Measurement of Force Dependence of Receptor-Ligand Bonding Using a Novel Forced Unbinding System

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Measurement of Force Dependence of Receptor-Ligand Bonding Using a Novel Forced Unbinding System

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ABSTRACT

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Measurement of Force Dependence of Receptor-Ligand Bonding Using a Novel Forced Unbinding System

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The force dependence of receptor–ligand bonding has been extensively studied in recent years. Many ultrasensitive force techniques have been used to measure receptor-ligand bonding interaction in biophysics. Microcantilevers have easily tunable and variable spring constants over other techniques. This work presents in detail the measurement methods of glass fiber microcantilever spring constants. Microcantilevers made from E-glass fibers were mounted in viewing chambers and imaged under a microscope. Multiple sets of images for each microcantilever were captured and analyzed using custom LabVIEW IMAQ image processing program with the centroid tracking images processing. The centroid data were processed using a 20 point moving average filter which was the optimum number of average points in a moving average filter. The results of microcantilever spring constants from thermal fluctuation method measurement were compared with elasticity theory calculation. The measured spring constants of 3 - 7 mm-long fibers were within the relative error expected for the spring constant due to the fiber length and diameter uncertainties.

A novel forced unbinding system was developed for the receptor-ligand binding experiments. The system applied hydrodynamic flow from one micropipette to a glass fiber microcantilever tip which was brought into contact with a bead held in another
micropipette with suction pressure. The displacement of the fiber tip increased linearly as the water pressure difference increased which is the magnitude of applied pressure used to generate flow from a micropipette. The interaction of protein A and human IgG was measured using the novel forced unbinding system. Heat treatment of the Bovine Serum Albumin is necessary to reduce the frequency of nonspecific adhesion. The percentage of adhesive events decreased from 2.69 % to 0.57 % after the BSA was denatured. The protein A and human IgG binding interaction was investigated for specific adhesion experiments. The lifetime as a function of force was directly measured by the method that allowed near-instantaneous application of forces. The response of protein A-human IgG bonds to force agreed best with catch-slip transition mechanics but the Bell model could not be excluded without further experiments.
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CHAPTER 1: INTRODUCTION

1.1 Cell Adhesion Molecules

1.1.1 Cell adhesion

Cell adhesion is the process by which cells attach to one another or to the insoluble extracellular tissue matrix that surrounds cells. Cell adhesion is made possible by proteins that are expressed on the cell surface. These cell-surface proteins are called cell adhesion molecules [1].

Cell adhesion is essential in maintaining multi-cellular structure and a requirement for multi-cellular organisms such as animals or plants. Cell adhesion is involved in signal transduction [2], and adhesion processes of cells [3]. Cell adhesion plays a critical role in inflammation [4, 5], cancer metastasis [6, 7], blood clotting [8] and the development of new blood vessels [9, 10]. In immunology, specific antigen-recognition is one pathway for the initiation of immune responses. For example, some types of circulating blood cells can adhere to cells lining blood vessel walls if a bacterial infection needs to be eliminated or if the blood needs to form a clot to prevent bleeding [11, 12].

1.1.2 Types of adhesion molecule

Mammalian cell adhesion molecules can be grouped into four main families based on homologous protein sequences and overall three dimensional structure: the selectins, the integrins, the cadherins and the immunoglobulin super family [3].

1. The integrin family is made up of transmembrane adhesion proteins that chiefly mediate the adhesion of cells to the extracellular matrix. An integrin molecule is a
glycoprotein that consists of non-covalently-attached alpha and beta subunits. Integrins link extracellular matrix to the cell’s cytoskeleton which consists of threadlike microfilaments composed mainly of the protein actin [13, 14]. Integrins mediate a diversity of cell adhesive interactions, directing cells to live or die and signaling different levels of mechanical response [15-17].

2. The selectin family is made up of cell-surface carbohydrate-binding proteins that chiefly mediate transient cell-cell adhesion in the bloodstream. Selectins bind to their ligands (carbohydrate molecules) which mediate the interactions between white blood cells and endothelial cells in leukocyte adhesion [18, 19]. Their transient high-strength connections initiate capture of fast-moving white blood cells at vessel walls under flow [20]. There are three members: E-selectin on endothelial cells, L-selectin on leukocytes and P-selectin on platelets and endothelial cells [21-24].

3. The cadherin family is made up of transmembrane adhesion proteins that chiefly mediate calcium-dependent cell-cell adhesion in multicellular organisms [25]. The family has three important members: E-cadherin in epithelial cells; N-cadherin on nerve cells; and P-cadherin on cells in the placenta and epidermis. Cadherins joined by cis and trans interactions group together in arrays of bonds [26]. The rupture strength of cadherin shows obviously different states that suggests multiple mechanical functions [27, 28]. The “diverse adhesive functions” [28] of the bonds between the “full five-domain fragments” [28] of E-cadherin
protein indicate distinct properties of different speeds, “from transient-weak interaction to durable-strong cohesion, depending on the binding state” [28].

4. The Immunoglobulin super-family members are 70-100 amino acid glycoproteins that are characterized by one or more copies of an immunoglobulin (Ig)-like domain that is found in antibody molecules. They are commonly involved in the recognition, binding, or adhesion of cells interaction for immune defenses in the immune system [29].

1.1.3 Antibody and antigen

Antibodies, also called immunoglobulins (Ig), belong to the immunoglobulin super family. Antibodies are glycoproteins produced by B lymphocytes, a class of white blood cell. There are five different classes of antibodies in mammals named IgA, IgD, IgE, IgG, and IgM. The major class of immunoglobulin in blood is IgG. Antibodies bind to foreign targets or invading organisms such as bacteria, viruses and toxins, inactivating them to protect the host from infection [30]. Molecules and parts of molecules that can induce the production of antibodies and to which antibodies bind are called antigens.

