specificity as specific interactions with PI(3,4)P₂ receptors were also detected, with average adhesion forces around 59 to 77 pN. Hence, the structural features of the hydrophobic face, including the presence of bulky residues and the size of the hydrophobic side chains within the face's interior appear to be important for conferring PI(4,5)P₂ receptors binding selectivity and avidity. Hence, the bulky residues around the hydrophobic face are critical determinants of PI(4,5)P₂ receptors binding selectivity and avidity, which is consistent with my observations in the preceding chapter that non-electrostatic factors are also important in the BAAPP- PI(4,5)P₂ interaction.

Figure 6.12. Adhesion force histogram of P4 sequences in which bulky residues at the edge of the hydrophobic face with smaller residues.

Each data set consists of more than 500 force-distance curves.

6.4. Conclusions

In this chapter, I have systematically investigated how different structural features within the NS5A AH BAAPP domain influence PI(4,5)P₂ binding specificity and avidity. The summary results are presented in Table 6.1. Both features were determined by atomic force microscopy-based force spectroscopy measurements. The binding specificity was investigated by comparing peptide binding to a panel of PIP₂ receptors, including PI(4,5)P₂, PI(3,5)P₂, and PI(3,4)P₂. The binding avidity was quantitatively evaluated by measuring the most probable adhesion force based on >500 force-distance curves.

Table 6.1. Summary of peptide-PIs binding forces

		Peptide Sequence	PI(3,4)P ₂ (pN)	PI(3,5)P ₂ (pN)	PI(4,5)P ₂ (pN)
Original Sequence	WT AH	SGSWLRDVVDWICTVLTDFKTWLQSKL	N/A	N/A	216.77
	P4	CDVVDWISTVLTDFTWLQSKL	N/A	N/A	328.93
	AH Δ1-6	DVVDWICTVLTDFKTWLQSKL	N/A	N/A	278.34
	AH Δ1-9	DWICTVLTDFKTWLQSKL	N/A	N/A	309.39
Lysine Deletion Mutation	P4 – K20A	CDVVDWISTVLTDFA T WLQSKL	N/A	N/A	N/A
	P4 – K26A	CDVVDWISTVLTDFTWLQS A L	N/A	N/A	N/A
	P4 – K20AK26A	CDVVDWISTVLTDFA T WLQS A L	N/A	N/A	N/A
Pincer Composition	P4 – K20R	CDVVDWISTVLTDFF T WLQSKL	N/A	N/A	240.02
	P4 – K26R	CDVVDWISTVLTDFTWLQS R L	N/A	N/A	194.16
	P4 – K20R26R	CDVVDWISTVLTDFF T WLQS R L	N/A	N/A	85.82
Disrupt Hydrophobic Face	P4 – L23D	CDVVDWISTVLTDFTW D QSKL	N/A	N/A	125.84
	P4 – L23K	CDVVDWISTVLTDFTW K QSKL	N/A	N/A	159.26
	P4 – L23T	CDVVDWISTVLTDFTW T QSKL	N/A	N/A	185.84
	P4 – L23A	CDVVDWISTVLTDFTW A QSKL	N/A	N/A	251.13
Remove Bulky Residues around Hydrophobic Face	P4 – F19A	CDVVDWISTVLTD A KTWLQSKL	77.30	N/A	129.51
	P4 – W22A	CDVVDWISTVLTDFTK A LQSKL	58.86	N/A	116.4
	P4 – F19AW22A	CDVVDWISTVLTD A KT A LQSKL	64.91	N/A	111.16

