Analysis of Activation Induced Cytidine Deaminase using Atomic Force Microscopy

by

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Abstract

Activation induced cytidine deaminase (AID) plays a large part within the pathway of immunoresponse. It does so by inserting mutations, during DNA replication, in B cells (immune system cells). This results in a more diverse set of antibodies in each subsequent generation. However, mutations that cause infinite replication with no cellular apoptosis, can lead to cancer. AID has been implicated in cancers which appear independent of the expected environmental causes, such as smoking or UV exposure.

The method for AID mutations involves binding to single-strand DNA. Using Atomic Force Microscopy (AFM) we obtained both the geometry or topology, as well as the nanomechanical information about this mutation process and AID. In this thesis, I present the first AFM images of AID, showing direct structural information about the protein. Although the measurements are done *in vitro*, physiological conditions have been approximated in order to get an accurate analysis of the protein system. To allow imaging in buffer, suitable substrates were tested and identified which would bind the protein sufficiently in this high ionic strength environment. Optimized plating and scan conditions for AID in both wet and dry conditions are described, which allow high resolution imaging to be performed that is not often seen in biological systems. Through the many scans and procedural changes it was established that although difficult it is possible to gain an image of AID through the use

of AFM. Using the some of the forces with the protein, an image of HOPG anchored AID was able to be obtained

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List of Abbreviations and Mathematical Constants

AFM	Atomic Force Microscopy
AID	Activation Induced Cytidine Deaminase APOBEC3
CSR	Class Switch Recombination
SHM	Somatic Hypermutation
SEM	Scanning Electron Microscopy
osmol	Osmolarity
K	Kelvin
N	Newton
nN	Nanonewton
pN	Piconewton
DLVO	Derjaguin-Landau-Verwey-Overbeck
DMT	Derjaguin-Muller-Toporov
OCT	Optimal cutting temperature
invOLS	Inverse optical lever sensitivity

MPa	Megapascals
GPa	Gigapascals
LC	Liquid chromatography
ESI/MS	Electrospray ionization mass spectrometry
TERS	Tip-enhanced Raman spectroscopy
PDMS	Polydimethylsiloxane
Π	Osmotic pressure
i	Van 't Hoff Factor
k_b	Boltzmann constant (1.3806×10^{-23} JK^{-1})
N_A	Avogadro's number (6.022×10^{23} mol^{-1})
T	Temperature of system
M	Molarity of solution
φ_i	Osmotic coefficient of solution
n_i	Number of ions into which the solute dissociates in solution
F	Force acting upon the AFM tip
k_c	Spring constant of the AFM tip
x	Cantilever deflection
m	Mass of AFM tip
F_0	Amplitude of the tip frequency
Q	Quality factor of AFM tip
ω	Angular frequency of the driving force

ω_0	Angular resonance frequency
$F_{t,s}$	Tip-sample interaction force
A	Hamaker constant
z_c	Tip-sample rest distance
Z	Instataneous tip-sample distance
ρ	Density of the fluid medium
b	Width of the AFM cantilever
Γ	Hydrodynamic function
$\hat{W}$	Fourier transform of the cantilever displacement
R	AFM tip radius
ϵ	Dielectric constant of the medium
ϵ_o	Permittivity of free space (8.854×10^{-12} Å^2 s^4 m^{-3} kg^{-1})
$\frac{1}{K_d}$	Debye length
σ_t	Surface charge on the AFM tip
σ_s	Surface charge on the sample
E'	Effective Young's Modulus
E_s	Young's Modulus of the sample
E_t	Young's Modulus of the tip
E^*	Reduced Young's Modulus
a_o	Intermolecular distance between two objects
EI	Flexural rigidity of AFM tip

p_c	Mass per unit length of AFM tip
$w(x,t)$	Transverse deflection of AFM tip
F_{hydro}	Hydrodynamic dampening force
F_d	Force driving the tip oscillation
L_c	Cantilever length
A_o	Area of applied force
Ig	Immunoglobulin

Chapter 1

Introduction

1.1 Cancer: a biophysical approach

Cancer is one of the leading causes of death in society today. Many current reports and papers explain what can and cannot lead to cancer. Even cutting edge research, is quickly disproved or modified after a few months [1]. Although a simple risk reduction through the use of selected diet or reduced exposure to radiations and chemicals will help, that should not be the only concern. The pathways through which disease are initiated often pre-exist within the human body.

1.1.1 DNA-based approaches to studying cancer

A path of interest with regards to research is DNA conformational change and how it affects DNA interactions. Exploring variations of the tertiary structure [2], new tetrahedral conformations, which take a tetrahedron scaffold of DNA were developed to understand how the newly adjacent strands would interact as well as develop within the supporting media itself. Understanding the way that these altered structures behave within the sample is not the same as understanding how they would function

within the body. Nonetheless, knowledge of changing interactions and their effect on the protein system around the DNA could lead to some alternative forms of treatments and cures for disease.

Mutations within the DNA itself can impact intermolecular interactions, particularly π stacking [3]. Studies of DNA mutations have yielded great insight into the nature of the bonds within the DNA itself and between DNA and proteins. This has also allowed a greater exploration into the process in which the proteins fold as well as what can lead to their denaturation and possible mutations.

Muramatsu et al. have identified where mutations arise within the genetic code [4] and observed a link with genes coding for immunoglobulin. During this study there were specific sites noted within the genome where errors were frequent. Studies began to focus upon these points of change and explore why they came about and what made these certain points ideal for mutations. These mutations not only occurred within similar locations, but they usually formed in similar base pairs, suggesting a pathway for errors or a genetic flaw to be passed down through generations.

1.1.2 AFM-based approaches to study DNA-protein interactions

Atomic force microscopy (AFM) allows for direct visualization and measurements of DNA and protein molecules, including the measurement of intermolecular binding forces.

To more directly probe protein interactions with DNA, protein conformations have been studied using an estrogen binding sensor [5]. This was possible by using an estrogen binding template and binding estrogen to the AFM probe. This allowed the study of the changes caused by protein binding with the DNA strand and the alterations to the conformations and binding patterns of the DNA. For this study, a

microchip was fitted with an estrogen binding agent and an AFM was used to probe the surface. It was compared to the original tip without the probe binding to the estrogen receptor. This allowed the group to determine how much binding affects the conformation geometry and binding properties. This study provided great insight into how much impact the enzymatic proteins can have upon the DNA and within the body itself. To better understand the pathway, the group attempted to develop an AFM substrate and conditions to allow the proper characterization of DNA and its conformational changes. These systems are designed to best mimic the natural conditions of the body. Although it cannot be perfectly copied many studies have enabled the system to be as close to physiological conditions as possible [6–11].

The other important factor for a substrate in this process is the binding affinity of the sample and the substrate. This simply refers to whether the substrate and sample are compatible with one another and have favourable intermolecular interactions. The most common substrates used for biological work are either a charged mica sample or a highly ordered pyrolytic graphite (HOPG) substrate [6, 12]. The consensus from biological plating revealed that a charged surface allowed the protein or DNA charged segments to bind more effectively to the surface for easier location and analysis. Mica is unique: depending on its preparations it can be tuned to be either positively or negatively charged. Positively charged mica is the most commonly used within DNA and protein studies. Uncharged Mica has very low binding affinity towards the proteins and does not give good surface-analyte interactions for analysis of most biological samples. The existing studies also state that the conformation and energy of the proteins and DNA are altered after the sample is mounted on the substrate.

Researchers have been working towards alternate methods of plating these biological samples such as nanolithography. Nanolithography is a very popular method,

which removes a layer of substrate, then the DNA is plated using a thiol linkage to keep the attachment in place to allow for analysis. [13]. Although the data obtained was very interesting, the large alterations of the ssDNA (Single Strand DNA) did not allow an accurate DNA formation and instead altered the chain lengths and thicknesses. This did not allow the proper analysis of an unaltered sample but it did open a new methodology for protein analysis based upon different bond sites as well as helix width alteration. This information can give us great insight into how to alter proteins in order to deactivate them or to slightly change their overall effectiveness within the system.

The last interesting approach that will be discussed here is analysis within a controlled atmosphere. One group prepared a cryogenic AFM, dropping the temperature to almost freezing to allow analysis of enzyme activity and potential for protein folding within a snapshot in time [14]. The issue with this arises when looking at whether the material has become more brittle due to the low temperature or if the conformation is altered due to these environmental changes. The colder temperature may decrease bonding activity as well as conformation affinity and protein binding lengths. Another point to look at is whether the mechanical properties taken from this AFM could be considered valid when it is close to freezing and the sample is most likely more rigid and firm compared to materials under normal conditions. However the benefits from this method would be the slowing down of the enzymatic activity for potential images of the bound protein/DNA system and obtaining a snapshot of what this looks like, although peak enzymatic activity will occur at physiological conditions. This method also provides greater preservation and consumes fewer resources (the sample can be reused).

1.1.3 Solution effects on protein and DNA structure

Proteins and DNA contain charges, and therefore the ionic strength [15] of a solution will impact their conformations [16]. In the extreme case of proteins in ionic liquids, the specific ion interactions can be identified and linked to protein structure [10]. Researchers have set up methods to observe the protein-DNA system of interest under conditions closer to physiological conditions [16]. This includes taking into account the ionic strength effects of the buffers. For example, one group has explored the charge gradient between the sample and the AFM probe in an ionic solution of the buffer. What is interesting about this study is the amount of charge build-up from electrostatic interactions among the proteins. This is mediated by the buffer solution ions, as seen by measuring the charge difference between the tip and the sample. By slowly lowering the tip from the point of zero interaction to when the tip begins showing some interaction, we can see how the electrostatics are effected by the ionic solution [17].

1.2 Atomic Force Microscopy

Atomic force microscopy is a method of nanoscale analysis to determine surface [18] and nanomechanical properties [19], with the possibility of measuring kinetics through imaging [20, 21]. One of the main benefits of this method is the ability to do many things within one simple scan. Another major benefit is when it comes to biological samples. Analysis in both wet and dry conditions make this an extremely valuable technique [22]. For this project we worked towards getting topographical and eventually some physical properties such as stiffness and binding force.

Atomic Force Microscopy (AFM) is a type of scanning probe method which passes a tip over the surface to gather information about the sample [23]. Upon analyzing

the sample the AFM uses a laser reflection off the tip to determine the features of the surface of a sample. The reflected laser is received by a photorecepter and, using standardized tip calibration [24], an image of the surface topography can be generated. In some cases this method is able to get to the atomic level of resolution giving as refined results as one could need for biological systems [18].

At a basic level what is observed is how the tip of the AFM is being received by the sample. This can be repulsion, attraction, or even adhesion to the samples surface [25]. Knowing the radius of the tip (generally 10-50 nm) [26], we can roughly measure the size of the sample in the x, y and z orientations as well as gather some of the physical properties through the complex analytic software and data collection apparatus. When everything is calibrated and working well, and the sample allows good contact between the tip and the material, it is possible to obtain a high resolution low noise image as will be demonstrated in the conclusions portion of this thesis.

In Figure 1.1 we present a generalized setup of the AFM. The following steps are taken each time the AFM is used: first a tip is loaded in the cantilever holder. This has to be aligned properly or no data will be obtained or even worse the tip will be damaged. Second, the laser is aligned to the tip, then the detector, and various calibrations must occur depending on the scan type to follow. It is important to receive the maximum possible signal when aligning so that the photodiode can obtain the most accurate data to determine exactly how the tip has moved across the sample. A main component of this setup is the piezoelectric actuator (PZT) in figure 1.1 which is responsible for the movement of the sample while scanning.

A raster scan is a method that uses two passes to collect the data [27]. This is to ensure good contact is obtained and the image returned is accurate. This type of scan is somewhat like a person pacing back and forth, however instead of starting and returning to the same point, the person takes a step to the side after returning

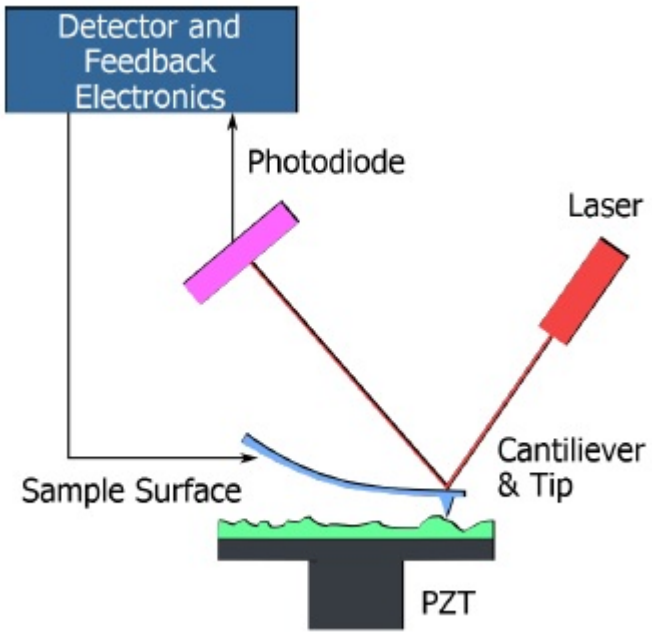

Figure 1.1: Block diagram of typical Atomic Force Microscopy setup with Piezoelectric Actuator labeled(PZT), Wikipedia Commons By Askewmind at English Wikipedia https://commons.wikimedia.org/wiki/File:Atomic_force_microscope_block_diagram.png

to their origin and repeats. This is done equal to the number of lines entered into the software depending on the resolution required. In geometrical terms, this scan does two passes in the (x) direction, collects data, then shifts in the (y) direction and does another two passes in the (x) direction.

The next thing to look at is the feedback loop. To do this one must understand the motion of the tip. Its best likeness is to a boat skipping the tops of the waves, it cannot only move up and down but it can move on various planes and the AFM tip can torque in many of these planes due to various forces like van der Waals [28]. As previously mentioned the laser being reflected is how data is collected and the detector collects information on all of the deformations using the photodiode array shown in Figure 1.2.

As the laser moves from quadrant to quadrant the intensity is measured and the

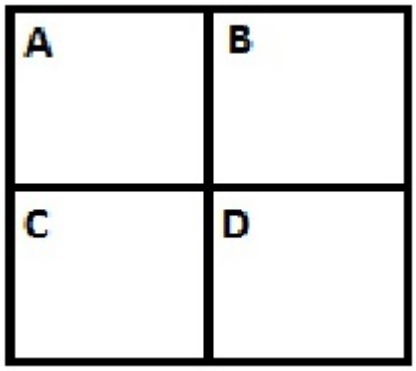

Figure 1.2: Display of the areas that the photodiode will Detect laser movement

vertical deflection value is determined using the following equation:

$$\text{Deflection} = (\text{A} + \text{B}) - (\text{C} + \text{D}) \tag{1.1}$$

The values of A, B, C, and D represent the intensity of the laser in each quadrant. To set a baseline for this value before an experiment is started, the user adjusts the set-point by moving the photodiode and sets it to the experiment's required deflection value. This will cause the internal adjustment factor to accommodate for this changed deflection. This is more important when using contact mode AFM then tapping mode, and will be discussed in detail within the following sections.