IgG isotypes have a basic structure as shown schematically in Figure. 1.1. Although the antibody is shows as a Y-shape, the arms are flexible and some antibodies have been shown to have a T shape [30]. They are composed of two identical heavy chains and two identical light chains which form two identical antigen binding sites, that are at the tip of each arm of the Y. Dark green lines represent heavy chains and light greens lines represent light chains. The two heavy chains also form both the tail and hinge region. These chains are bound together covalently by disulfide bonds between
sulfur-containing amino acids. The disulfide bond is about 2.05 Å in length with a typical bond dissociation energy of 60 kcal/mole (251 kJ/mol) [31]. The purple lines represent the disulfide bonds. Functionally, an antibody molecule can be divided into two fragments. The arms of the Y, containing the two antigen-binding domains that recognize specific foreign objects, are called the Fab fragment region. The base of the Y, which is composed of the two heavy chains that contribute two or three Ig domains, differs for different classes of antibody. This domain modulates immune cell activity, such as binding complement and binding to cell receptors on white blood cells. This region is called the Fc fragment region. IgG molecular weight is around 150 kDa [32].

Figure 1.1: Schematic representation of an IgG antibody.
1.2 Adhesion Bond Models

1.2.1 Receptor-ligand bonding

Cell adhesion can be thought of as an interaction between receptors on cells and ligands mounted on other cells or in the extracellular matrix. A receptor is a membrane protein that can form a specific but non-covalent bond with a specific target molecule (or ligand). A ligand can be any molecule that the receptor binds to. Receptors are often found on the exterior surface of a cell, and they can detect chemical signals originating from outside the cell. The usual convention is that a ligand is dissolved in solution or attached to the surface of a suspended cell that is free to diffuse and flow, while the receptor is attached to the stationary cell of interest. The binding between a receptor and a specific signal molecule ligand transmit can signal to the interior of the cell and initiate a response. Figure 1.2 shows a schematic diagram of receptor–ligand binding. The receptor-ligand binding is thought to involve a three-dimensional lock-and-key model where projections on the ligand molecule fit into complementary recesses on the receptor. The gap between a receptor and a ligand in the receptor–ligand binding interface is of the order of 0.2 to 0.5 nm. The sum of many non-covalent forces, including hydrogen bonds, hydrophobic and Van der Waals forces, and ionic interactions determine the overall strength and kinetics of the receptor-ligand binding.
When a ligand diffusing in the fluid encounters a receptor, there is a chance that they will bind together and form a receptor–ligand complex. Subsequently, the complex may break apart and there is a probability for this to happen in each short interval of time. When the number of binding (association) events per second is equal to the number of "unbinding" (dissociation) events, the reaction reaches a steady state, or equilibrium. The reversible reaction can be written as a chemical reaction Eq. (1.1):

$$ R + L \overset{k_f}{\underset{k_r}{\rightleftharpoons}} C $$

(1.1)

Here $R$ represents receptor, $L$ represents ligand and $C$ represents receptor–ligand complex. $k_f$ is the forward reaction rate for this process and $k_r$ is the reverse reaction rate [33]. The lifetime of the complex, $C$ ranges from milliseconds to months in the absence
of external forces. The average lifetime depends on the reaction rates, but each binding and unbinding event is a random process and a distribution of lifetimes will always be found. The rates for dissociation can be changed by applying external forces [34]. Forces ranging from a few tens to hundreds of piconewtons are usually sufficient to greatly speed up the rate of dissociation.

1.2.2  Bell model

A relationship between the dissociation rates for receptor–ligand bonds and applied external force was proposed in 1978 by G. I. Bell, based on transition state theory [35-37]. He posited that the reverse reaction rate \( k_r \) is an exponential function of applied force as shown in Eq. (1.2)

\[
k_r(f) = k_r^0 \exp \left( \frac{r_0 f}{k_B T} \right)
\]  

(1.2)

where \( k_r^0 \) is the reverse reaction rate without any applied force, \( k_B \) is Boltzmann’s constant, \( T \) is absolute temperature, \( f \) is the applied force per bond and \( r_0 \) is the “reactive compliance”, a parameter with units of length that describes how strongly the dissociation rate changes with applied force. When \( r_0 \) is small, the receptor-ligand dissociation rate depends weakly on applied force. When \( r_0 \) is large, however, dissociation rate depends strongly on applied force [38].

From Kramers” transition state theory [39], which is original source for Bell Model, Evans and Ritchie extended reaction kinetics in liquids to bond dissociation and proposed that the reverse reaction rate \( k_r \) has a general form [40]:

\[
k_r(f) = v_0(f) \exp \left[ \left( -U_0 + \Delta U \right) / k_B T \right]
\]

(1.3)
where \( \nu_0(f) \) is frequency of return to the dissociation barrier (which, in general, is a function of applied force), \( U_0 \) is the energy barrier height, and \( \Delta U \) is the change of energy barrier height induced by the applied external force. The exponential is a Boltzmann factor giving the probability of escape from the energy well. In the Bell model, the \( U_0 \) part of the exponential is separated out and combined with \( \nu_0(f) \) to get the zero force reverse reaction rate \( k_r^0 \) and \( \Delta U = r_0f \). The main idea of this simple model is that the interacting molecules in the bound state can escape over an energy barrier to the transition state by thermally activated diffusion.