Specific structural features that were investigated include removal of the pincer residues, changing the pincer composition, disrupting the central residue of the hydrophobic face, and removing bulky residues from the edges of the hydrophobic face. Based on this approach, it was discovered that nearly all mutations preserve PI(4,5)P₂ binding specificity except for removing either or both of the bulky hydrophobic residues. Subtle changes in the PI(4,5)P₂ binding avidity were revealed based on the different mutation series. It was possible to substitute the lysine residues with arginine residues in the pincer but the adhesion force decreased. The effect of substituting one lysine residue with an arginine residue moderately reduced the adhesion force, whereas substituting both residues had a greater effect on decreasing the adhesion force. On the other hand, replacing the central leucine residue in the hydrophobic face had variable effects depending on the amino acid substitution in the following order: hydrophobic > polar > cationic > anionic (from

strongest to weakest adhesion force; range is 126 to 251 pN). Hence, the hydrophobic face can tolerate some degree of mutation and still preserve PI(4,5)P₂ binding specificity with reasonable avidity. On the other hand, the bulky residues on the edge of the hydrophobic face are necessary for both binding specificity *and* binding avidity. Loss of either the phenylalanine or tryptophan residue resulted in a major loss of avidity as well as weak binding to PI(3,4)P₂ receptors as well. Collectively, the findings in this chapter demonstrate the importance of the pincer composition and especially bulky residues around the hydrophobic face in modulating PI(4,5)P₂ binding specificity and avidity. At the same time, numerous strategies are demonstrated to modulate PI(4,5)P₂ binding avidity while maintaining specificity and it will be interesting to explore such mutations in the context of HCV viral genome replication efficiency as well as towards developing more sophisticated bioinformatics approaches to identify similar amino acid sequences in other viral and non-viral targets.

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Chapter 7

Conclusion and Future Outlook

In this Conclusion and future outlook chapter, the conclusions of the results sections and the findings in this thesis are introduced and summarized. In the first part of this dissertation, we investigated the interaction between solid binding peptides and the inorganic materials. Next part, the binding affinity between antiviral peptides and phosphoinositide molecules was determined. Last part of this dissertation, we proposed the improved AFM platform in order to monitor the binding forces between the NS5A AH BAAPP domain and phosphoinositide receptors. For the future work, biorecognition processes with lipid bilayer platform using force spectroscopy studies are proposed. Phosphoinositol molecules are one of the most important cellular membrane components, but lipid bilayers and their interaction also play critical roles in the biological event. Based on previous studies, the role of a variety of antiviral peptide on the lipid bilayer membrane will be studied as well as the changes of mechanical properties of the membrane.

7.1. Conclusion

In this dissertation, the investigation of biorecognition processes which govern the binding forces between biomolecules has been proposed from the application of force spectroscopy measurement with chemically functionalized AFM tips. The specific goal was to explore how to determine the interaction between two molecular pair using force spectroscopy and their applications. Force spectroscopy, AFM represents one of the most promising methods for monitoring biorecognition systems, allowing to measure binding forces between target biomolecules with nano-scale sensitivity in physiological conditions. Moreover, the force spectroscopy provides information on the binding mechanism with dissociation rate and activation free energy for biomolecular interaction. To achieve the goal of this dissertation, we conducted the force spectroscopy measurement with several biomolecules, such as the engineered peptide, the antiviral peptide and intercellular membrane components. Also, a variety of experimental parameters, such as contact time between molecular pairs, the density of molecules on AFM tip and buffer condition were adopted to optimize the measurement.

In chapter 4, the binding affinity between solid-binding peptides (SBPs) and inorganic materials (gold, silicon oxide, titanium oxide and aluminum oxide) was studied by force spectroscopy with functionalized AFM tips. The role of the material substrate itself in triggering the adsorption and conformational response of a peptide molecule remains less explored and there are few studies that compare the interaction of one peptide with different inorganic substrates. To explore these interactions, we used Quartz crystal microbalance-dissipation (QCM-D) technique and force spectroscopy measurement. In QCM-D, SBPs binding affinity to inorganic solid substrates was evaluated, in force spectroscopy, the binding force of a single SBPs molecule was determined. SBPs affinity to gold was significantly higher than to the other oxide substrates. In addition, on three of the four substrates, peptide attachment induced helix formation which appeared to stabilize the bound peptide layer. In this work, a combination of QCM-D and force spectroscopy allowed us compare the peptide adsorption kinetics and binding forces between SBPs and the different inorganic substrates with corresponding changes in the SBPs secondary

structure. Regarding the results, the substrate-specific conformational change suggests that helix induction is important for the stabilization of bound peptides.