1.2.1 Contact Mode AFM

Contact mode AFM was the first and the most basic method Binnig et. al. published whilst looking to establish a new scanning probe method [29]. Initially when the tip was being lowered toward the sample, van der Waals and electrostatic forces act as seen in Figure 1.3. Initially these forces attract the tip to the sample, however as the distance between the tip and the sample closes another force takes precedent and becomes the dominating force. This repulsion is called Born repulsion due to the

electronic orbital overlap of the tip and the sample [25].

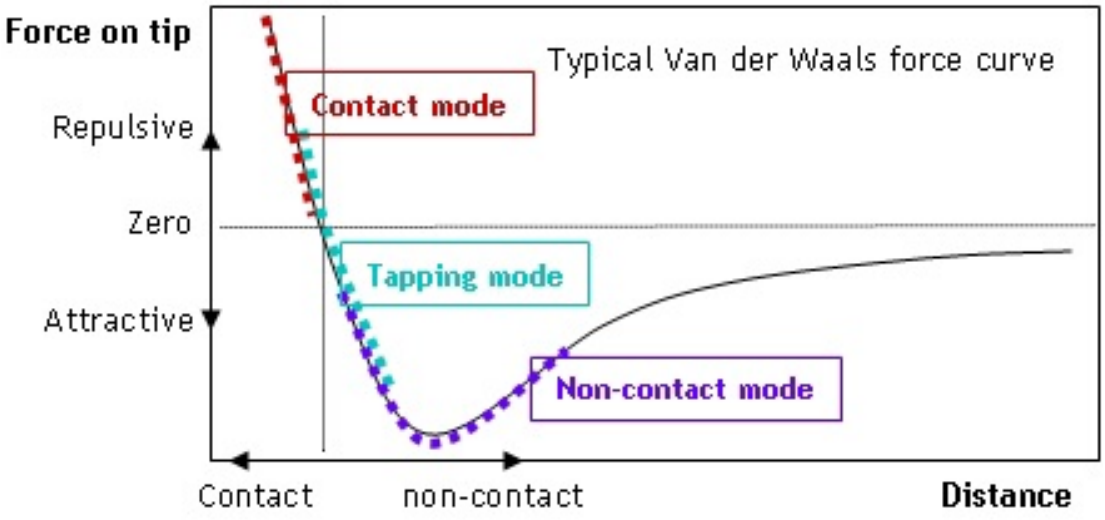

Figure 1.3: Force-distance curve map showing the force differences on the AFM tip in each type of imaging.Contact:always contact, Tapping: Periodic contact, Non contact: No contact relying on long range VdW. Public Domain (Wikimedia Commons, created by user KristinMolhave

Once in contact with one another, the repulsion can be on a magnitude of 10^{-9} to 10^{-10} N [30]. Hooke's Law can be used to explain the forces acting upon the tip through the following:

$$F = -k_c x \tag{1.2}$$

where k_c is the spring constant of the AFM tip (typically on the order of magnitude of 0.1 N/m) F is the force acting on the tip, and x is the cantilever deflection value.

As previously mentioned, upon calibration the set point can be adjusted before the scan and if the desired results or contact with the sample was not obtained, it can be adjusted between, or during a scan. Luckily the software is complex enough and well written to accommodate for these changes following equation 1.2 [29]. During a scan the tip will pick up alterations in the sample surface and this laser deflection is detected by the photodiode. This will be analyzed by the feedback loop and it will accommodate the alteration in set point. The AFM will correct for this by applying

voltage to the z-axis piezoelement causing the apparatus to move to correct the height and then back to the desired set point. These voltages applied to the PZT are analyzed and converted to the standard AFM topography image.

1.2.2 Tapping Mode Atomic Force Microscopy

Tapping mode AFM, as the name suggests differs from the previous method in that contact with the sample is not continuous. With this method, the tip has a resonance frequency that is used to make periodic contact with the sample surface as the probe moves up and down over time. Its resonating frequency is obtained through a thermal calibration and a tuning function of the AFM upon the tip. This fine tuning results in the tip making periodic contact, or tapping upon the surface of the sample [23].

This method is useful for many reasons, the first of which being the reduction of tip twisting due to the reduced sample contact, and this reduces the lateral deflection noise [23]. Another reason this method is ideal is that constant tapping does not drag the tip across the surface, which can damage both the tip and sample. Finally it helps to reduce the unknown features detected on the sample which are more frequently seen in contact mode [31]. The feedback loop for tapping mode is slightly different from that of contact mode. Instead of monitoring the tip deflection, the amplitude of the tip oscillation is detected and the height variations are mapped with comparison to the initial amplitude set point. At this point the analysis method is the same as contact mode, to which the voltage is applied to the z-axis piezoelement to restore the set amplitude.

García and Paulo [32] note that tip motion during tapping mode displays a trend toward dominant attractive forces observed in Figure 1.3. The following equation takes into account the tip excitation force, the effects of hydrodynamic damping of

the tip in the medium, and the elastic response of the tip:

$$m\frac{dz^2}{d^2t} = -k_c z - \frac{m\omega_\theta dz}{Qdt} + F_{t,s} + F_0 cos\omega t \tag{1.3}$$

where ω is the angular frequency of the driving force, ω_0 is the angular resonance frequency, F_0 is the tip driving force, $F_{t,s}$ is the net-attractive force between the sample and the tip, Q is the quality factor of the tip, and k_c is the spring constant of the tip. The tip-sample interaction ($F_{t,s}$) in equation 1.3 can be expressed mathematically, with the simplifying assumptions of the AFM tip as a sphere and the sample as flat surface [32], and can be calculated from

$$F_{t,s}(z_c, z) = -\frac{AR}{6(z_c + z)^2} \tag{1.4}$$

where A is the Hamaker constant, R is the tip radius, z_c is tip-sample rest distance, and z is the instantaneous tip-sample distance, which is illustrated in Figure 1.4.

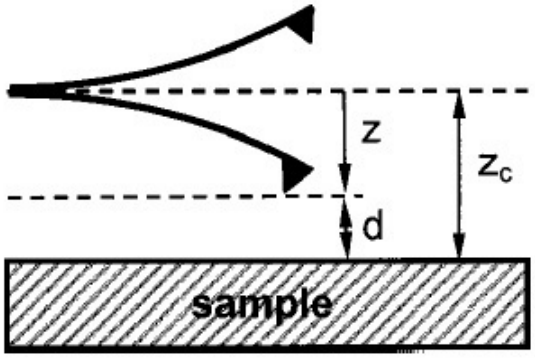

Figure 1.4: AFM tip operating in tapping mode, showing the tip-sample distance at rest (z_c) and while oscillating (z). Adapted with permission from Ref. 32.

Tapping mode imaging for this particular experiment is advantageous due to its specific traits mentioned above. Reduction in lateral movement and protection of the sample and the tip are particularly important for this thesis as biological samples of

this kind are generally quite soft and delicate. The minimal contact while tapping allows for high resolution data to be collected with far less risk to the sample [32].

1.2.3 In Fluid Atomic Force Microscopy

Much like in air, Atomic Force Microscopy has also been designed to obtain high quality data in fluid. [33]. This method has great advantages when being applied to medical or biological samples [34], such as individual cells [35], and in this case proteins [36], and being able to view them in a more natural environment. Within fluid both tapping and contact modes are available. This is mainly due to the shear force that contact mode places on the sample, making it more likely to tear or rip.

When switching from analysis in air to analysis in fluid there are other factors that must be considered to set up the AFM. Firstly is considering the difference the fluid will have on the tip. As the tip is in constant oscillation, its hydrodynamics will greatly differ from that value in air. It should be intuitive that if the frequency of the tip will lower in fluid with a low Reynolds number, it will also lower the quality factor (Q in equation 1.3) [22]. Although no definitive conclusions regarding tips in a fluid medium have been made, two considerations were brought about by Baró and Reifenberger in their book *Atomic Force Microscopy in Liquid: Biological Applications* [22].

The first of these is the hydrodynamics of an oscillating tip in a liquid medium. In this environment, oscillation of the AFM tip within a fluid causes a resistance much like you feel when in a pool. This is known as the hydrodynamic load per unit length, and can be calculated as [24]:

$$\hat{F}_{hydro}(x,\omega) = \frac{\pi}{4}\rho\omega^2 b^2 \Gamma(\omega)\hat{W}(x,\omega) \tag{1.5}$$

where ω is the driving frequency of the AFM tip, ρ is the density of the fluid medium, b is the cantilever width, $\hat{W}$ is the Fourier transform of the cantilever displacement at position x, Γ is the hydrodynamic function.

The second consideration is how the tip interacts with the sample upon approach, which can be modelled using Derjaguin–Landau–Verwey–Overbeck (DLVO) theory [37].

$$F_{ts}(d) = F_{DLVO}(d)\frac{4\pi R}{\epsilon\epsilon_0 K_d}\sigma_t\sigma_s e^{K_d t} - \frac{AR}{6z^2} \tag{1.6}$$

where R is the AFM tip radius, z is the instantaneous tip-sample separation distance, A is the Hamaker constant, $\frac{1}{K_d}$ is the Debye length, ϵ is the dielectric constant of the medium (e.g., water), ϵ_o is the permittivity of free space ($8.854 \times 10^{-12} A^2 s^4 m^{-3} kg^{-1}$), σ_t is the surface charge of the AFM tip, and σ_s is the surface charge of the sample.

In contrast, tip-sample interaction during contact can be modelled using Derjaguin-Muller-Toporov (DMT) theory [37]

$$F_{ts}(d) = F_{DMT}(d) = \frac{4E'\sqrt{R}}{3}(a_O - z)^{\frac{3}{2}} + F_{DLVO}(a_O) \tag{1.7}$$

where E' is the effective Young's Modulus of the sample, a_O is the intermolecular distance, and FDLVO(a_O) is the solution to equation 1.6 at a tip-sample separation equal to a_O.

Basak and Raman [37] attempted to create a specific model which accounted for the system being a viscous, polar liquid, and the sample being elastic in nature. It was expressed mathematically as:

$$EI\frac{d^4\omega(x,t)}{dx^4} + P_c\frac{d^2\omega(x,t)}{dx^2} = F_{hydro} + F_d + F_{ts}(Z_c - \omega(L_c,t)) \tag{1.8}$$

where EI is the flexural rigidity of the tip, which is a measure of the resistance of

the tip while undergoing bending, P_c is the mass per unit length of the tip, $\omega(x,t)$ is the transverse deflection of the tip, F_{hydro} is the hydrodynamic damping force seen in equation 1.5, F_d is the force driving the tip oscillation, Z_c is the tip-sample rest distance seen in Figure 1.4, F_{ts} is the tip-sample force as seen in equations 1.6 and 1.7, and L is the cantilever length.

1.3 Ionic Solution effects on the Protein

Ionic strength can be defined as the measure of the ion concentration in solution. This can be represented by the following formula:

$$I = \mu = \frac{1}{2}\Sigma c_i {z_i}^2 \tag{1.9}$$

Where c_i is the ionic concentration in mol/L and z_i is the number of charges on the ion [15]. Ionic solutions can have interesting effects on the system, such as adding a charge to mica. Ions can even add a partial charge to HOPG and hence alter its affinity to certain system [38]. As in a study here HOPG was able to adsorb some ionic liquid systems to the surface using an electrochemical approach. Other studies reveal that HOPG can be altered by passing current through the system, which also induces charge that enable wetability and better ionic liquid absorption to the surface [39].

Here it should be noted that the systems can be better explained using the Derjaguin–Landau–Verwey–Overbeck (DLVO) theory [40], which shows that the protein and the substrate surface interactions have an increased effect as the radius decreases. This, as seen in equation 1.6 relies upon a z^2 function which is more easily explained by comparing the system to that of Coulombs law, which determines the energy between two charged systems. In this case we can see the system between the charged buffer solution and the protein interacting with the substrate system. In the Coulombs law

equation the force between charged particles scales as r^2, the same functional for as in the DLVO equation this r^2 is closely related to the z^2 in the DLVO equation, making it a distance relation equation and thus understanding the forces within the system easier to understand.

AFM in fluid allows for scientifically notable interactions to be analyzed [22]. Even the fluid used to keep the sample hydrated can be altered and observed. Using ionic solutions can have a great effect upon the protein response and would alter the system's nanomechanical properties. This is due to the electronic properties of protein being affected by the ionic system. An ionic liquid is one where the salts are not well coordinated resulting in them being in a liquid state below standard temperatures, basically a salt that is in a natural liquid state. This system type will change the binding and folding of the protein in order to alter its geometrical state. This negative or positive charged system may also have an altered pH which will again alter conformation and folding of the system.

Zhao et al [10] discussed the various forces interacting upon a protein in ionic liquid systems. When diluted in aqueous solutions there is partial solubility allowing for individual ion interaction with the protein. This interaction depends on where these ions fall within the Hofmeister series, which lists ions and whether they salt in or salt out on proteins. Kosmotropic anions and chaotropic cations will stabilize the protein, while chaotropic anions and kosmotropic cations will destabilize it [10]. Figure 1.5 presents this schematically

1.4 Activation Induced Cytidine Deaminase

Activation induced cytidine deaminase (AID) is a newly discovered protein group related to those within the APOBEC family [41]. APOBEC abbreviated from Apolopopro-

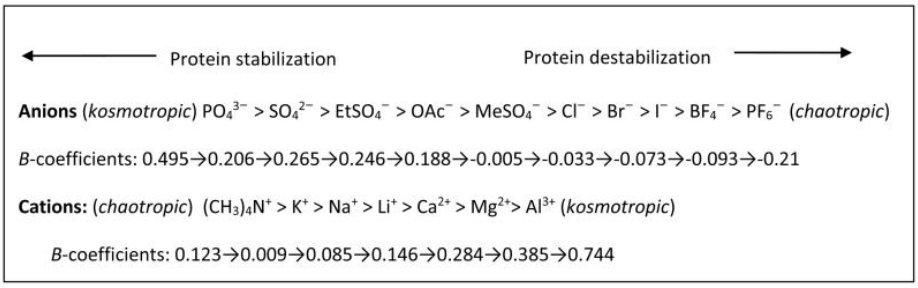

Figure 1.5: Hofmeister series as an order of the ion effect on protein stability [10]

tein B mRNA editing enzyme, catalytic polypeptide-like. As the name would suggest it is a protein that deaminates cytidine within DNA, but what makes it different from other proteins?