A force spectroscopy method was presented by Evans et al. in the late 1990s [41, 42]. In most force probe application experiments, a finite interval time is required to apply force. The applied force was, thus, treated as a ramp with \( f(t) = r_f \cdot t \), where \( f(t) \) is the instantaneous force, \( t \) is time and \( r_f \) is the loading rate with \( [r_f] = \text{force/} \text{time} \) [15]. The dissociation is related to the observed most probable rupture force, \( f_B \), which also depends on loading rate [43-45]. Using reliability theory, the relationship between the force and \( r_f \) can be expressed (for the Bell model) as:

\[
f = \frac{k_B T}{r_0} \ln\left(\frac{r_0}{k_r^0 k_B T}\right) + \frac{k_B T}{r_0} \ln r_f \quad (1.4)
\]

If \( f \) is plotted as a function of \( \ln r_f \), then the slope and intercept of the resulting straight line will be given by:

\[
\text{slope} = \frac{k_B T}{r_0} \quad \text{and} \quad \text{intercept} = \frac{k_B T}{r_0} \ln\left(\frac{r_0}{k_r^0 k_B T}\right) \quad (1.5)
\]
A plot of most probable force at break-up vs. logarithm of the loading rate should have line segments that correspond to sets of Bell model parameters, $k_r$ and $r_0$ [40, 42, 46, 47]. The Bell model parameters can be calculated from the slope and intercept of each line segment [48-50]. The detailed derivations of the above equations are given in one of Dr. Tees’ previous papers [51].

**1.2.3 Catch-slip bond model**

Receptor-ligand bonds are classified by how their dissociation lifetimes respond to applied tensile force. If bond dissociation lifetime is shortened when force on the bond is increased, the bond is called a “slip bond”. If bond dissociation time does not change when forces are applied, the bond is an “ideal bonds”. If bond dissociation time is (counter intuitively) prolonged by applied force, the bonds are called “catch bonds” [52]. A small force can extend the mean lifetime of catch bonds. Most actual bonds that exhibit the catch behavior become slip bonds for higher applied forces [53]. They are thus called “catch-slip” bonds [54]. A critical force value can be defined for this catch-slip transition: below the critical force value, the bond behaves as a catch bond and above the critical force value, the bond is a slip bond [55]. In equation (1.3), $\Delta U(f) = r_0 f$ in the Bell model) can be positive, zero and negative to represent slip, ideal and catch bonds respectively. The applied force can pull the system toward the transition state from the bound state by doing positive work to lower the energy barrier. The bond lifetimes are shortened -- the characteristic of slip bonds. If the applied force pulls the systems further away from the transition state by doing negative work, the bond lifetimes are prolonged - - the characteristic of catch bonds[56, 57].
Selectins and a bacterial protein called FimH have been shown to form catch bonds in recent research [58, 59]. Increasing force first prolonged and then shortened the single bond lifetime for P-selectin and its ligand. This combination of both catch bond and slip bond behavior has been seen in AFM and flow-chamber experiments [60]. Both Selectin and FimH catch bonds mediate shear-enhanced adhesion [56]. Catch bond behavior has been proposed as a mechanism to explain why selectins require a threshold shear to support cell adhesion. The bonds between leukocytes and selectins will bind more strongly when the cells tether from the flow stream and transition to rolling adhesion as shear increases to a critical value [61]. The bonds between bacteria and FimH will bind more strongly when the cells change rolling adhesion to stationary adhesion.

1.3 Methods for Applying Force to Bonds

Since molecular bonds must be able to resist forces that pull the cells apart, force dependence of these receptor–ligand bonds plays a vital role in tissue architecture, cell movement and cell communication. Over the last two decades, a number of methods have been used to apply forces to receptor-ligand bonds and to manipulate individual molecules to study the force dependence of dissociation rates.

1.3.1 Atomic force microscopy (AFM)

Atomic force microscopy (AFM) is a well-established technique which allows direct force application and measurements for single molecule force spectroscopy (SMFS) [62-66]. This technique applies forces to receptor-ligand bonds using a sharp needle attached to a calibrated cantilever beam [67-69]. The scanned probe technology can measure interfacial forces, in addition to producing three dimensional images of the
profile of a surface [35, 70]. Force is applied through the needle-tip, and the cantilever deformation induced by the force is monitored using a laser technique. A laser beam is reflected off the cantilever deflection onto a quadrant photodiode allowing angstrom-scale accuracy for force measurement [71-73]. The force at the moment of bond dissociation can be measured from the degree of bending of the cantilever beam according to Hooke's law. The accuracy of such measurements is highly dependent on the determination of the cantilever spring constants [74, 75].

### 1.3.2 Optical tweezers (OT)

An optical tweezers system uses a highly focused laser beam to trap and manipulate particles varying in size from 100 nanometers up to a few micrometers [76-79]. Optical tweezers have been used in biological sciences to trap particles, cells, viruses, yeasts, and bacteria over the past three decades [80, 81]. Trapped particles are dielectric objects with an higher refractive index than that of the surrounding fluid medium [82]. The particles are affected by light scattering and gradient forces [83, 84]. Optical tweezers trap particles using the gradient force, not the scattering force. An infrared laser beam is brought to a point focus with the high numerical aperture objective lens of a standard research microscope [85]. A simple ray optic and momentum transfer argument can be used to explain the optical trapping when the particle size is much bigger than the wavelength of light [86]. The direction of the light (and hence its momentum) will change when the incident light passes through the particle with a higher index of refraction. The change of light momentum before and after refraction leads to a sideways restoring force toward the center of Gaussian beam and an in-line restoring force towards
the focus. In this way, the Gaussian beam acts as an “optical tweezers” or a 3 dimensional trap that can be used to pull or push the particle. Traps with a Gaussian intensity profile lead to a Hooke’s Law restoring force. An optical tweezers setup includes the following components: a laser with filters, a beam expander, a mirror or Spatial Light Modulator (SLM) for creating multiple movable traps, lenses and mirrors that are used to steer and focus the trap beam in the sample plane, and a microscope combined with a video camera. A near infrared laser was chosen since most biological materials have a low light absorption in the near infrared wavelength, thus preventing damage. In our lab the Optical Tweezers system set up used an 800 mW 1064 nm infrared laser. A high numerical aperture (NA) objective lens \( \text{NA} = n \sin \theta \) where \( n \) is index of reaction between sample and objective lens and \( \theta \) is half angle of light captured by the objective lens) is required for a stable trap with a gradient force that is sufficiently large [87, 88]. The positions of the optical traps were manipulated to apply forces on actin filaments to study the actin myosin mechanical properties [89].