In chapter 5, we focused on establishing an AFM platform to evaluate the interaction between peptides and phosphoinositide molecules, which are a type of glycan that is important for numerous biological processes. In particular, the interactions between a synthetic peptide corresponding to the hepatitis C virus NS5A protein's N-terminal anchor and PI(4,5)P₂ molecules were determined with piconewtons level. The force spectroscopy experimental approach is based on covalently attaching the peptide to a gold-coated AFM tip. The parameters of the force spectroscopy measurement were optimized with respect to the peptide coating density and contact time. Based on the optimized settings, we then investigated the binding affinity of the platform along with the effect of ionic strength, revealing mechanistic insights into the factors governing the specific interaction. After that, we demonstrated that the platform is suitable for screening candidate inhibitors that block the interaction between peptide molecules and PI(4,5)P₂ molecules. The results indicated that the peptide-PI(4,5)P₂ interaction is primarily related to non-electrostatic interactions and a precise quantitative readout of the binding affinity could be obtained by comparing multivalent interactions and monovalent interactions. As demonstrated by the successful pharmacological disruption of the peptide-PI(4,5)P₂ interaction, this platform represented potential significance for drug screening applications and can also yield further insight into the molecular determinants of basic amino acid PI(4,5)P₂ pincer domain interactions.

In chapter 6, the AFM-based platform in order to investigate the interactions between the NS5A AH BAAPP domain and phosphoinositide receptors were reported. The synthetic peptide comprising the NS5A AH BAAPP domain and phosphoinositide molecules including PI(4,5)P₂, PI(3,5)P₂, and PI(3,4)P₂ were adopted to determine the biorecognition system and the binding affinity and avidity were quantitatively evaluated by measuring the most probable adhesion force based on collected force-distance curves. Based on this approach, a shortened, template peptide with enhanced binding avidity, both the presence and composition of the pincer residues, disrupting the central residue of the

hydrophobic face and bulky residues surrounding the hydrophobic face were engineered in order to facilitate high-resolution deciphering peptide-PI(4,5)P₂ interactions. The results indicated the importance of the pincer composition and especially bulky residues around the hydrophobic face in modulating PI(4,5)P₂ binding specificity and avidity. It led us to identify that rational mutation of the BAAPP domain can modulate the avidity of the peptide-PI(4,5)P₂ interaction, and the bulky residues surrounding the hydrophobic face play a particularly important role in modulating both binding specificity and avidity.

7.2. Future Outlook

There is a strong potential to continue study in this direction on the basis of the outcomes presented in this dissertation. In this future outlook section, two specific opportunities are introduced and highlighted the potential of force spectroscopy measurement for supported lipid bilayer platforms and their interaction with a variety of engineered or antiviral peptide.

- i. Investigation of the nanoscale organization of supported lipid bilayer and monitoring the mechanical properties of supported lipid bilayer using atomic force microscopy.
- ii. Force mapping measurement of the supported lipid bilayer with peptides and proteins.

7.2.1. Supported lipid bilayer

Supported lipid bilayers (SLBs) are robust membrane-biomimetic model systems that is compatible with studying the biophysical and biochemical properties of biomembranes^{1,2}. SLB is also utilized for biotechnology applications with nanoscale. Recently, there has been lots of interests in SLBs as model membrane systems, consisted of single or multiple component lipid. Especially, membrane-peptide interactions play a pivotal role in biological event including signaling, membrane fusion and maintaining of the cell architecture²⁻⁵. Generally, SLBs can be formed on glass or silica based substrates

through vesicle fusion method but, in this dissertation, solvent-assisted lipid bilayer (SALB) formation method will be adopted with various chemical composition^{6,7}.