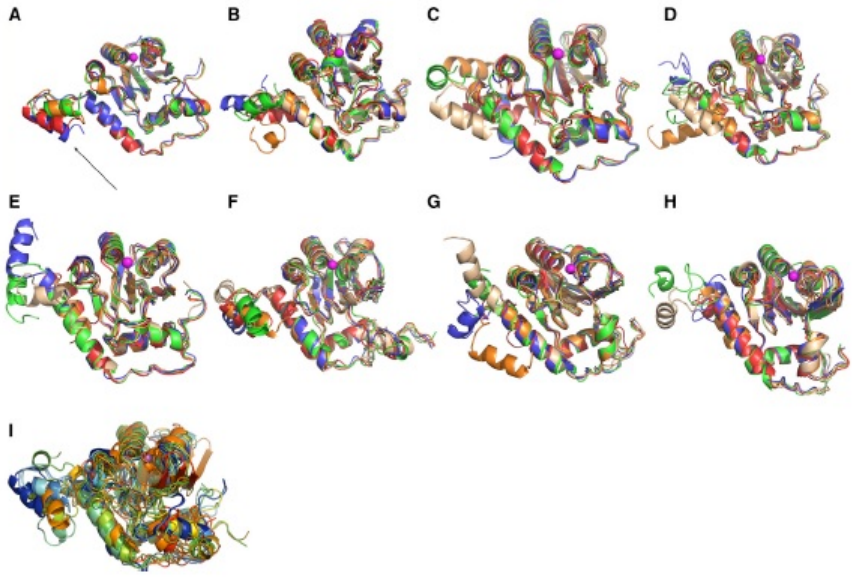

Figure 1.6: Computer models of AID, based of different calculation models prepared by Justin King of the Larijani Lab, used with permission from Ref [42]

These proteins are found within vertebrates and have developed many different variants over time. Computer models prepared by the Larijani lab can be observed in Figure 1.6; these models will be discussed later in this thesis. The farthest ancestor

to which AID may have diverged from was the lamprey, which uses a variable lymphocyte receptor method of immunoresponse [43]. Although only relatively recently discovered and placed within this protein group (1999) it has become a hot spot of immunoglobulin and cancer pathway research.

Originally most immunoglobulin activity was associated only with the class switch recombination (CSR) or somatic hypermutations (SHM) within the V region [44, 45]. The V region of the gene, is a portion of the gene designated to be altered during the various mutation steps to generate various Immunoglobulin forms to allow protection of the body. Alternation of these V regions by SHM, takes place during B-cell early maturation within the lymphoctyes. The 24000 kDa monomer, is the most common orientation of AID, but can also be found sometimes as a dimer and quite rarely as a tetramer. Their respective volumes are 15 nm^3, 47 nm^3 and 87 nm^3. Studies began to isolate the origins of the mutations within the IgM to IgA using B-cell mouse lymphoma line CH12F3-2 [46]. This addition along with various stimulant ligands were able to produce a 60% efficiency transfer rate of CSR.

Once formed, the Ig leave the bone marrow to be further altered for their specific purpose using SHM and CSR. Initially these were thought to be the only actors, but it was later discovered that AID was a necessary component [44]. This was determined using studies on mice with and without the AID gene. Within this study it was observed that immunoglobulins which needed mutations to be effective were not formed within those mice missing the AID gene.

A study Muramatsu published in 1999, established that AID was predominately produced and activated within the lymph nodes, which would be intuitive. What was more interesting is that there was a mild expression also present in the spleen. The Northern blot method of analysis showed no AID activity from other organs [4].

Upon this cataloging of the AID origins, more questions arose as to whether SHM,

CSR and AID were all independent functions of each other. A study was prepared to which mice, using Mendel's laws for genetic combinations, produced controlled as well as AID deficient mice. These were raised having no visible defects from the test and control groups. Analysis of the immune response showed no deficiencies within the various immuno response components, which include the T and B cells, macrophages and graulocytes, these were used as surface markers by Muramatsu et al(2000) [4].

Overall with the study of these mice litters there was no difference until the Ig levels were analyzed. Results displayed a much lower Ig level and nonexistence of certain Ig CSR derivatives. This study showed the importance of the AID enzyme within the lymphnodes to produce varying antibodies for the immuno response to be effective. So far 11 AID/APOBEC variants have been catalogued within the literature with many other research groups looking to expand this list [41].

As we can see from Table 1.1, the function of APOBEC 1 is well known while there is still much to be learned about APOBEC 2 and 4. What makes these proteins interesting is their functionality within the body. This group of proteins are specifically designed to introduce mutations within the DNA/RNA replication, more specifically deaminating a cytidine to a uradine [43]. Although this seems counter productive to the specific body needs, it does this to help build different immunoreceptors. The process through which this is accomplished uses two main methods: somatic hypermutation, and class switch recombination. The purpose of these mutations is to create varying antibodies to help protect the body.

This replication/mutation process, however, is monitored by various genetic "erasers", which correct dangerous mistakes prepared within the mutations [47,48]. If some mistakes are not corrected, various genetic disorders can arise or even an infinite reproduction of mutated cells with no process for apoptosis. Mutation correction systems are highly efficient, however there are still errors. This can give us a partial explaina-

Table 1.1: Table of AID/APOBEC variants

Name	Genomic location	Exons	Deaminase domains	Expression	Cellular localization	Editing activity	Target
AID	12p13	5	1	Activated B cells, testis	Mainly cytoplasmic, acts in the nucleus	DNA	Immunoglobulin gene
APOBEC1	12p13.1	5	1	Small intestine	Cytoplasmic/nuclear, acts in the nucleus	RNA, DNA	Apolipoprotein B mRNA
APOBEC2	6p21	3	1	Skeletal muscle, heart	Cytoplasmic/nuclear	Unknown	Unknown
APOBEC3A	22q13.1	5	1	Keratinocytes, blood	Cytoplasmic/nuclear	DNA	Adeno-associated virus, retrotransposon
APOBEC3B	22q13.1	8	2	Intestine, uterus, mammary gland, keratinocytes, other	Predominantly nuclear	DNA	Retroviruses, retrotransposons, HB
APOBEC3C	22q13.1	4	1	Many tissues	Cytoplasmic/nuclear	DNA	Retroviruses, retrotransposons, HB
APOBEC3DE	22q13.1	7	2	Thyroid, spleen, blood	Unknown	DNA	Retroviruses
APOBEC3F	22q13.1	8	2	Many tissues	Cytoplasmic	DNA	Retroviruses, retrotransposons, HB
APOBEC3G	22q13.1	8	2	Many tissues, T cells	Cytoplasmic	DNA	Retroviruses, retrotransposons, HB
APOBEC3H	22q13.1	5	1	Blood, thymus, thyroid, placenta	Unknown	DNA	Retroviruses
LOC196469*	12q23	1	2	Pseudogene	-	-	-
APOBEC4	1q25.3	2	1	Testis	Unknown	Unknown	Unknown

tion as to why cancer is so abundant to this day. This also motivates the extensive study of the AID gene since its first discovery.

1.4.1 AID pathway

Another question that arises when talking about AID concerns the numerous base pairs that are present: how is it decided where the protein will attack?

To address this question we look into a mechanistic study by Casellas et al [49] trying to gain understand of HIV and its many mysteries. It was observed that AID attacked at a common spot each time it was observed in loops of RNA.

We can observe from Figure 1.7 that as replication of strands are occurring the AID likes to attach to the loop that is coming off due to the separation of the strand.

This area is favorable due to the increased accessibility for the AID. AID then begins removing an amine group from the cytidines in this area.

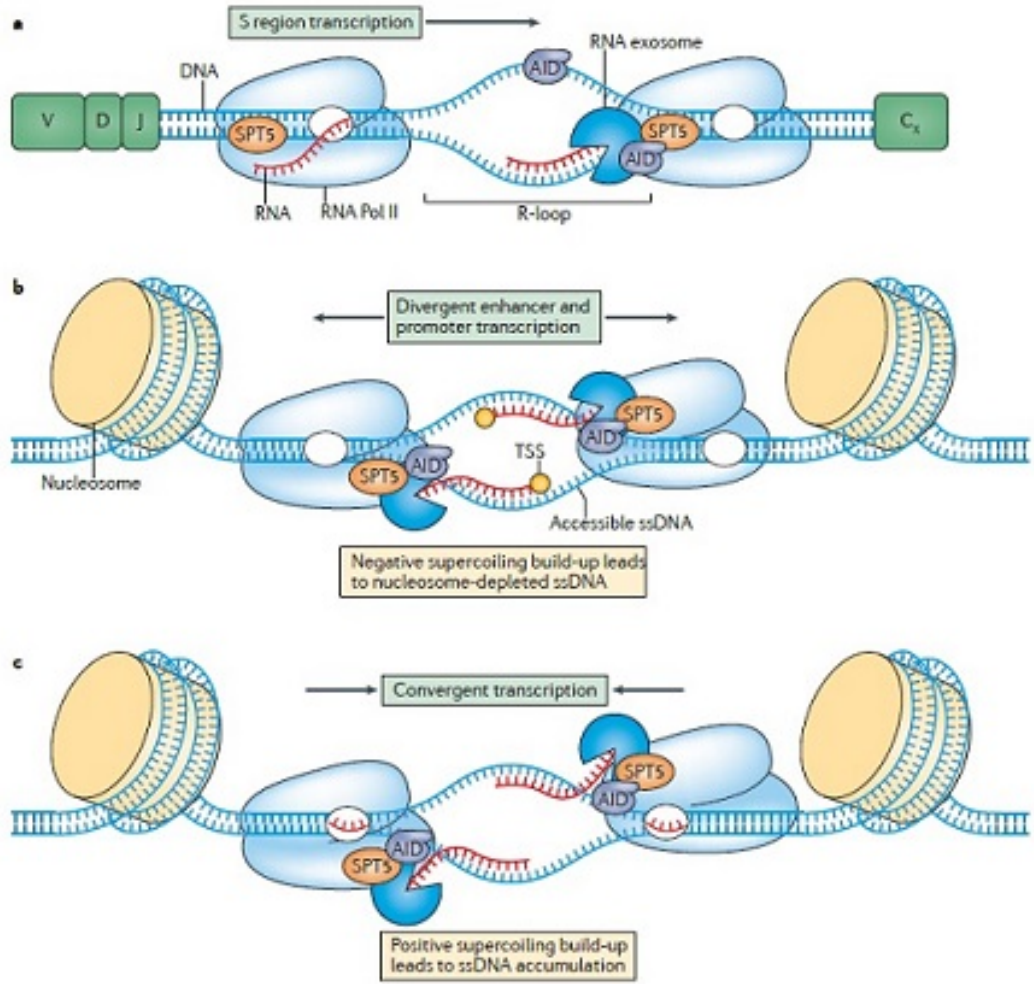

Figure 1.7: a | Transcription of switch (S) regions creates R-loops that cause stalling of RNA polymerase II (Pol II), early transcription termination and RNA exosome recruitment. b | Divergent transcription at enhancers and promoters creates nucleosome-free DNA and single-stranded DNA (ssDNA) structures, in which cognate RNAs may associate and become substrates for exosome degradation. These activities are proposed to increase accessibility to activation-induced cytidine deaminase (AID). c | Convergent transcription mediated by RNA Pol II may lead to the formation of RNA exosome substrates by the build-up of positive DNA supercoiling. CX, constant region of either heavy or light chain; D, diversity; I, intron; J, joining; TSS, transcription start site; V, variable.Used with permission from Ref. [49]

What we can see from Figure 1.8 is the regions where the processes that pair with AID are favored to act. From the schematic it is easy to see the various segments within the immunoglobulin gene. The ones of interest are those labeled V, D and J standing for variable, diversity and joining segments respectively. Each of these portions are favored by a different system which relies on the APOBEC brethren AID to activate and perform its roll within the body.

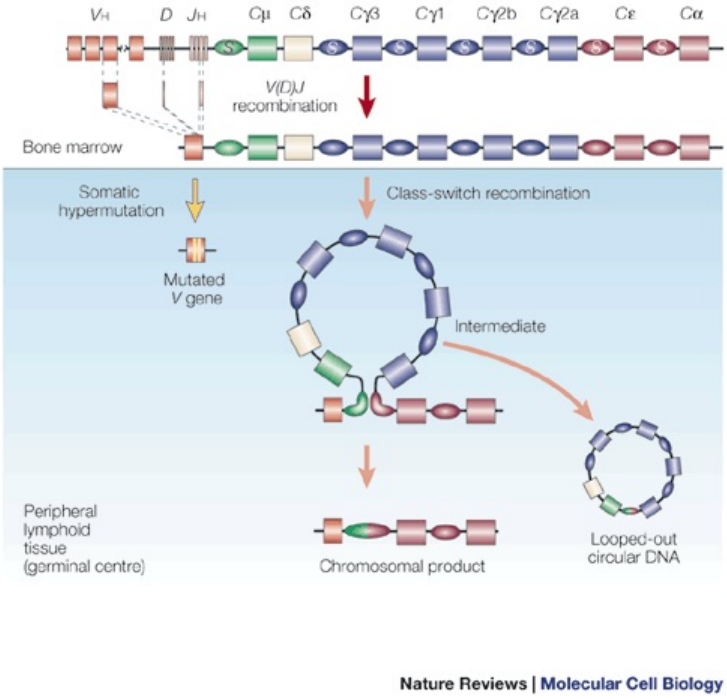

Figure 1.8: The mouse immunoglobulin heavy-chain locus is shown. Rectangles and ovals represent exons and switch (S) regions, respectively. V(D)J recombination occurs in the bone marrow, whereas somatic hypermutation and class-switch recombination occur in the peripheral lymphoid tissues. V(D)J recombination selects one segment for each of the V, D and J segments from respective pools of gene fragments and combines them into a single variable (V)-region exon. Somatic hypermutation introduces frequent mutations in the rearranged V exon, providing a pool of B cells with diverged antigen specificity from which high-affinity immunoglobulin producers are selected. Class-switch recombination brings the downstream constant (C) region exon in the proximity of the V exon by deletion between Smu and another S region upstream of the target C region. Deleted DNA is released as a circular DNA. Used with permissions Ref [45]

1.5 Somatic hypermutation and Class Switch recombination

The process of somatic hypermutation (SHM) is to alter the immunoglobulin chain within the replication process [45]. This is an important process with most vertebral creatures. Using this process which has been evolving with the species we have been able to fend off a variety of diseases and outside attacks to the body. This process attacks the variable region of the immunoglobulin entity within the body and changes various portion to developed more specific antigens to the pathogens that are attacking the main body. As can be observed in Figure 1.8 it is easy to spot the location where the SHM occurs within the transcription process, and only affects the single cells of the system. From the figure it is observed that somatic hypermutation favors attack at the joining segments.

An interesting feature of SHM is the discovery of its two step process for alteration of the antibodies [50]. The first phase of this process is the alteration of a C:G base pair to that of a U:G Base pair. This is a process that requires the catalysis of the AID enzyme which become active due to the B lymphocytes, without AID presence as mentioned above there would be no SHM process within the body, the exception to the rule being hyper ImG syndrome [50].

The second phase of this process was discovered as a change in the A:T base pair. This occurs only under a certain set of variables. This second stage was hypothesized when an alternate outcome than what was expected came from the removal of a mismatch recognition protein (MSH2) from the mice in the study. Having AID protein and a suitable lesion remaining from the first step the A:T base pairs will be altered along with the C:G [51].

Class switch recombination also alters the immunoglobulin chain, however, through

a different mechanism than that of SHM. The key difference between the two methods of ImG recombination is the area of preferential attack. As we noted in the previous section we saw that SHM preferred to switch in the joining regions of the immunoglobulin, the CSR prefers to attack within the constant C region present after the variable region of the strand, as can be observed in Figure 1.8.