1.3.3 Biomembrane force probe

The biomembrane force probe was pioneered and developed by Evans and collaborators as an alternative device for measuring unbinding forces [90, 91]. This device uses a micropipette-aspirated red blood cell coated with receptor as the force transducer. The cell is touched to a ligand-covered cell or particle held by another micropipette [92]. In a modification of this method, a flat surface was substituted for the bead for more accurate position discrimination [93]. This device also was improved by attaching a microscopic latex bead coated with receptor to a red blood cell which is
pressurized by micropipette suction. A number of complementary ligands have been examined with this method [94, 95]. A piezoelectric device is used to bring the receptor and ligand-coated surfaces together and then pull them apart. The well-characterized red blood cell used as the biomembrane force probe is a sensitive, tunable force transducer since the tension in the membrane, and hence the stiffness of the transducer can be easily changed using suction from the micropipette [96, 97]. The dissociation force can be measured by calibrating the stiffness of the red blood cell [98]. The dissociation force can be determined by this method and the bond lifetime can be measured by analysis of intervals from an association event to the next dissociation event [99].

1.3.4 Hydrodynamic flow

Hydrodynamic flow techniques have also been used for applying picoNewton-level forces to adherent cells in suspension. While hydrodynamic flow techniques have been used in the past to dissociate doublets of suspended cells or particles [100-103], the principal hydrodynamic method for forced unbinding uses the drag exerted on cells interacting with surfaces in parallel plate flow chambers. The plate surface are coated with a low density of suitable receptors to bind the cells allowing single bonds to form [104]. Similar experiments can also be used to look at cell motion in micropipettes using hydrostatic pressure [105]. Force dependence of rates can be measured by calculating hydrodynamic forces corresponding to a given shear stress and observing the lifetime of bonds under that force [106-109].
1.3.5 Microcantilever

The microcantilever is an alternative spring-based device for applying biologically-relevant picoNewton-level forces to the receptor-ligand bonds. The microcantilever setup was developed in the laboratory of Richard E. Waugh and collaborators and was used for pulling membranes tethers from red blood cells and measuring the tethering force during lipid bilayer tether extrusion from blood cell membrane [110, 111]. Microcantilevers made of long, thin, glass fibers were also used as force transducers for applying picoNewton forces to E-selectin and sialyl Lewis$^x$ bonds in Dr. Tees’s previous paper in 2001 [51].

The microcantilevers were made using custom fiber glass with 3 µm outer diameter and 2-10 mm long in Dr. Tees’s previous work. The glass fibers were coated with the adhesion molecule E-selectin. A latex bead of around 10 µm in diameter was coated with the selectin ligand, sialyl Lewis$^x$. The experimental procedure is shown in Figure 1.3. One end of the glass fiber was fixed in one micropipette (not visible in the diagram) but the end shown in the diagram was free to move. A bead sucked onto another micropipette tip that was connected to a piezoelectric actuator was pushed toward the fiber. When the bead touched the fiber, receptor-ligand bonds could form. The actuator then pulled the bead back from the fiber for a set distance at a set velocity. If there was a bond between the fiber and the bead, the fiber was forced to follow the bead and the force on bonds increased as the fiber deflected. The fiber deflections could then be found using image analysis. The force on the bonds at the moment of dissociation is found from the product of the spring constant of fiber and the fiber displacement at the moment when the
bond broke. A range of force loading rate (force/time) $r_f$ was produced by the computer deflection corrected with hydrodynamic and fiber spring forces [112]. The average force at the moment of bond dissociation was plotted versus with $\ln r_f$. The slope and intercept of the plot allowed the Bell model parameters to be calculated.

Figure 1.3: Adhesion between a carbohydrate-coupled bead held in a micropipette and a E-selectin-coated fiber microcantilever. (a) Arrangement before force application; (b) Arrangement after micropipette retraction with a bond formed.

The microcantilever system has several advantages over other techniques. First, it has ultra-high sensitivity to applied force. It is relatively easy to make cantilevers with spring constant on the order of a few pN/µm. Second, PicoNewton scale forces can produce microcantilever deflections large enough to be visible under the microscope so that the deflection can be accurately measured using video microscopy. In addition, the force loading can be varied over a wide range by varying the fiber length (and hence spring constant). Precise and accurate measurement of the microcantilever spring constant, however, is crucial for its application as a force probe for forced unbinding studies.
1.4 Elasticity Theory of Cantilever Stiffness

Elasticity theory can be used to find the spring constant for a glass fiber cantilever with circular cross section. A cantilever beam is shown in Figure 1.4 [113]. One end is firmly attached and the other end is free to move. If a force \( F \) is applied at point \( x \) on the beam, a deflection \( d \) will be induced. The spring constant \( k \) can be calculated by the force and applied the amount of deflection according to Hooke’s Law:

\[
k = \frac{F}{d}
\]

![Figure 1.4: Variables used in microcantilever beam.](image)

For a uniform beam for which the centroidal moment of inertia, \( I \), is constant along the beam, the deflection \( d \) at a point \( x \) on the beam can be calculated using:

\[
d = \frac{F}{6EI}(3Lx^2 - x^3)
\]

where \( L \) is the total length of the beam, \( E \) is the Young’s Modulus. For a fiber of beam with a circular cross section, the centroidal moment of inertia for the beam is:

\[
I = \frac{\pi D^4}{64}
\]
where $D$ is the fiber diameter. Plugging this expression into Eq. (1.6) and solving for the spring constant one gets:

$$k(x) = \left( \frac{\pi D^4}{64} \right) \frac{6E}{(3Lx^2 - x^3)}$$  \hfill (1.8)

When the force is applied at the fiber tip, $x = L$, and the spring constant at the fiber tip becomes:

$$k_{tip} = \frac{3\pi ED^4}{64L^3}$$  \hfill (1.9)

The detailed derivations of the above equations are given in my Master’s thesis [114]. The spring constant depends on the material that beam is made of (through the Elastic Modulus of the material) as well as the dimensions of the beam. The spring constant can be adjusted by altering the length and diameter of the glass fiber according the above equation [115]. One can thus easily make cantilevers with expected spring constant on the order of a few pN/μm by selecting suitable length and diameter fibers.