The SALB process is a simple and adaptable approach that relate to lipid deposition in isopropanol, followed by solvent-exchange with buffer solution (**Figure 7.1**). Using this method, supported lipid bilayers can be directly formed on a variety of materials, such as glass, hydrophilic oxide substrate and gold^{8,9}. The SALB formation method enables that it is easy to approach the easy operation and high reproducibility with strong potential. By simplifying the biomimetic system, it is possible to produce the supported lipid bilayer platforms which sub-components of cellular membranes for research that interprets to clinical applications.

Figure 7.1. Solvent assisted lipid bilayer formation.

The first step of SALB is the preparation of lipids dissolved in isopropanol solution. In this step, it is not adsorbed onto the substrate. As water content increases in the lipid-isopropanol mixture gradually, micelle structures start to form. From this point, it can be adsorbed onto the substrate. When the isopropanol solution exchanges to water, the water content reaches to 100%, the form of liposomes are formed in solution and the lipid bilayers are formed on the substrate. Adapted with the permission from Hohner et al.¹⁰

7.2.2. AFM study of lipid bilayer formation

Typically, SLB study is analyzed using surface sensitive measurement techniques such as acoustic or optical biosensors. However, AFM also can be used to investigate the biomechanical properties of SLB and their interactions. In the proposed work, more detailed the analysis of lipid bilayer formation and measurement of the mechanical properties of lipid bilayer using force spectroscopy will be demonstrated.

From previous studies, two main approaches to investigating the SLB systems with AFM have been performed by other researchers^{11,12}. The first approach is that AFM imaging of the surface of SLB, which represented topographical characteristics including roughness, height of bilayer patches and lateral extent of domains. Also the surface topography changing of lipid bilayer due to chemical treatments, such as ethanol¹³, immunodeficiency peptide^{14,15}, cholesterol^{7,16}, model peptide WALP23^{17,18} can be determined (**Figure 7.2**).

Figure 7.2. AFM height images from characterization of dye-excluded domains.

(a) Topography image of SLB formed with 70 mol % DOPC lipid and 30 mol % cholesterol, 50 x 50 μm of scan size, (b) topography image of a representative dye-excluded domain, 10 x 10 μm of scan size, (c) Height image of a lipid bilayer defect influenced by 1 mM MβCD treatment, 5 x 5 μm of scan size. Adapted with the permission from Tabaei et al.⁷

The second approach is the measurement of the mechanical properties on SLB using force spectroscopy¹⁹. In this approach, the AFM tips approach and contact to SLB surface and applied force increasingly until SLB ruptures and the AFM tips contact to hard substrate (**Figure 7.3**). The rupture of SLB surface is revealed by a well-defined jump in the approach curve, which can be determined the penetration of the AFM tip through the lipid bilayer, defined as break-through force. The changes of mechanical properties due to chemical treatment or experimental conditions, such as the effect of pH condition, ionic strength of buffer solution, incubation temperature have been determined using force spectroscopy measurement. However, still there are several ambiguous factors in SLB study using force spectroscopy including defect region of SLB surface, time-lapse study of SLB formation and real time measurement of effector molecule treatment.

Figure 7.3. Representative force-distance curve on a SLB.

In a typical cycle, the AFM tip approaches to the bilayer (red line, approach curve) up to a force limitation. The z-scanner is subsequently retracted (blue line, retract curve) at the same loading rate. During the approach curve process, the AFM tip and SLB have no interaction (a), the lipid bilayer is elastically deformed by AFM tip until it ruptured (b), the AFM tip ruptures the lipid bilayer, which is plastic penetration of the AFM tip.

Therefore, in this proposed work, we will design the customized fluidic chamber for SLB formation using SALB method. The chamber is well-fitted with our AFM system and fluorescence microscopy. It enables the fundamental study of SLB formation and real time investigation of biomechanical changes due to chemical treatment using both AFM and fluorescence microscopy simultaneously. Based on force spectroscopy with SLB data, the experimental parameters can be optimized for SLB studying, it can utilize to obtain the reliable result for biorecognition system together with SLB and peptides or proteins.