CSR generally requires two rounds of cell division. Another requirement for this function is double strand breaks. This is a necessity in order to complete the mechanism of CSR.

An interesting point to note is that CSR and SHM have no relation or need for the other but both are entirely reliant upon the AID to allow their functionality to occur [45, 52].

1.6 Summary

From this chapter it is easy to see how important AID can be. Performing a leading role within our immune system, it is a necessary component to our survival. However this excellent enzyme is also a double sided sword, allowing mutations within the replication process which can, within the replication loop, alter the stop codon. With this alteration it can lead to an infinitely replicating cell, also known as cancer. Without apoptosis this can go on indefinitely without some outside interference.

This protein will be analyzed through the use of atomic force microscopy creating a substrate suitable for the analysis of the desired protein, and developing the proper setup and parameters. Our work lays the groundwork for future related studies and may contribute to an eventual understanding of the mechanism of AID. This will help future scholars find the pathway by which both our immune system and cancer works. This information can help establish a potential understanding for the improvement of

the current cancer treatments and give insight into the disease frequency itself.

Chapter 2

Methods

2.1 General Considerations

The choice of materials and equipment used throughout the thesis work was essential to the experiment. Ultrapure water (0.055 μScm^{-1}) was used to ensure no outside contaminants altered the results of the samples and keep the integrity of the samples intact as some contaminants may denature the protein. Calcium chloride 99.9% purity was used to make the charged mica for analysis, HOPG and mica (Thermo Scientific) were used as substrates and to enable the ideal setup for experimentation. Calcium chloride was selected due to its availability and it had been used similarly by other group members. The samples of AID were obtained as wild-type human AID in phosphate buffer, provided by the Larijani lab(School of Medicine, Memorial University) [53]. An AFM (ND-MDT) was used for some initial experiments but as the MFP-3D AFM(Asylum Research) proved to be more effective for this work, this became the primary AFM used throughout this thesis work. Two AFM were tested to see which would provide the highest resolution images, however due to some technical issues present in the ND-MDT at the time is was not used. The tips used for

this experiment were Au/Cr tips (micromash) and both tapping (NSC35) and contact mode tips (NSC37) were used. Finally the software use to process the data collected was Igor pro 6.37 (WaveMetrics, Inc).

2.2 Substrate preparation

Optimization of the substrate is a critical portion of complete biological analysis using AFM. Many things need to be considered before moving forward like will the substrate alter the properties of the sample. For example will it have grooves where the sample will become altered or misshaped? Another possibility is that the substrate is soft and will absorb some of the impact from the analysis method in the case AFM.

In using the AFM, we will be examining different charged and uncharged substrates to see which will hold the sample and alter it the least in order to give us the best analysis results. The main substrates to be included will be mica, charged mica and HOPG. These substrates were attached to the glass slide using standard double sided tape. Both the mica and the HOPG had the top layer removed using this tape. This was performed by gently pressing upon the surface and then removing the top layer before measurements. This was done to ensure no anomalies were present such as dirt, dust or other debris upon the substrate which would interfere with both the sample and the data collection.

The freshly exposed layer was imaged using AFM to ensure there were very few cracks or large steps within the surface to cause the analysis to be flawed due to imperfections. Small (micron-sized) defects in the substrate itself can cause the tip to drop a significant height and may end up damaging the tip so that it will be unable to be used in future experiments. Even nanoscale features on the substrate could be problematic, as imaging of proteins and DNA requires the detection of very small

differences in sample height.

Once the layers had been removed, mica was further altered to ensure a positively charged surface. To complete this the cleaved sample was soaked in 0.1 M Ca^{2+} solution. After a brief time within the solution the surface was rinsed to remove any excess salt and ions, after which the surface was again inspected for errors or abnormalities. Once the surface had been approved, it could be then used for sample plating.

2.2.1 AFM Parameters

When beginning to start scans it was important to establish the parameters that would provide the highest quality images. Unfortunately these parameters may vary greatly between samples under wet or dry conditions. Wet conditions are closer to the physiological state, but the sample being semi mobile can lead to challenges in data aquisition. Dry conditions are generally the easiest to measure, however the sample can become quite brittle at times.

The first thing that should be considered is the scan type. In this case, a series of experiments were performed to establish whether contact or tapping, are best for the protein system. Contact mode it is a technique where the tip barely leaves the surface, making it an excellent technique for accurate height images, however if the tips have the resolution, they are too stiff to be pulled across a soft samples surface. Even under dry conditions the ideal tip will frequently destroy the sample and thus not yield any data.

A stiff tip being dragged through the sample can visibly damage the sample, but the real damage is the collection of much of this debris from the sample and substrate upon the tip. It is not easy to remove debris or sample material from the tip, and so the tip cannot be used for any more scans. For these samples the ideal tip will need to

be small, and able to analyze within the nm range and be soft enough not to harm or destroy the sample, whilst still being stiff enough to provide the necessary resolution. With this often impossible compromise for a contact mode tip, tapping mode will be the ideal scan type for the protein system using the available tips. Tapping mode tips also need to be stiff to provide high-resolution data, and in fact they are often stiffer than tapping mode tips. The risk of scraping material from the sample onto the tip is much lower, however, and there is usually no indication of sample damage after tapping mode imaging. The stiffness for the contact mode tips NSC37 was 0.3 N/m and for tapping mode the NSC35 with force constant of 16 N/m.

The second consideration in developing the AFM method was the scan speed. Normally experimentalists would think that going slower would offer a better result as the probe can have time to respond to each small feature, however in this case it was determined that a faster scan allowed for a higher resolution sample image. It was noticed that with the slower scans the protein had more time to change position and therefore make a rougher image, subject to greater thermal drift. The ideal scan rate for these materials was between 1.5 Hz and 1.78 Hz, with a 5-15 nm change in tip height as determined by optimizing scan speed to minimize noise. This was the case in both dry and wet conditions of the sample. Another advantage with faster scan times is that when analyzing a very sensitive system it will give a quick analysis before the sample can either break down or change itself away from the state in which it is meant to be observed.

The third thing that must be determined is the resolution of the scan lines and number of points per line measured within the imaging area of the experiment. I kept both the Scan Points and Scan Lines to the same number. If the Width:Height parameter is not set to 1, then the Points and Lines will scale appropriately. For example if the Width:Height is 2:1, then a resolution of 256 would give you 256

Points per 128 Lines.

Scan Points and Scan Lines are always the actual Dispite appearances an AFM image is not just a picture that appears on the screen after a brief time. Each pixel of the image correspondes to an individual datum or set of data, like a pointillist artist would have to do for a painting. Anything below 256 points per line gives inadequate results. The maximum is not really firm, but for any resolution more than 4096, the burden of scan time (and its associated time-dependent errors such as thermal drift or sample degradation) will outweigh benefits of collecting more data.

The number of points has to be a binary number for efficiency of internal algorithms of the AFM software. Since this number can't be changed in the middle of a scan any new values will be applied to the start of the next scan. It should be mentioned that with each increase of the number of lines, the time needed for the experiment also increases. Normally at a resolution of 256 with a scan rate of 1 Hz an experiment will take about 8 minutes. At 1024 it can take roughly 16 minutes. For the experiments conducted on the AID system the best results were obtained with points and lines being set greater than 1024. It is here that we can more clearly see the edges of the sample once we reach a scan size of roughly 200 nm.

Once located, scans can be repeated as necessary which is often the case. For this work, a good strategy is gradually increasing the points and lines to a maximum of 2048. The reasoning behind this is as mentioned in the previous point the thermal drift and movement of the sample, the longer the scan the more time it will allow the sample to shift and introduce error. The best method to keep these ideal scan parameters, times were kept within 10-20 minutes. This required adjusting both points and lines as well as scan speed to keep these parameters in check.

The next feature to be adjusted as scans continued was the integral gain. This gain is in direct relation to the speed in which the feedback system will react to the

error signal in the scan. Normally for the size of scan that is worked with from 500 nm to 100 nm we use a range of 1-5 units for the integral gain. This drastically reduces the noise present within each line. The height difference between the sample and the substrate is minimal, and within a small scan area one is unlikely to encounter a very high feature. Therefore, we do not need a very tight feedback loop. Higher integral gain means that the feedback system responds to any small fluctuations, which can cause the vibration of the tip to be amplified.

The two main methods of scanning coined methodical and shotgun were checked to see which found information more effectively. The methodical approach we start at 2 μm and slowly zoom, using the "nice zoom zoom" feature which just like the crop button moves the scan area in and enhances the selected area keeping the aspect ratio the same. This was performed on areas that look like they have data until reaching a scan size 150-500 nm, then back to 2 μm and begin again.This is a function on the AFM that does a perfectly square zoom in on a location and shifts the tip to allow the location selected to be centered and in focus. This method took large amounts of time and in wet conditions rehydration would sometimes shift the area making it an impractical method of analysis for this system.

As for the scan itself it generally underwent a shotgun treatment, by this I mean if the sample was dry first we scanned a large area (2 μm) to see where we can notice either residual salt or some abnormality to the HOPG in most cases, then we began to perform a uniform decrease in scan area, basically zooming in on a point. From here wet and dry scans are generally the same, wet samples had a large droplet to confirm the area to scan. Then we continued scanning until something of interest came within the scan performed.

Generally we would zoom again to about 500 nm which would allow observation of the protein and be able to adjust for the size that is expected. Then we would

slowly shift around the area of the scan performing 500 nm scans until an artifact of interest appears on the screen, either something blurry that looks the right size or an area of fibrous looking materials. The sample is once again nice zoom zoomed and an area of 150-200 nm is scanned. At this scan size it is difficult to get an ideal image as at this size even small ambient noises can be observed within the scan. Therefore, scans performed either after class hours or at night were the most pristine. Even a shifting of a chair was picked up by the AFM so when a small scan was taking place a perfect still was necessary, seen in Figure 2.1.

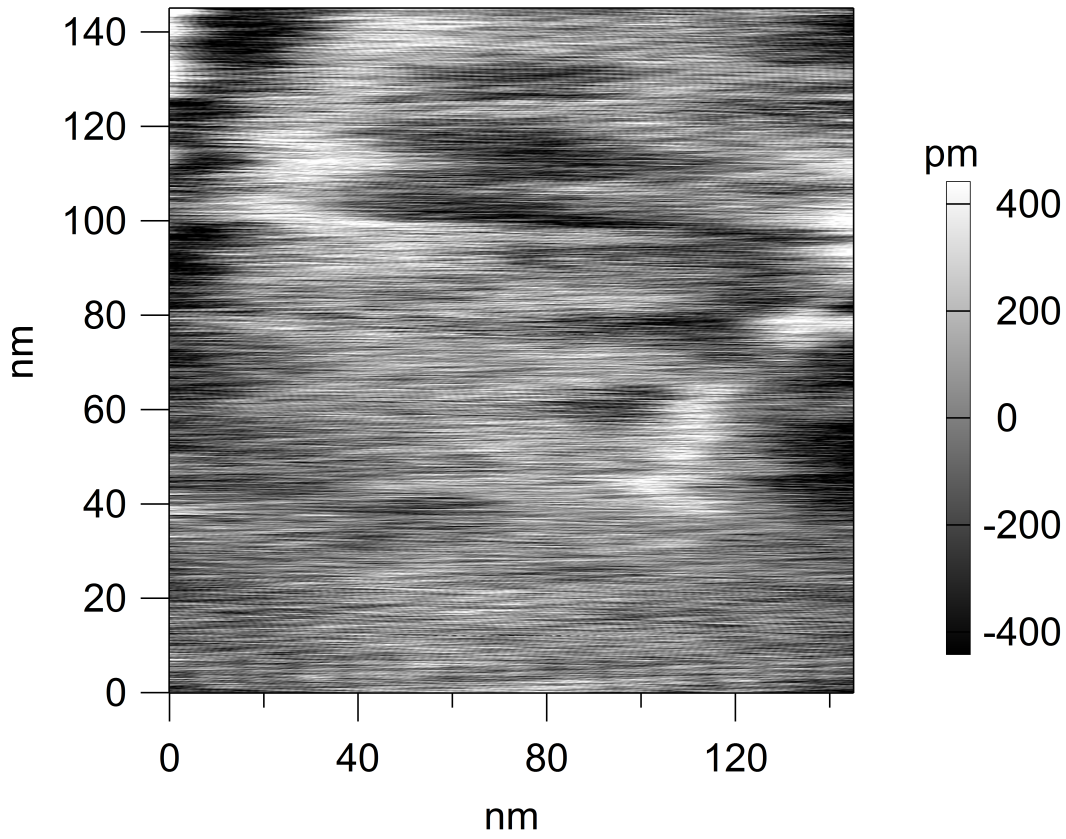

Figure 2.1: AFM images start the scan height at zero and the tip moves up and down on the sample. This scan with obvious loss of contact due to low scan speed, as well as performed during high traffic times at Memorial Regular class hours (deflection image).Note: This was lessened in wet conditions

2.2.2 Confirmation of tip size

In order to ensure the tip size is adequate for the sample we used a grading chipset and with this we were able to calculate the exact tip size based upon the angles present upon the size of the chip valleys as observed in the following figure 2.2.