Measuring the diameter of fiber cantilever accurately from a microscope image, however, is very difficult due the small size of the glass fiber cantilevers and the fact that under the micropipette the edges show up as a complex diffraction pattern that makes determining the true edge difficult. To be certain of the spring constant, it is thus very important to have an independent method of calculating the microcantilever spring constant.

1.5 Thermal Fluctuation Method

The harmonic oscillator Hamiltonian is
\[ H = \frac{p^2}{2m} + \frac{1}{2} m \omega^2 x^2 \]  

(1.10)

where \( p \) is the momentum of the oscillator, \( m \) is its mass, \( x \) is the oscillator’s displacement from its rest position. \( \omega \) is the angular frequency at resonance, which for a simple spring is given by

\[ \omega = \frac{k}{\sqrt{m}} \]  

(1.11)

where \( k \) is the effective spring constant of the oscillator.

According to the equipartition theorem from statistical mechanics[116], each quadratic term in Eq. (1.10) has an average value of \( k_B T / 2 \), giving:

\[ \langle \frac{1}{2} m \omega^2 x^2 \rangle = \frac{k_B T}{2} \]  

(1.12)

Plugging in Eq. (1.12) yields:

\[ \langle \frac{1}{2} m \frac{k}{m} x^2 \rangle = \frac{1}{2} k \langle x^2 \rangle = \frac{k_B T}{2} \]  

(1.13)

Rearranging allows us to solve for the spring constant [117]:

\[ k = \frac{k_B T}{\langle x^2 \rangle} \]  

(1.14)

where \( k \) is the spring constant of a given microcantilever, \( x \) is the displacement of the microcantilever from its rest position, \( k_B \) is Boltzmann’s constant and \( T \) is the absolute temperature. \( k_B T \) is the basic thermal energy unit in statistical mechanics. At room temperature, \( 1 \ k_B T \) is around 4.1 pN·nm [118]. This method (using Eq. 1.14) is known as the thermal fluctuation method for evaluating spring constants. The spring constant is found by measuring the variance of the cantilever’s tip position at given temperature. The
measurement of the microcantilever’s spring constant is, thus, achieved by recording video images of the cantilever motions under an optical microscope, then with the aid of computer, the centroid of each cantilever image in the video can be determined and the variance can be calculated in the time domain. The spring constant can then be calculated using Eq. (1.14). Although visible light cannot resolve two objects that are spaced closer than about half a wavelength apart (~200 nm at best), it is possible to determine the location of a centroid using optical methods to a precision that is limited only by noise in the image capture system [119].

Since the publication of the thermal fluctuation calibration method in 1993, it has been widely used to calibrate cantilevers for measuring receptor-ligand bonding properties. For example, an AFM-based force spectroscopy was calibrated with thermal fluctuation method to study cell detachment forces on living leukocytes which allowed force to be measured with resolution down to the level of individual molecules. The principle is used for the characterization of thermal fluctuation changes of an AFM before and after it is coupled to a rigid surface via a receptor-ligand bond. Using this relation, the elastic moduli of L- and P-selectin complexed with P-selectin glycoprotein ligand-1 (PSGL-1) was measured [120]. The catch-slip transitional bonds were observed in L-selectin-PSGL-1 and P-selectin-PSGL-1 interactions.

In previous spring-based techniques, the forces were applied to receptor-ligand bonds with some constant loading rate. The average or most probable force at unbinding was found to increase linearly as a logarithm of the loading rate in the force spectroscopy model. In most force probe application experiments, it takes a significant amount of time
to establish the desired force on the bonds since the elastic element must be stretched. The typical scale of these loading rates was that a 100 pN force needed 0.01-1 seconds time period to load. The dissociation life time is related to rupture forces which were the function of the loading rate.

It would be better to apply forces quasi-instantaneously on receptor-ligand bonds, instead of that a finite interval time is required to apply force. This would make possible a direct measurement of the force dependence of bond lifetime. The lifetime as a function of force could be directly observed instead of using the loading rate. A novel microcantilever unbinding technique system could be designed to allow near-instantaneous application of forces. In this Ph.D. dissertation research, a novel microcantilever unbinding technique system (with the advantages of speed of force application and of being able to determine the best model for force dependence of bond lifetime rather than assuming one) was built and shown to be useful for measuring bond lifetime as a function of force.
CHAPTER 2: SPRING CONSTANT MEASUREMENT RESULTS FOR FIBER MICROCANTILEVERS

The spring constants of E-glass fiber microcantilevers needed to be determined for receptor-ligand bonding experiments. The diameter is the most uncertain factor for propagating the error in the spring constant. This chapter presents glass fiber diameter measurements using scanning electron microscope (SEM) and optical imaging experiments. The spring constant of each glass fiber was measured using the thermal fluctuation method and compared with the value from elasticity theory.