7.2.3. Investigation of the interaction between SLB and peptides.

In this dissertation, the interactions between hepatitis C virus (HCV) nonstructural protein 5A (NS5A) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) were evaluated with a variety of experimental parameters (see chapter 5). Together with fundamental information of binding affinity between NS5A protein and PI(4,5)P₂, the interaction between various mutant amphipathic helix peptide from NS5A proteins and PI(3,4)P₂, PI(3,5)P₂ and PI(4,5)P₂ were investigated using optimized experimental parameters (see chapter 6). Likewise, phosphoinositol is one of the most important cellular membrane components, but lipid bilayers and their interaction also play critical roles in the biological event. Our the final goal of this study will be to expand the deep understanding of the various peptides interaction with lipid bilayer membrane and its contribution onto biorecognition system using force spectroscopy measurement.

In order to address the biorecognition process with peptides and lipid bilayer, three main essential points deserving the dependable result for lipid bilayer study have been presented;

- i. Lipid bilayers should be fully covered on the substrate. If the lipid bilayer has a defect or full of holes, the AFM tip can easily contact with the substrate or substrate-lipid edges and become contaminated¹¹. Generally, in this case, the inappropriate force-distance curve will be achieved. It is absolutely imperative

that the lipid bilayer has no patch island and be formed continuously for such force spectroscopy measurement.

- ii. New analysis technique must be applied for force-distance curve with lipid bilayer formation²⁰. In contrast with typical force-distance curve, there is a break-through point in force-distance curve with lipid bilayer. The AFM tip firstly contacts with bilayer, then due to the electrostatic and hydration forces, the bilayer is deformed elastically until the AFM tip penetrates the bilayer formation. During the process, the approach curve shows a sudden jump shape. It is a break-through force which is dependent on the characters of the lipid bilayer including chemical composition.
- iii. The interaction between the desired peptide and lipid bilayer is highly dependent on the chemical composition of lipid, chemical properties of buffer solution and temperature²¹⁻²³. Therefore, the optimized experimental condition is crucial for the measurement.

In this context, the preliminary study for lipid bilayer using AFM was performed (**Figure 7.4**). We firstly confirmed the lipid bilayer formation with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipid (Avanti Polar Lipids, Alabaster, AL) using SALB method and the customized fluidic chamber for AFM machine. Some region showed the defect of bilayer formation (**Figure 7.4(b)**), the bilayer was fully covered on the most of region successfully (**Figure 7.4(a)**). Also the force-distance curve with 4.5 nm of rupture depth and 3.5 nN of break-through force could be observed in lipid bilayer formation area. This result indicates the lipid bilayer can be formed on glass substrate by the fluidic chamber and it is the promising platform for the bilayer study with AFM.

On the basis of the preliminary study, the role of a variety of antiviral peptide on the lipid bilayer membrane can be investigated as well as the changes of mechanical properties of the membrane. Due to the nanoscale AFM technique, the experimental approaches provide the superlative platform to determine the interaction between lipid and peptide controlling the lateral organization of the cellular membrane component. By

applying the customized fluidic chamber, both AFM and fluorescence microscopy can be combined for characterizing the lipid bilayers and their interactions.

Figure 7.4. Preliminary study of the supported lipid bilayer using AFM.

The lipid bilayer is fully covered on the substrate, the line profile shows the flat line. In bilayer region, 3.5 nN of the break-through force can be detected (a), AFM image of defect region of the lipid bilayer, the line profile represents the approximately 5 nm of depth, it is the good proof of the lipid bilayer formation. There is no break-through force in the force-distance curve on non-bilayer region (b).

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6. Kim J, **Kim SO**, Cho NJ. Alternative Configuration Scheme for Signal Amplification with Scanning Ion Conductance Microscopy (SICM). *Review of Scientific Instruments* 2015; 86, 023706
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