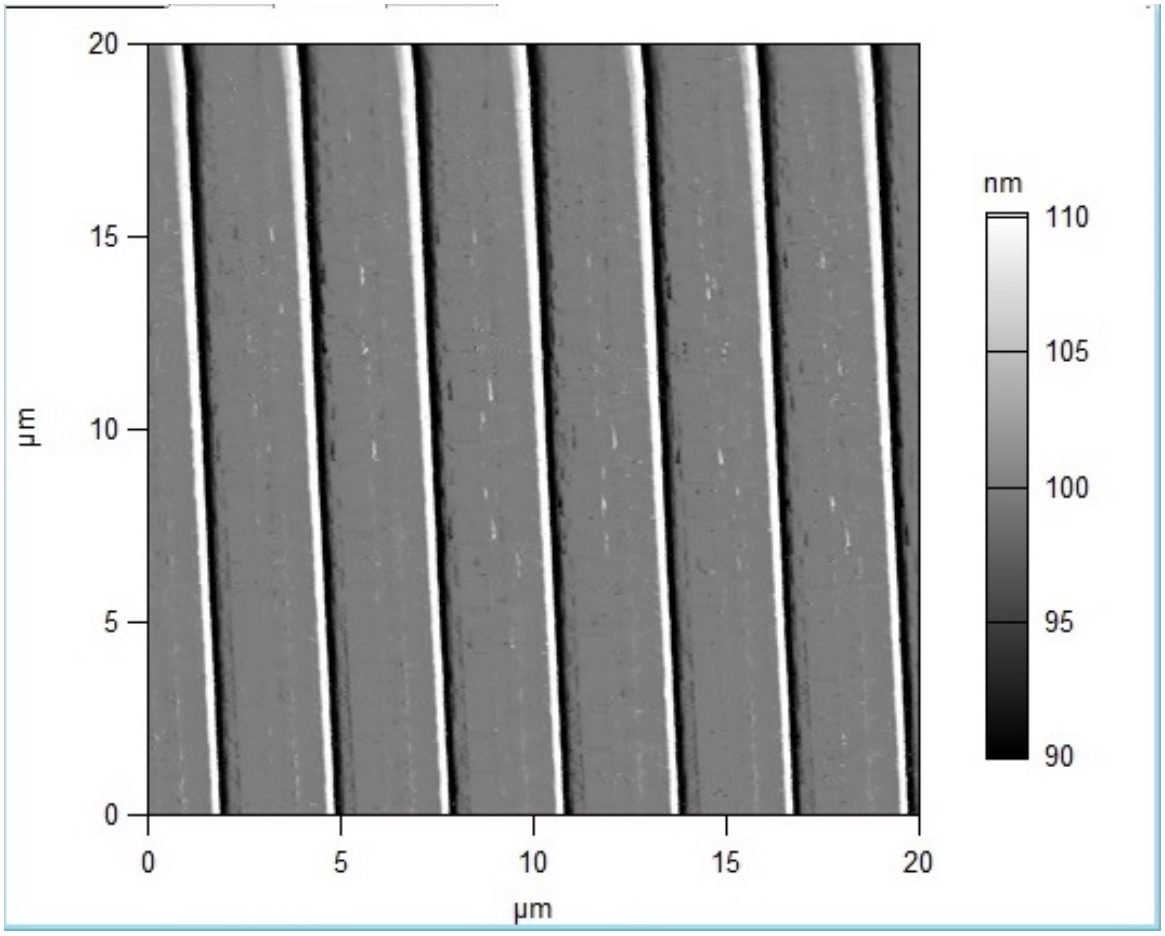

Figure 2.2: Chipset to confirm tip size based on calibrated troughs

Confirming tip size is an important aspect to determine the actual spatial structure of the substrate and compound in question. What is interesting to note is that the tip width would cause the calibrated ridges to alter. For example if the tip was larger it would cause the ridges to also appear wider to an extent, and if it was smaller it would be closer to the actually calibrated value. Another note would be that the

angle of the sides will also affect this measurement giving a more or less accurate result based on the slope of the sides of the tip used 2.3.

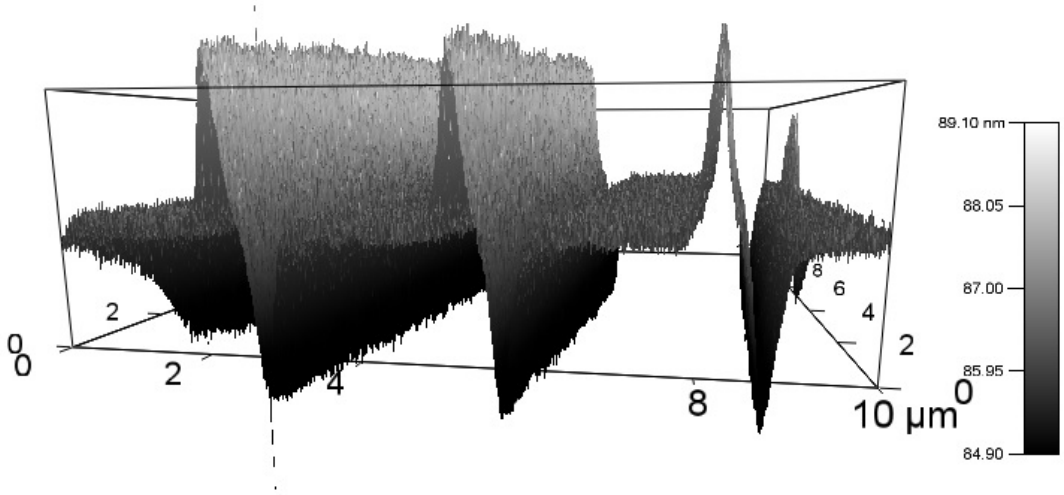

Figure 2.3: Chipset to confirm tip size based on calibrated troughs, side view to confirm accuracy of peak heights

Chapter 3

Surface Optimization and Selections of Substrates

3.1 Sample preparation in wet and dry conditions

Once the substrates had been checked and approved the sample was selected out of a variety of AID samples provided by the Larijani lab (Memorial University), the sample was removed from ice and placed on the benchtop to allow it to gently melt. This protein is highly sensitive so applying a heat source to assist with the melting would shock the protein and cause it to degrade within the system. We were advised by the Larijani lab to allow them to slowly thaw for this reason. Once the sample had finished thawing, it was gently shaken by hand to ensure uniformity within the sample as the layers can separate upon thawing, and applied to the substrate surface.

Sample sizes were varied (3-15 μL of the analyte) and it was determined that the lowest volume did not bind well to the substrate as re-hydration was necessary within several minutes. The highest volume was too great and an unnecessary waste of the analyte as well as resources, and it also ended up causing salt within the analyte to

be more persistent and in some cases led to contamination of some of the tips used within the experiment.

A range from 6-12 μL of the analyte was used, most consistently was 10 μL due to the HOPG's hold upon the protein enabled an ideal area of analysis. The volume of sample was placed on the substrate and left to adhere for 20 minutes, constant re-hydration of the analyte was performed using ultrapure water, it should be noted that this was only necessary on lowest volume of sample.This time period is necessary to ensure the charged portions of the protein/DNA mixture had time to meet with the charged surface and adhere before being wiped away using a kim wipe to reduce salt formation.

Although much of this process is similar in wet conditions the sample prep requires more attention. Upon the day of analysis the fresh substrate was prepared and the sample was removed from the freezer to allow the gentle thawing. Once thawed and shaken, the required sample volume was placed upon the substrate to allow adhesion to begin. As water will be added constantly through the experiment to not allow the sample to become dry and keep conditions as close to physiological as possible it was still necessary for the 20 minute wait time for the adhesion to the surface, experiments began at 20 minutes after plating upon the substrate. Once the scans began constant observation of the tip is necessary to ensure that the droplet does not recede back to the tips and the sample remains wet. This is necessary to ensure the protein are as close to physiological conditions as possible during analysis.

3.2 Salt effects on AFM data collection of biological samples in wet and dry conditions

The constant hydration in the experiments described above prevented large salt crystals from forming which would impede ideal surface adhesion to the substrate. Once this time period had concluded the excess solution was removed with a kimwipe or filter paper, which helped removed the excess salt from the surface of the material. If this step was not performed large snowflake-like artifacts become visible under the AFM as shown in figure 3.1.

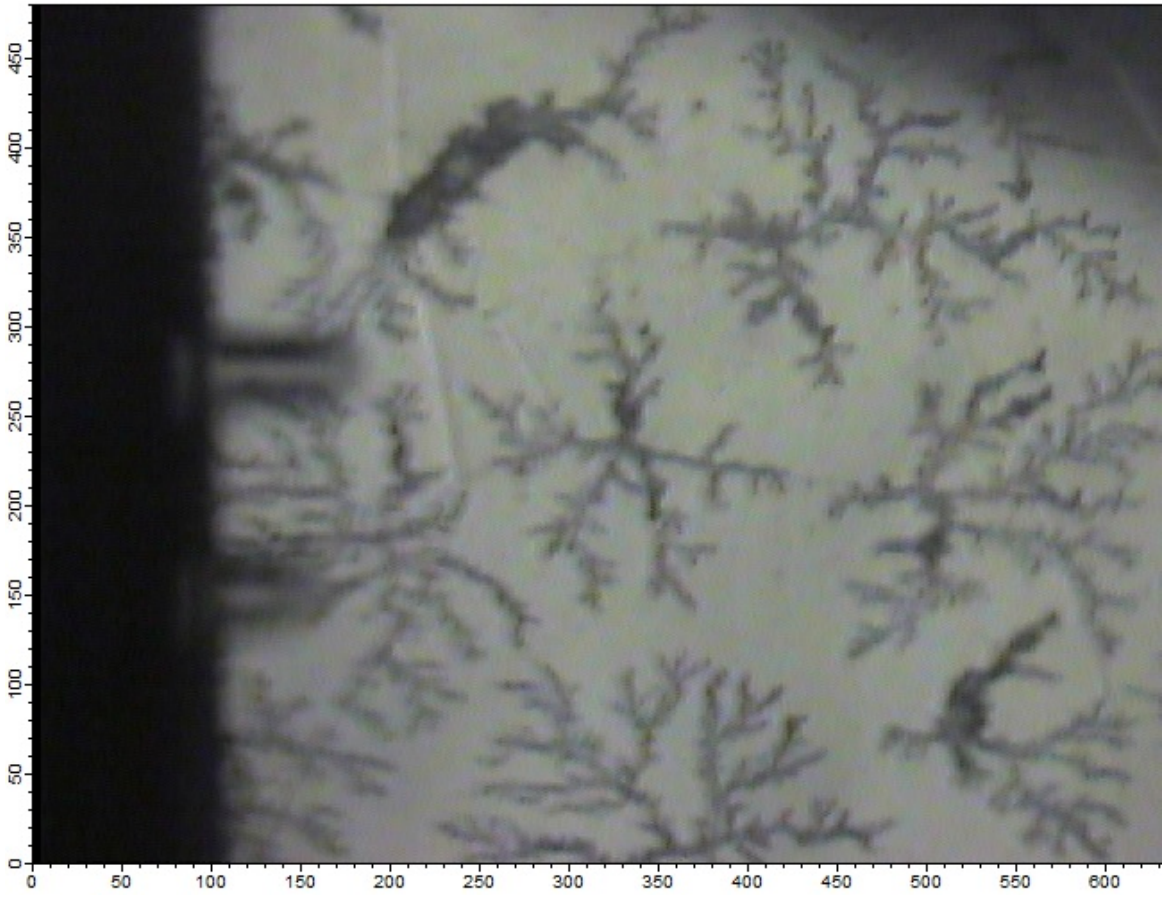

Figure 3.1: Effects of salts when improperly removed taken with optical micrograph

The effects of salt are also visible in constant fluid conditions. The reason for this

is due to the salt present within the buffer mixture which does not congregate upon the tip itself. If this occurs salt masses are the only image present in the scan. Once formed the salt is resistant to rehydration and difficult to remove from the sample. This is due to the large volumes of liquid required which can interfere with the tip. To correct for this salt buildup as observed in the following figure 3.2. If it does happen there are processes that can be undertaken, one of which involves "freshening" the tip. The process involves either raising greatly or removing the tip and gently rinsing with ultra pure water, once water is collected upon the tip and no damage is done,(pressure from certain methods of water addition can break or damage tips) the excess water is removed from the tip and the experiment can continue. This freshening process

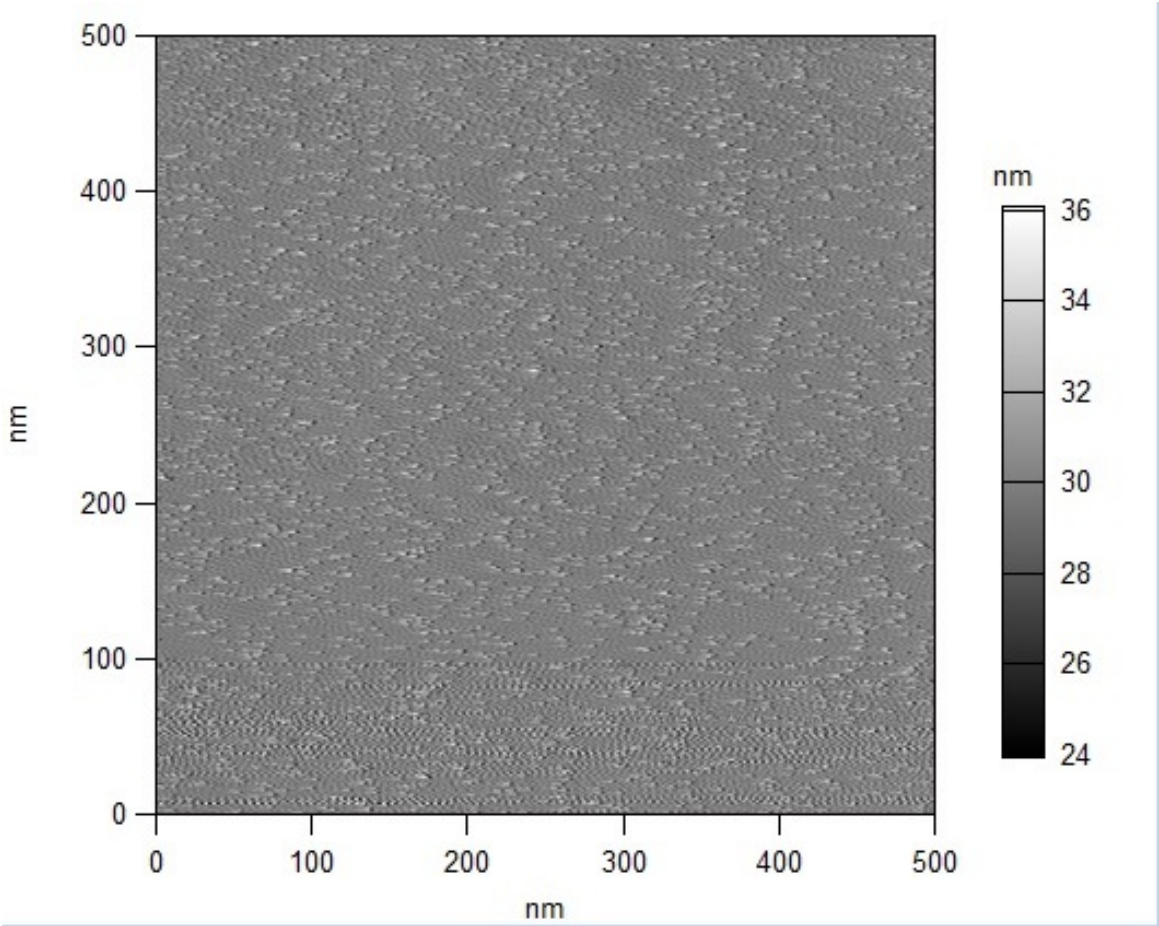

Figure 3.2: Salt effects on the tip in wet conditions

is sensitive to the collection of data to which water is added to the sample and the tip is re-calibrated to account for the error present because of the salt. The scan is continued and the results are equivalent to that before the salt disposition occurred. Once the experiments are completed the tip is removed and gently cleaned and dried to be returned to the tip box for use in another high resolution experiment. For most of these experiments the second method was used for the sake of time.

3.3 Adhesion of sample

Through the experiments it was observed that uncharged mica did not hold the sample as well as the charged mica and HOPG had the strongest adhesion, which will be discussed shortly, as well as the majority of the salts able to be removed with the kimwipe in the dry analysis. Analysis under AFM led to the observation of many small fibers, although too large to be the protein in question it did allow a benchmark to compare the proteins which can be noted in the figure 3.3. These fibers were attributed to proteins and enzymes used in the isolation of AID.