2.1 Materials and Methods

2.1.1 Fabrication of glass micropipettes

Capillary glass (Friedrich & Dimmock, Millville, NJ) with 0.5 mm inner diameter (ID) and 0.9 mm outer diameter (OD) was used to form micropipettes using a vertical Needle/Pipette Puller (David Kopf Instruments; Model 730, Tujunga, CA) which is shown in figure 2.1. The details of pulling and forging are given in my Master’s Thesis[114]. The first and second pull heat settings were 10.2. A solenoid setting of 1.2 was used for the second pull, which was not limited in extension. In Figure 2.1, the light in the upper left corner is the hot heater filament melting the capillary glass during the first pull.
Figure 2.1: David Kopf vertical Needle/Pipette Puller 730 for micropipettes fabrication
Reproduced with modification from my Master’s thesis [114].

After the micropipettes were made, the micropipette tips were further adjusted using an MF 200 Microforge with a MF200-H2 filament (World Precision Instruments Inc., Sarasota, FL) under a Zeiss dissecting microscope (Stemi 2000-c, Bridgeport, NJ) with illumination from a Fiber Optic Illuminator (Dolan-Jenner, Industries, Inc. Lawrence, MA). A picture of the setup is shown in Figure 2.2. The internal diameter (ID) of the micropipette tips was observed optically under a microscope (Nikon Eclipse TE
300, Fryer Co., Huntley, IL) with 20x magnification. The micropipette tips images were captured by a custom made LabVIEW image program named “capturesaveimage.vi”. The internal diameter (ID) of the micropipette tips was measured using “capturesaveimage.vi” program with one or two pixels (0.256 ~ 0.51 μm) error. The ID of micropipette tips used for measurement experiments of glass fiber microcantilevers spring constants ranged from 11 to 15 μm. The ID of the micropipette tips used for applying hydrodynamic force to bonds ranged from 7 to 12 μm.

Figure 2.2: Micropipette tip adjustment with microforge. Reprinted from my Master’s thesis [114].

2.1.2 Fabrication of microcantilever

E-glass fiber sections (MO-SCI, St. Louis, MO) were laid on a tissue-culture dish lid, picked up by micropipettes using static electricity, and inserted into the tips of micropipettes by hand under the Zeiss dissecting microscope with fiber optic illumination
as shown in Figure 2.3. The E-glass fibers were glued in place with UV curing adhesive (Norland Optical Adhesive 71; Norland Products Inc. Cranbury NJ) and irradiated with 365 nm UV illumination (Spectroline E-series; Spectronics Corp., Westbury, NY, USA) for more than one hour to allow the UV adhesive to cure completely. The resulting mounted E-glass fibers were trimmed to lengths ranging from 3 mm to 7 mm using a sharp pincer (model 511-A, Ted Pella, Redding, CA) in order to investigate the relationship between fiber length and the spring constant of the fiber. The lengths of fibers were measured with a ruler under a Zeiss dissecting microscope. An assembled fiber microcantilever is shown as Figure 2.4. Microcantilevers were stored in a custom-made micropipette holder with a plastic dust cover until they were needed for experiments.

Figure 2.3: Inserting an E-glass fiber into the tip of a micropipette. Reproduced with modification from my Master’s thesis [114].
Figure 2.4: Assembled fiber microcantilever.

2.1.3 **Design of viewing chambers**

2.1.3.1 **Flow viewing chamber**

A great deal of effort was devoted to producing a viewing chamber that was suitable for the microcantilever experiments. A waterproof flow viewing chamber was developed for the experiments to assess microcantilever spring constants. The chamber was composed of two layers of lexan plate attached using silicone sealant (DAP Inc., Baltimore, MD, USA). A schematic drawing of the viewing chamber without fibers is shown in Figure 2.5. Both top and bottom plates contained a square viewing window at the center. The dimensions are also shown in the figure 2.5. Glass cover slips (22 mm x 22 mm; SPI Supplies, West Chester, PA) were attached over the square viewing windows to complete the construction of the viewing chamber. The cover slips were fitted into countersunk shelves surrounding the central window so that the chamber would be flush even when placed on the microscope stage. Two 0.06 " holes in the bottom plate were
used as an inlet and an outlet to allow liquid to fill and flow through the chamber. The holes were located at opposite corners of the center square viewing window to help avoid air bubbles in the chamber during filling. Two 0.043" holes in the top plate were used for mounting microcantilevers in the chamber. Silicone sealant was used to secure the micropipettes into the slots and to seal the gap around each microcantilever assembly in the slot. A groove on the bottom plate was filled with silicone sealant to make a watertight seal between the top plate and the bottom plate. The whole lengths of the fibers were mounted within the viewing chamber window. The silicone sealant was allowed to cure for 24 hours before the chamber was used for an experiment. Two syringes (BD, Florence KY) were connected with Tygon tubing to the inlet and outlet ports to inject the liquid into the chamber. The tubing could be clamped to prevent liquid from flowing out of the chamber. The biological buffer HBSS- (Hanks’ Buffered Saline Solution without Calcium, Magnesium or Phenol Red; Lonza, Walkersville, Inc. Walkersville, MD) was injected into the chamber and air was pushed out through the outlet channel. Before filling the chamber with HBSS-, the liquid was filtered using a 0.2 µm filter (Whatman Inc. Clifton, NJ) to remove bacteria and impurities larger than 0.2 µm. After the filling, the chamber was put in a tissue-culture dish (Fisher Scientific, Hanover Park, IL) and placed on the microscope stage for experiments. The tissue-culture dish made positioning and handling easier on the microscope stage.
2.1.3.2 *Thin viewing chamber*