Uncharged mica did not hold AID as effectively as both HOPG or the charged mica, fewer of these fibers we able to be observed over the area. It was also noticed that due to the hydrophobic nature of HOPG it kept a large droplet of water containing the sample, this allowed better containment for analysis. Mica on the other hand being hydrophilic the surface was easily wettable and allowed the sample to spread farther making the protein and the unknown fibers spread farther apart increasing the difficulty of analysis.

The HOPG was decided to be the substrate of choice due to the simplicity of use and reduction of human error which would be present when using the charged mica as a substrate. It should be noted here that this process was completed the day of

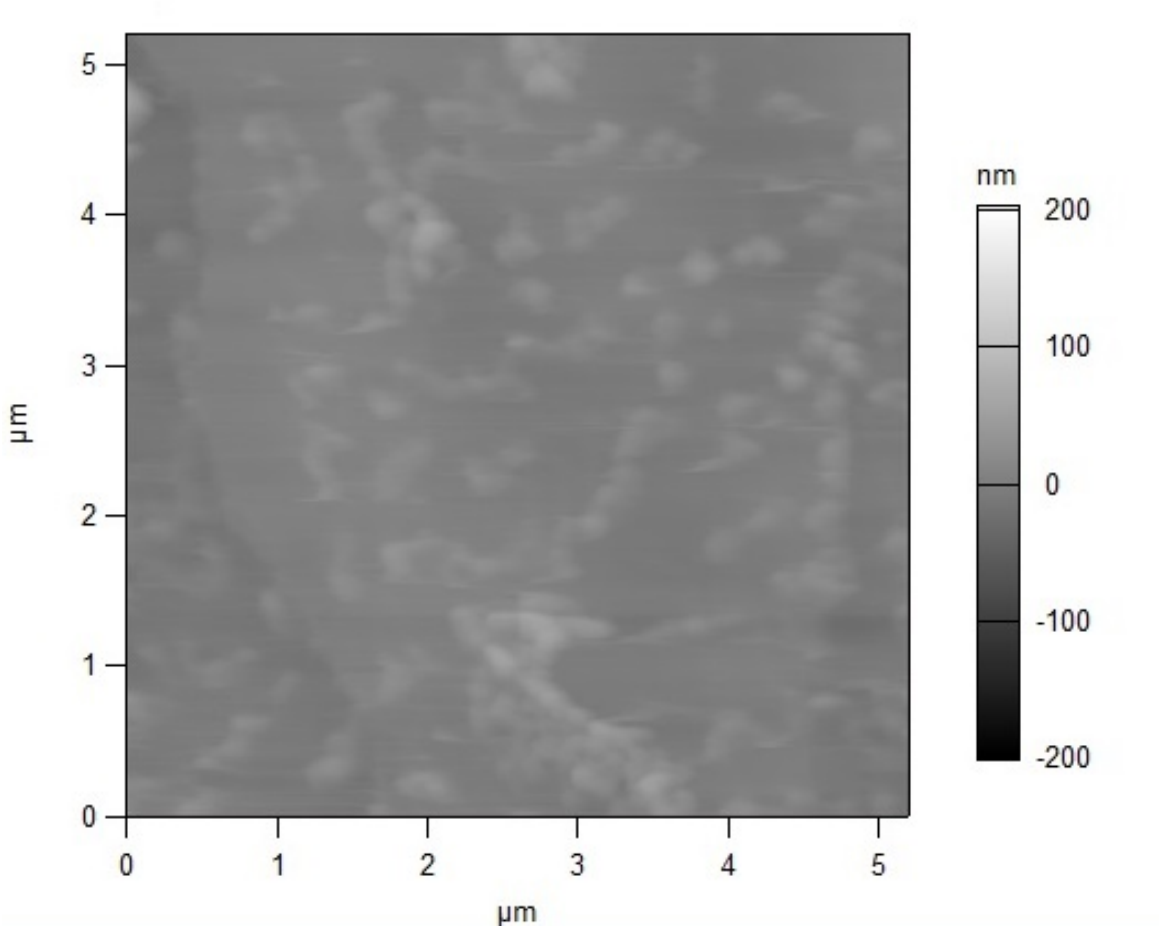

Figure 3.3: Fibers found in the solution used as substrate benchmark. (force micrograph)

analysis as to not allow any dust or debris to settle upon the surface of the sample in between plating. To test whether this would certainly affect the sample a plate of HOPG was removed and left in the sample holder. It was noticed that the humidity and dust began to settle after some time soiling the substrate.

In this experiment, I noticed that there were several factors that affected the adhesion of the sample to the substrate. The first being what force, if any, would draw the sample to the substrate surface, in the case of HOPG there is a hydrophobic effect, (explained in more detail in the following subsection). This allowed the water in the sample to be repelled and maintain a droplet shape, while the salts and fibrils

were drawn to the surface enabling the hydrophobic portions of the protein to interact with the substrate. This method did not rely on any significant charge to draw the sample to the surface and is less likely to alter the sample and keep it in its most natural conformation for analysis.

Mica in its uncharged state is hydrophilic and does not have a charge to draw specific portion of the fibrils. This is why very little to no adhesion with uncharged Mica was observed. In the charged case however with a chemically applied positive charge it enable the substrate to draw the negative portions of the protein to allow them to adhere to the surface. As the protein is overall positively charged this again should not alter the conformation too severely, however as it is meeting with a negative section it can cause some protein alteration.

The sample adhesion was not always simple. There are many factors that can be the difference between a successful plating and an entirely unusable sample. The main problem with adhesion in fluid is the assumption that as it will always be in fluid there is ample time for the sample to adhere meaning we can begin scans immediately after plating. This was not the case, when scanning rehydration had to be done on an angle and if the sample was not properly adhered to the surface it would be pushed off the substrate and onto the surrounding glass slide as observed in figure 3.4. So the 20 minute wait time was essential in both wet and dry conditions instead of wasting sample and substrate by being impatient. The wet sample was kept wet for 20 minutes before any scans began.

3.3.1 Hydrophobic/hydrophilic Effects

Many substrates and items in the world can be classified as either hydrophobic or hydrophilic; there are modifications that can be made to create a hybrid. What forces cause this to happen has been studied for a long time. A very common system

that is often studied is that of the lipid bilayer layer. A hyrophobic fat surface which has an uncharged surface and it is well known that water is a charged system which does not allow easy interaction to an uncharged system.

The common phrase is like attracts like, which in this sense the charged system of water and the buffer solutions within the sample. What is important about this is that the proteins within the sample have both hydrophobic and hydrophillic portions. Mica is a hydrophillic substrate which means that it is wettable. Water and the surface have a positive interaction the water can freely interact with the surface and little to no beading of the water is observed.

With HOPG however there is a hydrophobic interaction, with this interaction we see the beading of the water upon the surface of the substrate. This beading

Figure 3.4: Sample being pushed off of substrate due to pipette force. In this image from left to right we see, the glass slide, then the edge of the double sided tape used to adhere the substrate, and the black square is the substrate. Observe the grey presence of sample on the glass and the tape having being pushed off the substrate.

and the general repulsion of charges within the system allows the protein time to approach and attach to the surface of HOPG, its constant graphite composition the overall charge is a net zero and only allows an induced charge. This is due to the hydrophobic nature where something of a similar charge. The hydrophobic portions are draw to the surface and allow the binding of the protein.

3.3.2 Rehydrating the sample

The addition of ultrapure water to the sample seemed to be a simple aspect of this project however there were ample problems which arose just from the method and techniques used to add the water. The first issues came when it was noticed that a blank slide was being scanned for several weeks of preparation. After the first sighting of fibrils which were very small in number it was noticed that they were spread far across the substrate in what seemed like a constant spray. It was realized that the pressure from the pipette and the angle of addition caused issues in both wet and dry conditions similarly.

In wet conditions the sample was pressure washed off the substrate due to the angle of addition of the fluid. Unknowingly the addition of water to keep the sample rehydrated during the dry scans was off center in order to ensure the proper addition to the sample area, this became easier with HOPG due to the hydrophobicity forming a bubble used to ensure accurate addition. So the addition of fluid straight down while the sample was still adhering was essential.

Another issue present within the rehydration of the sample was over/underhydration. In some scans the area was increased to double the initial sample addition area, this is not due to the hydrophillic sample, but due to the flooding of the sample to keep it hydrated. This, along with quickly depressing the pipette plunger caused a large circle of sample to the far edges of the substrate and tape, with a blank portion

in the center. Seeing this, it was realized that the pressure from the pipette was an issues as well as flooding the sample with ultrapure water.

This problem caused salt to begin to form upon the sample due to the lack of solvent to keep it in solution. Not as much as was originally present on the slide, however there is enough present to distort the dry sample, and in wet conditions it causes the salt to crystalize on the tip which returned to the salt issues in the experiment. The solution to these issues was adding fluid very gently and in minimal amount more frequently.

Overhydration and pressure can be similar to that in the previous figure 3.4. One way to overcome this problem in dry conditions is slow addition of the sample volume over a 20 minute time frame. In fluid it is easy to keep the sample hydrated, once it is plated the AID sample is followed up with constant additions with low pressure every 2-3 scans with the AFM. Although over time salt will eventually deposit on the surface of the tip it was drastically reduced and the sample remained intact on the substrate.

Chapter 4

Imaging AID Using AFM Under Physiological Conditions

4.1 Introduction

Performing Atomic Force Microscopy (AFM) in fluid is highly advantageous over typical ambient imaging when studying biological materials, as discussed in Section 1.1. This chapter will outline the standard method of calibrating, introducing fluid, and imaging used in my experiments, which can serve as the standard method for performing in-fluid imaging of materials using AFM.

4.2 Experimental

4.2.1 Initial Setup

An atomic force microscope (MFP-3D, Asylum Research) was used to perform the in-fluid imaging of all samples. A gold-chromium coated AFM probe (HQ: NSC36/CR-AU BS, Mikromasch) with a spring constant of approximately 0.6 N/m into the AFM

tip holder, and then placed into the AFM head. The laser was aligned on the back of one of the AFM cantilevers and the sum signal was maximized, and then the laser deflection value was set to -1 V. It was important to ensure the AFM head was properly levelled in all experiments.

4.2.2 Calibration of the Tip (In Air)

Calibration of the AFM tips inverse optical lever sensitivity (invOLS) is based on the procedure outlined in the AFM reference manual. In short, a force-distance curve was performed on a hard surface such as a Si wafer or freshly cleaved mica. From the resulting force-distance curve, approach phase of the curve was highlighted, markers placed on two points along the flat, horizontal portion of the trace. Under the force tab of the master panel in IGOR software, the 'cal' sub tab was selected which is basically calibration for the scan. Under the 'Set-sens' pull down menu, select 'virtual defl line' was selected this created the baseline deflection of the tip which would allow us to determine the force of the protein in analysis.

This will set the virtual deflection value of the force curve, in such that this free-air portion of the curve where the tip is moving towards the surface will have a constant deflection value. Next, another force-distance curve just like above is performed. The indentation portion of the force-distance curve is highlighted, and markers placed on the indentation portion of either the approach or retract phase. From the same 'Set-sens' pull down menu 'Defl invOLS' was selected.

This sets the invOLS value for the calibration force-distance curve. This value is necessary in determining the proper spring constant of the tip. Next,the tip was disengaged and the AFM head raised to remove the tip from the sample, and the laser deflection value set to 0 V. Next, a thermal calibration of the tip in-air was performed, to determine the spring constant of the tip. If at this point the spring constant of the

tip was outside of the factory-provided specs, the tip was replaced with a new one and the calibration process was performed again.

4.2.3 Introducing Fluid to the Setup

When working in fluid, the laser deflection value had to be reset -1 V. Then the head was lowered until the tip was in contact with the surface of the sample. It was at this point the AFM head was lifted slightly to remove the tip in order to introduce water to the sample. Using a micropipette, approximately 5 to 10 μL of ultrapure water was added to the sample slowly to bring the water upward which encompassed both the AFM tip and the sample. For the best results it was important to allow the sample to equilibrate for approximately 15 minutes. After this time the sum signal and laser deflection values were readjusted to ensure the best results could be obtained.

4.2.4 Re-calibration and Tuning of the Tip (In Fluid)

Once the experiment required the AFM tip to be in fluid, recalibration was performed to ensure the best results were obtained. First, the tip invOLS was performed again, in the same manner as outlined in Section 4.2.2. Once this was done the analysis mode had to be changed to 'AcAir' (also known as tapping mode), one of the standard analysis modes available on the AFM. The laser deflection value to 0 V for this experiment, and the ideal set point was determined to be 800 mV. Finally once the tip was tuned and it had a distinct resonance frequency, this was saved as the drive frequency and the 'Centre Phase' was set allowing a gradual increase in drive frequency to occur. If the tuning was correct values greater than your initial set point could be obtained, however the aim was for a drive amplitude of near or slightly above 1.0 V. Once all of the calibration was complete the experiments could begin provided no power loss or software crashes to which all had to be redone.

4.3 Results from Experiments

Much of this project was checking and reevaluating parameters and setup to ensure that the desired image could be achieved with as little interference as possible. Up to this point it would seem that the goal of this project was not achieved. Unfortunately, no force data were collected on the sample, but based on size comparisons and general shape it seems like a glimpse of this elusive protein has been obtained, as shown in figure 4.1. Although the image is somewhat rough, certain key aspects of the protein

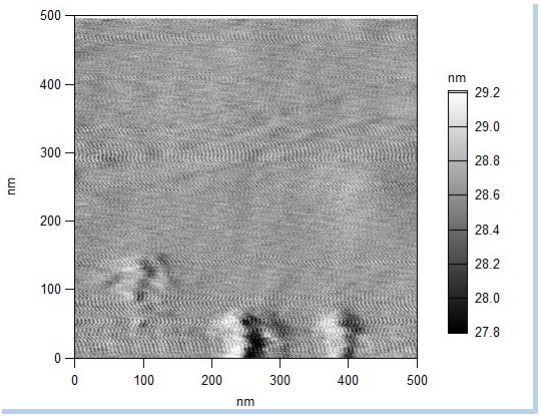

Figure 4.1: AFM image of Activation Induced Cytidine Deaminase at 50nmx75nm in size

can be seen which agrees with the computer modeling simulations of Larijani lab, who provided the AID sample. Through other literature previously mentioned in this paper we see that it matches what would be expected of an AID tetramer. These two factors lead to the conclusion that this is not just an artifact on the AFM that we had been seeing previously but is most likely the protein of interest.

As observed in Figure 4.3 with a slight counter clockwise rotation we can overlay the computer model over the scanned image. What is interesting to note is the slight bent "y" shape. This is clearly visible in both the computer models and the AFM image. Another thing of note is that the thicker areas of the AFM image of AID, those with a larger height difference from the substrate, we see matches with the large

clusters of helices present within the model, and the thin areas within the image, those with lower height range, we see very short packing in the same area of the computer model.

Further evidence for this being the protein of interest was observed with a scan of the Z-axis observed in figure 4.2. With this analysis of the shown section in figure 4.3 b we see that the height of the protein is comparable to computer and online databases for the protein. As well to ensure that we have the sample and not the substrate a FT was performed on a scan of the HOPG and clearly showed the substrate layers versus that of sample. With this discovery a review of previous scans for similar sized features were to be reviewed and compared to this image. Now that an image with some certainty had been found, past scans may be able to provide more information as well as evidence.