A thin viewing chamber was designed for use with the 100x magnification objective lens experiments. The depth of focus is smaller for the higher magnification so the microcantilevers needed to be closer to the bottom surface of the chamber than in the flow chamber described above. The thin viewing chamber was used to check the calibration of glass fiber spring constant at higher magnification.
A schematic drawing of the thin viewing chamber is shown in Figure 2.6. To build this chamber, a 22 mm x 22 mm square microscope cover slip (01023-AB; SPI Supplies; West Chester, PA) was placed on a rectangular microscope slide (3” x 1” x 1 mm; VWR Scientific; West Chester, PA). Two strips of single-sided adhesive tape (Scotch transparent; 3M, St. Paul, MN) were placed parallel to one another on the square cover slip with an approximately 5 mm gap in between the strips. Different length E-glass fibers were laid on the tape strips. Two strips of double-side adhesive tape were adhered to the two strips of single-sided tape to secure the fibers. The thickness of the single-sided tape is approximately 58 µm. The thickness of double-side tape is 80 ± 5 µm thick. A round cover slip of 18 mm diameter (01025-AB; SPI Supplies; West Chester, PA) was placed on the double-sided tape to complete the chamber. HBSS- solution was injected into one end of the narrow channel that contained to fibers to fill the chamber by capillary action. Clear nail polish was used to seal the ends to prevent evaporation. If an air bubble appeared inside the chamber, the chamber and microcantilever were discarded.
2.1.4 **Optical microscopy**

Some fiber spring constant measurements and all of the molecular binding experiments were observed under an inverted optical microscope (Nikon Eclipse TE 300, Fryer Co., Huntley, IL) with a 40x bright field objective lens (Plan Fluor, NA 0.6, Nikon, Japan) and a 20x bright field objective (Plan Fluor, NA 0.45, Nikon, Japan). The fiber motion inside the viewing chamber was recorded using a CCD video camera (Cohu, Fryer Co., Huntley, IL) which was connected to the side port of microscope. The camera frame rate was 30 frames per second. The video camera sent images to a video monitor (Sony Trinitron) through a Video Cassette Recorder (Sony SVO-9500MD, Fryer Co.,
Huntley, IL). All images are captured in real time using custom made LabVIEW image capture programs (National Instrument, Austin, TX).

The fibers’ thermal fluctuation motions in a flow chamber or a thin viewing chamber were also observed under another inverted optical microscope (Eclipse TE2000; Nikon Instruments Inc.; Melville, NY) with a 40x objective and a 100x oil immersion objective. A video of fiber motions was recorded using a monochrome digital camera, (PL-B761F, PixeLink, Ottawa, Ontario, Canada). The PL-B760 series cameras operate at 69 frames per second (fps) in free running mode at a resolution of 752 x 480. The maximum frame rate depends on resolution, region of interest size and exposure time. The frame rate could be increased by increasing the pixel averaging, by decreasing the region of interest or by decreasing exposure time. PixeLink’s PL-B760 Frame Rate Calculator application was used to choose the conditions needed for a given frame rate by varying the region of interest size and exposure time. The relationship between the image size and the frame rate (frame/second) is shown in formula below:

\[
\text{Frame rate} = \frac{26666667}{(MAX(\text{width,661}) + 43) \times (\text{height} + 5)}
\]  

(2.1)

The region of interest on fiber images was repeatedly tested to find the proper size for a high frame rate. A region of interest that was a horizontal band 360 pixels wide and 40 pixels in height was chosen in all videos. Use of this region of interest allowed the frame rate to be increased to 743 frames per second.

2.2 Calculation of Spring Constants from Elasticity Theory

The E-glass fiber microcantilevers were to be used to measure the force dependence of receptor-ligand bond dissociation rates. The spring constant of glass fiber
needed to be characterized to determine the force applied to the receptor-ligand bonds.

Spring constants for glass fibers with circular cross sections were calculated from
elasticity theory using the equation (1.9):

\[ k_{tip} = \frac{3\pi ED^4}{64L^2}. \]  

(1.9)

The spring constant at the fiber tip depends on the composition of the fiber \((E)\),
the fiber cross sectional diameter, \(D\), and fiber length, \(L\). The E-glass fibers used in the
experiments had the fixed composition and cross-sectional dimension with an elastic
modulus \(E = 7.40 \times 10^{10} \text{ N/m}^2 [121-123]\). The length and diameter were the two main
sources of random. The spring constant uncertainty was calculated using the standard
error propagation formula [124]:

\[ \frac{\delta k}{k} = 4 \frac{\delta D}{D} + 3 \frac{\delta L}{L}. \]  

(2.2)

Segments of glass fiber, several millimeters long, were used in these experiments.
Initially, the length was measured under a microscope using a ruler, but it was later found
that it could be determined much more accurately with a microscope eyepiece reticle
under 10x magnification in our Nikon TE 300 microscope. It is straightforward to
measure the length of the fiber (with an error of \(~1\%\)). The fact that the length is cubed in
Eq. (1.9), however, means that that error is magnified to \(~3\%). The diameter, \(D\), which is
nominally 4 µm in the specifications for the fiberglass material obtained from the
manufacturer, remains a major source of uncertainty. Since that uncertainty is multiplied
by a factor of 4 in the estimation of the spring constant, this is a serious concern and the
most uncertain factor. In order to have as accurate and precise a measurement as possible,
the diameter of glass fibers was measured by SEM and light microscopy as described below.

2.2.1 **SEM measurements of glass fiber diameter**

Custom-made E-glass fibers (MO-SCI Corporation, Rolla, MO) with a nominal diameter of 4 μm were cut into sections, sputter-coated with gold and mounted on a plate with a double-sided carbon tape. The fibers were then imaged using a Scanning Electron Microscope (SEM, Jeol, JSM 5300, Tokyo, Japan). A micrometer scale was also imaged using the SEM as a length measurement control.