With this finding and comparing it to the paper produced by the Larijani lab have a better understanding of the best possible model for AID. Originally, the lab had developed 9 potential models that fit with the energy and binding affinities. Most importantly they made sense with the purpose the protein is meant to serve. Unfortunately, to have a better understanding and study reaction rates and the mechanisms behind AID, 8 models are far too many and would require computing power and time beyond any feasible means. Fortunately with the AFM image taken it can be compared to the models for shape and size to narrow down the potential candidates to study.

As observed in the figure 4.3 two models have stood to match the obtained figure of AID present which are models C and F. The others had features that did not stand next to the AFM image. Of those two images both have the bent “Y” shape as previously mentioned as well as the thick and thin parts to match. However, the best match seems to be model F due to the larger spacing of the cleft of the Y making it a

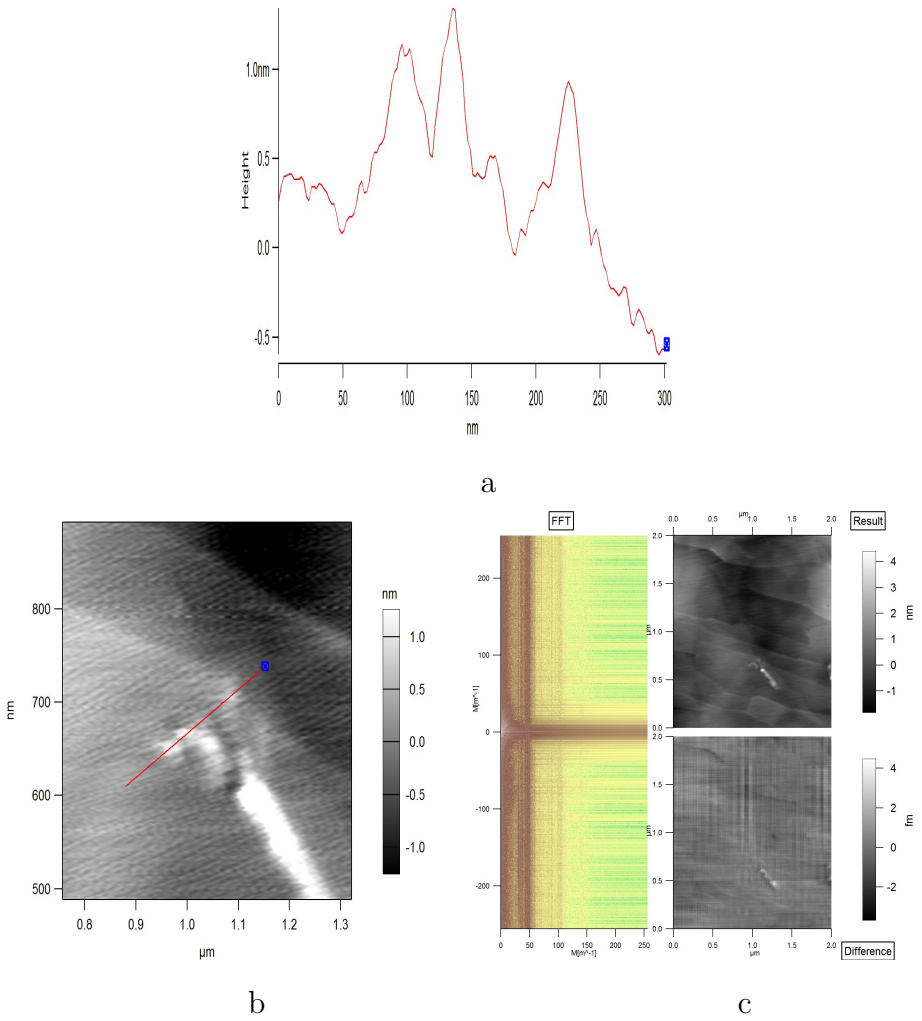

Figure 4.2: a: AFM image of the protein taken along the Z-axis to provide more accurate representation of the protein size. Using the asylum software we took a line across the protein to determine thickness and compared this with the theoretical protein size b: Section of the protein analyzed used to confirm the thickness of the protein with respect to cited databases. Image of where the cross section was taken. c:Fourier Transform of the substrates to display easy distinction between layers of substrates and analysis material .

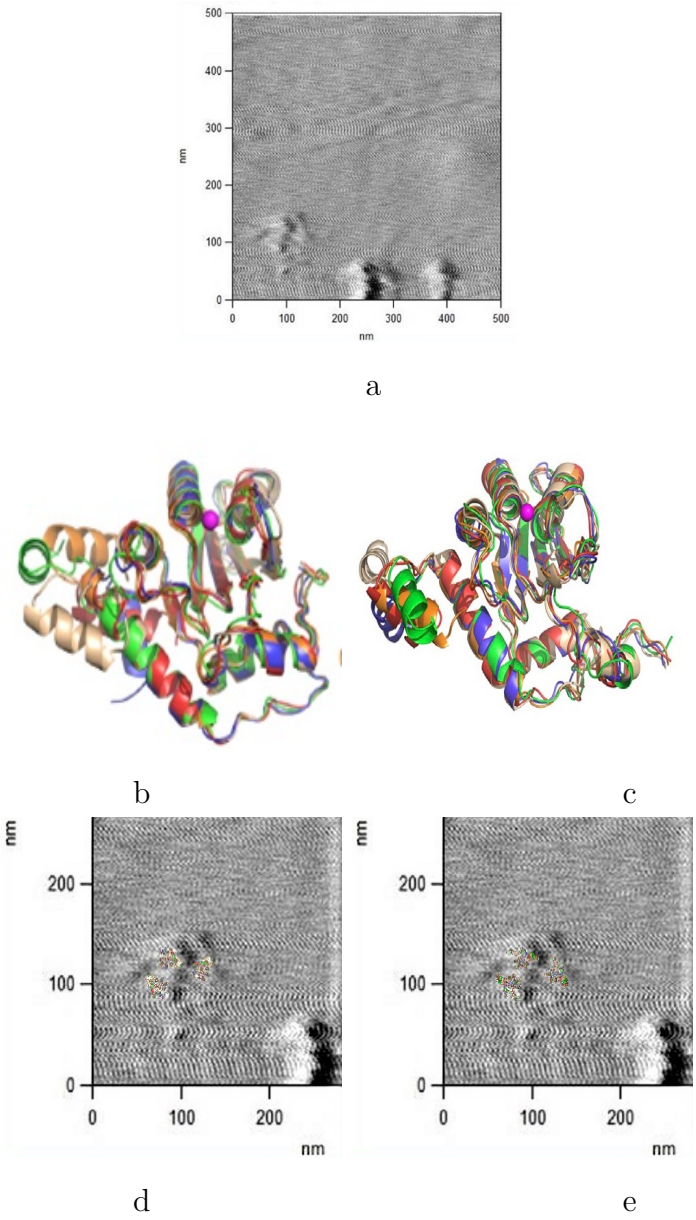

Figure 4.3: a: AFM image of a tetrameter of Activation induced Cytidine Deaminase. b: 3D molecular model of Activation Induced cytidine deaminase produced by Justin King of the Larijani Lab labeled C. Used with permission from Ref. [53] c:3D molecular model of Activation Induced cytidine deaminase produced by Justin King of the Larijani Lab labeled F. Used with permission from Ref. [53] d: A super imposed image of the 3D model C over the AFM image of AID. Adapted with permissions from ref [53] e: A super imposed image of the 3D model F over the AFM image of AID. Adapted with permissions from ref [53].

more accurate match to the actual AID tetramer. With this information although the AID was mounted and not entirely in its natural state we can establish that this is the most plausible model for AID. This is a great leap forward for this project enabling future experiments to essentially be tailored to this model and be able to obtain more accurate and specific data.

After reviewing previously collected data as observed in figure 4.4 we can see that there are similar features to the image in figure 4.2, although an optimal orientation is not always obtained. What we have gained from reviewing other data is that this protein was not a chance finding, and has been imaged several times under a range of conditions. With this news it would allow for more accurate data to be obtained in the future. The baseline parameters and experimental setup allow for the protein to be released onto the substrate, adhere and maintain its properties in fluid to allow an image to be taken. With the local humidity summer seems like the best time for this analysis to take place as there is longer lasting hydration of the protein of interest.

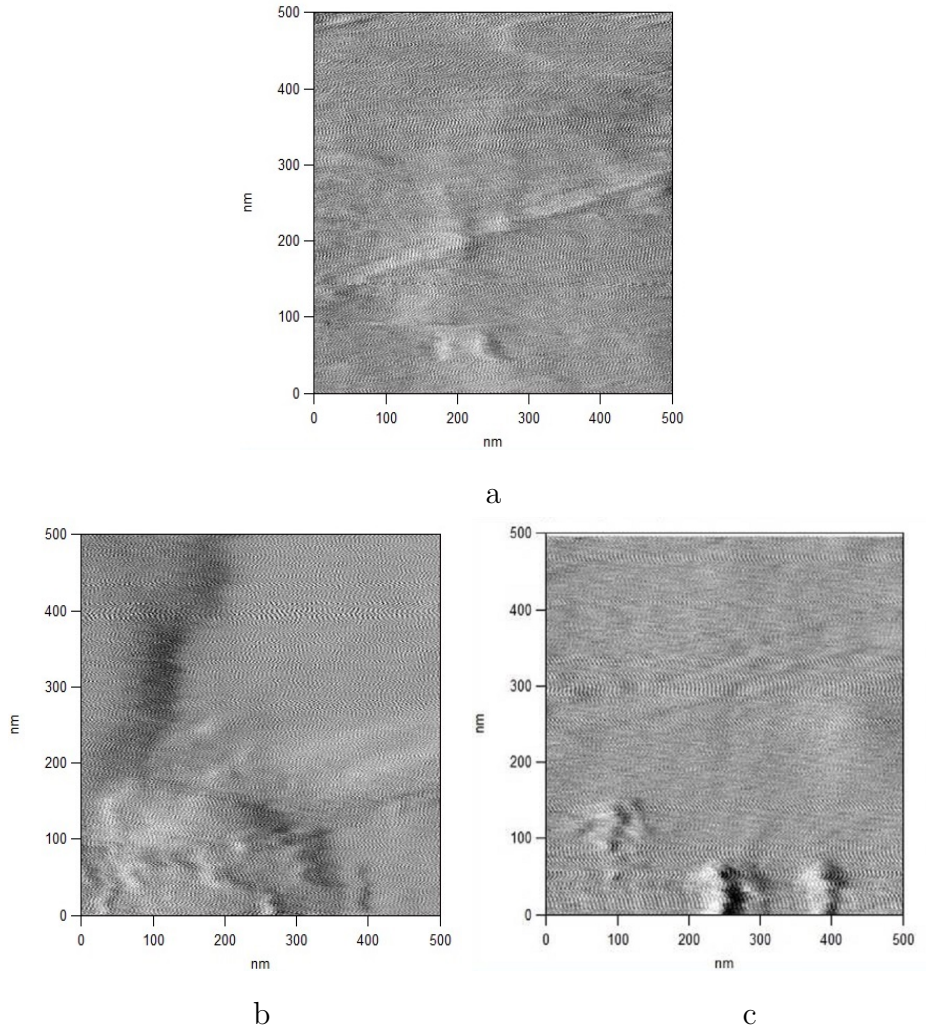

Figure 4.4: Amplitude images showing features which match the expected conformation of AID. a: Artifact presence did not allow for accurate analysis or size comparison as there was interference from an outside material, although judging by the scale and general size this could be AID in an alternate orientation. b: Amplitude data of a grouping of protein clumps, at first glance look like interfering fibrils however after further analysis look much like the protein of interest. c:This is the selected image and comparing A and B with this they are of similar size shape and scale, but the sharpness and orientation differ not allowing A and B to be ideal for further analysis
.

Chapter 5

Conclusions

Within this project, many challenges were faced, an AFM image of AID was successfully obtained. Headway in this field can lead to many advances within cancer research, as well as within the immune system [54]. Future research would target a complete understanding of the conformation properties of the protein. With this information an understanding of the energy needed for this process to occur [55], as well as being able to stimulate or slow the process down to prevent some mismatching leading to mutations with no apoptosis within the cell or at the very least a pathway of understanding the mechanism. These cancerous cells will often metastasize and spread across the body after forming an infinitely growing tumor in a certain location. [56] It seems like an ideal method of understanding the baseline reaction for the replication of DNA.

Another pathway this research can lead to is developing better immunoresponse techniques. By obtaining a better understanding of how the immune system works and develop an immune system simulation [57]. From this research many things have been established like setup for the future of this project. Having spent time completing the baseline research, the project can move forward for future work on this project.

There have been many hurdles to overcome, but with the errors of setup, device parameters and substrate selection overcome, great potential for moving forward now exists. Some of these hurdles took many weeks to overcome, such as the problem with the washing away the salts and the proteins. Other large hurdles were within the selection of the tips used for the AFM, and finding a tip that will not destroy the sample but still achieve the resolution needed. Upon doing this it was established that usually tip resolution was 3 times the size of the tip, so the minimum tip size to observe a monomer of the protein would be 15 nm so a tip this size or smaller was necessary. The problem with tips this size is that they are either very fragile and expensive, or they are designed for alternate purposes than that of analysis and damage the sample [26]. This makes the purchasing of super fragile expensive tips a delicate process. If there is a purchased tip and it becomes contaminated that is wasted resources as well as wasted time.

In Figure 5.1 some of the projects problems and how they have been overcome with persistence and dedication to the project are presented. In figure 5.1 a the formation of salts present within the buffer solution are shown, which prevented any chance of finding the protein of interest. Image b shows what excess force through rinsing resulted in, a pressure washing of the slide and removal of all potential deposited proteins. C was an example of what residual proteins and fibrils present in the sample provided would look like on the slide. In preparation of the sample these materials are used to prevent the protein breakdown and can sometimes make it into the final sample through the filter. Image d displayed that as the buffer solution begins to evaporate some salts can be deposited upon the tip leaving us with artifacts of constant size scattered around. The dip in this case being deformed gave a false image. In our final image, we see a return of water to the tip and the crystals no longer present. This removed the given artifacts from the image allowing for a clearer

more representative image.

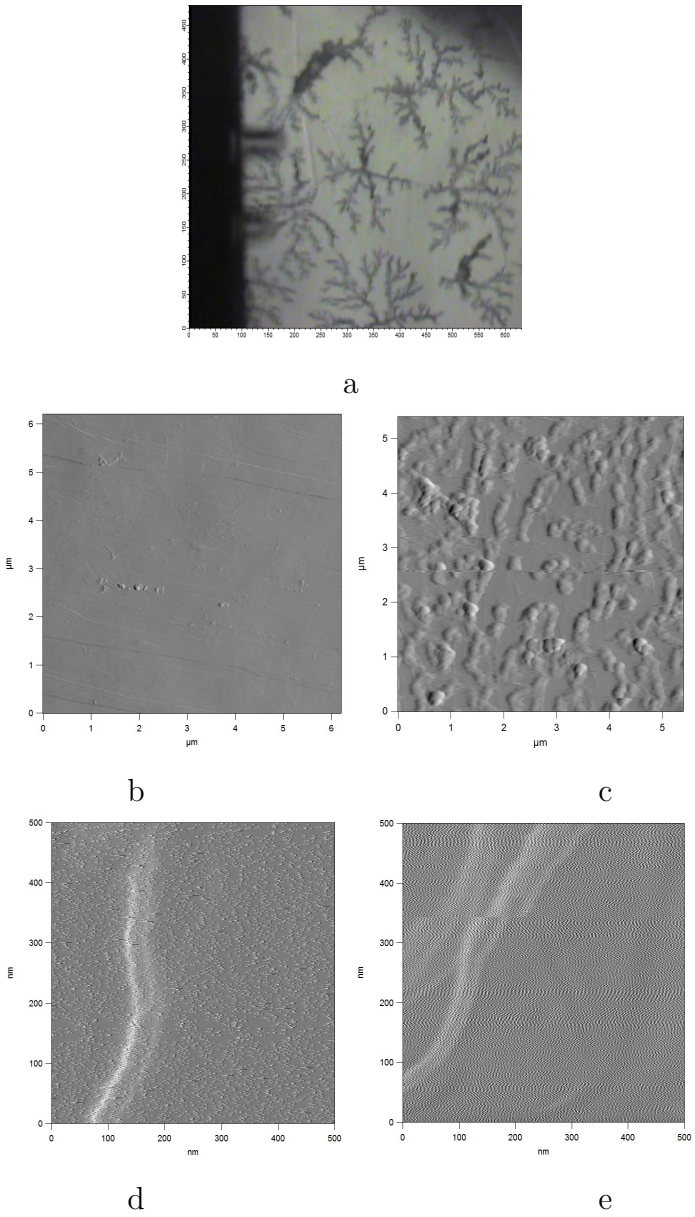

Figure 5.1: a: Salt deposited from sample buffer solution. b: Blank slide from lack of fibril adhesion. c: Fibril adhesion solution later used as a benchmark for other parameters. d: Salt buildup on tip causing artifact presence in scans. e: Tuning of the tip calibrating the tip which removes artifacts

5.1 Atomic Force Microscopy and Analysis

Within this project there were many aspects that had to be accounted for before any analysis could begin. First the size of the material to be analyzed, in this case a very small protein often known to form monomers, dimers or tetramers needed to be considered [58]. The size of these materials were between 15-47 nm^3 for the monomer and dimer respectively; the tetramer is closer to 90 nm^3. Furthermore they are easily denatured or destroyed by slight variations from their natural environment. In order to overcome these issues it was necessary to explore how these proteins should be analyzed, and what tips would be necessary to complete this very difficult task. The main issue was whether tips small enough to get to this resolution were available and if would they capable of locating the protein.

To address the first problem a tip capable of such a fine resolution, it was determined a Gold Chromium tip with a resolution of approximately 35 nm, as shown in Figure 2.2. Even with this fine tip it is difficult to pinpoint any features due to the minimal size of analysis being double the tip resolution. This prevented certain evaluation of a single protein strand. Another interesting thing to point out is that in fluid measurements the resolution will also dip due to the constant movement of the sample and the medium it is in [21, 59]. In order to correct for this, a faster scan of the sample was performed to match the amount of movement capable of the sample and the increased viscosity of the fluid medium [60], as well as a cooler setting [61] to prevent protein denaturation as well as protein activity within the fluid and the changing environmental conditions.

Wet versus dry analysis is a completely different scenario. When in dry conditions locating and observing protein structures at the desired size occurred more frequently than in wet conditions [62]. The main issue being that once dry we notice biological samples loose much of their interesting properties and this alteration will

give improper results [63]. The most drastic of which being the change within the elasticity of the sample [19, 64]. Another problem would be the lack of physiological conditions, this will cause a denaturing of proteins in most cases, this can be either changing their overall conformation to that of another less effective one due to the charge removal with the salts [65], or a completed degradation of the protein due to high heat or a more extreme pH environment [66].

Another common issue within the dry sample was the salt that is present (see Figure 5.1), these salts will be removed from the sample before any analysis takes place. By keeping the sample hydrated for 20 minutes to allow migration of the protein to the substrate we can then use a kimwipe to draw the excess solvent away. The ease of analysis that is present within the dry sample prep is often the trade off for not having an accurate representation of the sample, but for this analysis dry conditions will not do. It is essential to keep the sample hydrated as well as leaving the various physiological salts present. This can often cause a problem because of the salts that will eventually remain upon the tips.

This can cause artifacts to form within the analysis. Other issues that are present within the hydrated sample is the hydrophobic nature of the substrate [67]. This will often cause the fluid to slide from position on the sample and improper addition of ultra-pure water could push the sample clear off the substrate, with the re-hydration over and over this could also dislodge the sample from the substrate. The pressure of the pipette and excess volumes of water can also dislodge the sample (see Figure 3.4). The utmost care must be taken when adding water or re-hydrating the sample, because not only might the sample on the substrate shift, but the whole slide under the AFM tip.

This shift can cause many issues, a major one being the destruction or damage to the tip, which are delicate and expensive to use. Another issue would be to the scan

itself. If you are zoning in on a spectacular location and a rehydration is necessary, a shift of the slide will cause you to loose your place. This increases the potential of not finding the analyte you are looking for. A great issue present with using the ultrapure water, or water in general is the viscosity [68]. As it is not a thick fluid, this can allow the sample that is not already adhered to the surface to become mobile or even remove previously attached sample. This would make the protein highly mobile and difficult to find within the fluid using simple AFM analysis.

A correction for this is to use a greater viscosity liquid such as glycol [69], which is used within the sample preparation and is commonly used to preserve various DNA and protein samples [70]. If the system has greater viscocity is should slow down the enzyme movement and once located should ease in the analysis process. It should have no negative effects upon the protein itself. The high viscosity of the fluid will allow hydration, as well as adherence to the surface of the substrate. With its low volatility as well re-hydration will be less frequent and will remove some of the extra issues accompanying much of the wet sample analysis. It may cause the protein to seem more rigid due to higher viscosity of the solution [71], but for the most part it will make the analysis easier due to the lack of mobility of the sample.

5.2 Project Accomplishments

This project was important for understanding the AID pathway and how it interacts within systems. This is one of only a few studies on AFM using this method. Due to AID having such a positive charge, few methods are able to ideally image the sample. Even though it is one of the only adequate methods, there are still very few papers presented with AFM data. This is due to the very sensitive nature of this protein and its buffer system. Overcoming the system and the conformation analysis is a feat

in itself, and as it has yet to be done elsewhere this is breakthrough methodology for analysis of the protein.

Some groups have been able to obtain images of denatured monomer but never an image of AID in its natural conformation. Being able to keep the protein in its natural state, or at least as close as possible while being plated was a struggle. This project allowed me to explore all of the AFM knowledge and setup a system that was able to do more than just detect the protein, but also enable an accurate height image of the sample.

Through this experiment the substrate system has been optimized for best results. Luckily, the impure sample provided, allowed some charge fibrous materials present to enable a comparative study for each of the substrates analyzed. Although similar results were observed with the charged mica and HOPG the process of preparing the mica introduced too much error potential. It also made analysis a struggle within the time frame of the AFM availability. A summary of the substrates can be observed in the following table.

Substrate List			
Substrate	MICA	Charged MICA	HOPG
Sample Adhesion	No	Yes	Yes
Sample Preparation	No	Yes	No
Sample Area	Very Large	Medium	Small

Table 5.1: Comparative table of substrates

5.2.1 Potential Applications

Some benefits from undergoing this research is the hopes that a greater knowledge of the mechanism of this enzyme will lead to a better understanding as to why it can also cause mutations that are not beneficial in nature at all [48]. An example of this would be any of the various forms of cancer relating to the AID lymphnode recombination. [72] Upon laying the groundwork with this research, we hope to be able to gain an understanding of the mechanism to which this enzyme reacts, its conformation when it begins to cause its switching within the various systems within the body, as well as its favorable attack points. Although research has observed the mutations generally occur within a replication loop [73], what causes the infinite replication to go without repair still remains a mystery.

My substrate analysis and AFM optimization provides a template for in fluid biological analysis of AFM [74], and a baseline protocol for imaging AID. After determining which type of substrate system would work best for the material in question, a simple procedure to be followed could make this a more effective method of analysis. This could potentially speed up the analysis time and remove the error which could be associated with the increased setup and plating times of the sample, as well as the AFM setup. This would greatly benefit the biological analysis due to the high risk of degradation associated with proteins outside of physiological conditions [75].

The main goal after establishing the substrate work as well as the AFM parameters is we may be able to better classify the nanomechanical properties of the enzyme itself [76]. Once this data can be optimized and collected, a world of information could be extracted allowing for a possible prevention to the start of a dangerous infinite cellular growth, which is cancer [77]. The mechanical properties although difficult to obtain, advances within the AFM technique itself as well as improved technology are leading to a better analysis method [78]. The ground work of substrate analy-

sis will further aid this research towards the end goal of a better understanding of the pathway and a more effective treatment, or preemptive treatment of this terrible disease.

5.3 Future Goals

The main goal that would be important to meet would be more detailed AFM analysis, with the system we were using various optimization steps had to be taken to ensure the best possible results were obtained. Confirmation that the sample was indeed present upon the substrate which would give the ideal results and cohesion to the sample. With all of these necessary steps, the quality of the data collected was not as high as it was planned to be. Some solutions to this problem would be using much finer tips and introducing some alternate parameters to the experiments themselves. A main future goal would be obtaining mechanical properties of this protein. This should be easily attainable with the groundwork in place, once sharper tips are obtained.

For most of the project the tip size used was 35 nm which should have given a rough image of the monomer itself. Rather than wasting very expensive and high quality tips, it was more ideal to use a cheaper option that would still yield some results. This was the best option overall due to various unforeseen circumstances with the AFM. This lead to broken cantilevers and the entire tip being removed due to a glitch or error within the feedback loop not alerting to contact with the surface. Not only would the cost of these ultrasharp tips have been a factor, but breaking them would be very costly for one but the dulling factor of these tips is quite high leading to more replacements being necessary. Even if the sample was not stiff enough to dull the tips the salts present within the system would adhere to the tip and the residue would reduce the functionality and precision of this highly calibrated tip.

Other future goals for this project would be to introduce some new parameters and experiments to obtain several different scenarios. One would be to have the sample in near physiological conditions and complete the analysis [42]. This goal may be unattainable due to the many variables talked about throughout this thesis, however with some of the imaging techniques that are being developed with certain AFM conditions it is believed that this should be possible in the near future. With scan times for some experiments being less than 4 seconds per scan it should be able to tract some drastic changes within the protein through the various images collected.

This technique is mainly used for larger systems, but once it has been further explored and developed for the use in A) sharper and stiffer tips, and B) very soft samples it will be much more effective. In the case of the former, the tip would be observed undergoing large volumes of stress due to the frequency and speed of the data collection [79]. This will readily dull the tip making the data collected unreliable. If the tip remains sharpened it will in time also become coated with substrate or various portions of the sample and will again become unreliable in the data collection [80]. The other issue would be the damage to the sample such as in the case of a reusable substrate designed for protein analysis. If this was used with the ultra hard/sharp tips they would most likely end up with scratches or damage upon the substrate [81]. Many times these substrates could be very time consuming and/or expensive to make, and this would be a very difficult con to overcome for usage of this method [16].

As for very soft samples, depending on the sample size it could either puncture or rip the sample leaving you with no results and much wasted resources [82]. The other possibility with soft samples is if they are small the tip will miss them constantly, which will make them roll out from under the tip, much like peas to a fork. This will prevent any mechanical or topographical information to be collected. Once development within this technology occurs these problems should be easily overcome. This

will become the ideal method for analyzing mobile samples or those with high rates of decay. It will allow quick assessment before the samples degrade into their components and become inactive. Unfortunately, this is the case with the AID sample, it will degrade over time making this method ideal once it becomes more developed [16].

Another option for the future of this project would be the development of a cryogenic form of analysis [14]. The features of this method that draw attention is the analysis while the sample would still be technically flash frozen. The sample will be locked into its natural state allowing for topographical and details about the orientation of the system to be collected. The issues that arise with this method are the mechanical properties cannot be assessed and the protein interactions will not be able to be measured as the conformation is locked and all the natural properties other than orientation and topography will be altered.

Another large issue that may still prevent the orientation and topography to be measured would be the freezing of the sample will often cause it to be more brittle and may shatter upon beginning the analysis [14]. A slight compromise would be using a colder system that restricted the movement of the sample to enable an effective analysis, but still having some softness and somewhat regular properties. Another advantage of less than cryogenic conditions is that not only are some of the natural properties still present and easier to analyze, but it also would increase the lifetime of a volatile protein system. It would prevent the protein from being brittle due to being frozen, but it would allow it to be more stationary and increase the longevity by a significant margin. This would allow a larger window and potential for a more in depth exploration of the entire system and its various conformation both on its own and when engaging with the protein itself.

Another parameter that would be interesting to explore would be the system interaction with various points upon the DNA replication process. As explored

above it has been established that AID prefers to attack upon the replication loop of DNA/RNA [47] and this would be an interesting point of study. By exposing the AID to loops of various size and shape upon the RNA strand we can observe how the protein changes when interacting with the DNA itself. [47] Once a forced loop is prepared, we can prepare an in vivo solution and flash freeze the sample, although this would most likely require the cryo AFM for analysis. This would be due to it being a rapid process and it would take long intervals of time in order to find a protein interacting with the loop of the replication process. [17]

A method that could be used in this case would be to attach an encoded loop [83] to a tip for analysis, and use this tip to scan a normal surface [20]. In doing this it is possible to obtain the structural information about the loop attached by how much the thermal calibration is altered upon the scan [84]. Information based on how the auto tune is changed based upon this new appendage at the end of the AFM tip could also be collected. Once various measurements have been taken the tip can then be used to interact with the AID system to see how the properties of the loop are altered and where the alterations will occur if they will occur in this system. Altering the loops size and shape would be a key component of these studies in order to gain a more in depth understanding of the preferential attack of AID and what it needs in order to be able to bind to the replication loop of a system [85].

Another future aim for this experiment would be to compare the binding and surface properties of the various immunogenes present within different species. It has been well established that the earliest ancestor with an active immunoresponse would be the lamprey, although very different from that of humans it still works in a similar fashion [11]. Being able to compare these two systems and see what makes them differ, as well as their similarities could open some doors within the knowledge we can get from this response and why unwanted mutations like cancers occur [86].

Other studies have shown the naked mole rat having a very low or negligible rate of cancer within its species. Another immunoresponse system to be observed would be one within this family and working up the evolutionary chain to the naked mole rat [86]. Unraveling this mystery could provide significant insight into understanding and responding to various forms of cancer within the human body. This will all begin with the understanding of the physical properties of the protein that is often a necessary evil, providing protection against diseases but can eventually lead to the most threatening disease of all.

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