Seven fibers were investigated to evaluate the actual glass fiber diameter using SEM imaging. A scanning electron micrograph of one E-glass fiber is shown in Figure 2.7 (a). An SEM image of a calibration scale at the same magnification is shown in Figure 2.7 (b). Each pair of lines in this calibration scale is spaced 10 µm apart. The calibration scale allowed the pixel-to-micrometer conversion to be determined. The seven fibers measured had an average width of 81.6 ± 6.6 pixels (measured using Paintbrush for Windows). This corresponded to a fiber width of 6.1 ± 1.02 µm. The measurement value is considerably larger than the nominal 4 µm value obtained from the manufacturer. The SEM images were still rather fuzzy at these edges and this leads to continuing uncertainty about whether the measured diameter is too large compared to the actual diameter value.
2.2.2 Light microscopy measurements of glass fibers

The diameter of glass fibers was investigated under an optical microscope (Eclipse TE 200; Nikon Instruments Inc.; Melville, NY) with a 100x oil immersion objective lens. The optical microscopic image of a glass fiber is shown in Figure 2.8 (a). A diffraction pattern was seen at the fiber edges. The darkest positions were taken as the fiber edges. The pixel intensities of all horizontal cuts through the fiber are plotted in Figure 2.8 (b). The data in the plot were obtained by a LabVIEW program named “vertical average x pixels value.vi” (see Appendix 1). The locations of the two intensity minima were taken as representing the fiber edges. The average distance between the two intensity minima was calculated using a LabVIEW program named “twominD.vi” (see Appendix 2). The micrometer scale was also imaged as a length measurement control. The distance between the bars on the scale is also 10 µm.
Figure 2.8: (a) A optical microscope image of a glass fiber. (b) Pixel intensity for the average of all horizontal cuts through the captured fiber image in Figure 2.8 (a).

In order to determine how changes in focal plane affected the apparent width of the fiber, glass fibers were imaged at 1.0 ± 0.1 µm intervals on the focus knob, so that each frame was separated from the next by 1 µm vertically. The fiber could be said to be in some semblance focus for up to 12 µm of vertical travel (i.e. 12 frames). A series of images of the glass fiber as the focal plane was changed are shown in Figure 2.9. The measured diameter of two different glass fibers increased as the focal plane approached the midpoint of the fiber, as shown in Figure 2.10. The measured diameter ranged from 3.8 µm to 6.2 µm. The diameter from the images increased, and then plateaued between 4.1 µm to 4.3 µm which should be closest to the real fiber diameter. It then continued to increase, but the image was clearly out of focus. The fiber width from an optical microscope measurement should, therefore, be 4.2 ± 0.2 µm, assuming that the distance between the minima accurately corresponds to the fiber diameter. SEM and optical
measurements produce divergent estimates of the fiber diameter. It is necessary to have another independent measurement method of the microcantilever spring constant in order to settle which diameter is more accurate.

Figure 2.9: The series images of glass fiber 1 as the focal plane was increased

Figure 2.10: Microcantilever measured diameter increased as focal plane moved through the fiber
2.3 Thermal Fluctuation Measurement of Spring Constant

Spring constants of glass fibers were also measured by finding the variance of the cantilever’s tip position at a given temperature according to the Thermal Fluctuation method (Equation 1.14):

$$k = \frac{k_B T}{\langle x^2 \rangle}$$  \hfill (1.14)

The measurement of glass fiber microcantilever spring constants was achieved by tracking the fiber position as a function of time and finding the fiber centroid variance due to thermal fluctuations. The measurement method is independent of the geometry of the cantilever. The spring constant of glass fiber could be obtained at any time after the microcantilever had been constructed. The experimental method is described in detail as follows. All measurements were made at 25°C.

2.3.1 Image analysis

The variance of the fiber centroid position was calculated from images of the fiber’s Brownian motion. Images of the fiber’s motion inside the viewing chamber were analyzed using custom-made LabVIEW image processing programs to find the centroid of the fiber microcantilevers. Images were read frame by frame. The intensity along a horizontal line passing through the fiber using the full unthresholded image was determined. The x centroid was determined from features on the graph (as described below). This procedure was repeated for the whole y range to get the average x centroid of each frame. The intensity profiles were different depending on the plane of focus. For example, as shown in Figure 2.11, a central peak showed up in the horizontal cut pixel intensity graph for certain focal planes. The x centroids could be found from the intensity
peak locations. The two intensity troughs representing the fiber edges were identified. For other focal planes, the outer shoulder intensities were higher than the central peak intensity value, so it was not possible to simply find the location of the maximum intensity. To correct for this, the two troughs were used as references, the location of the fiber intensity peak sandwiched between them was determined and used as the fiber centroid’s horizontal location for variance calculations. A LabView program “peak.vi” for image analysis (Appendix 3) was developed to find the fiber centroid location for this circumstance. Figure 2.12 shows a flow chart of this image processing program.

![Figure 2.11: (a) Captured image of a microcantilever tip. (b) Pixel intensity for average of all horizontal cuts through the captured fiber image in Figure 2.10 (a).](image)
Figure 2.12: Flow chart of the image processing program “peak.vi”

Another commonly seen intensity graph was shown above in figure 2.9 (b). The profile featured the usual pair of troughs and also a pair of peaks. It was more commonly seen that the intensity graph had a pair of trough or a pair of peaks than graphs that had just one central peak. The left and right intensity peak or trough locations were determined by finding corresponding maxima or minima in the graph plots. The midpoint of the peaks or troughs locations was calculated by the average value of maximum or minimum intensity pixel numbers from both fiber edges for each horizontal cut through the image. The whole y range of horizontal cuts was scanned to get the average intensity peak or trough locations from both fiber edges of one image. The x centroid was the
average intensity peak or trough locations from both fiber edges. The LabVIEW image processing program “twomax.vi” (Appendix 4) or “twomin.vi” (Appendix 5) was used to find the centroid of fiber microcantilevers for this case.

This image analysis process was repeated for all frames in the video to get the x centroid for the fiber for each frame, and calculate the mean and variance of the x-component of centroid position of all the frames. These programs were developed to read the images frame by frame, plot the graph of intensity along a horizontal lines passing through the fiber using the full unthresholded images. This method of finding centroids provides a more accurate spring constant measurement of microcantilevers compared to a previous program “d mod fast.vi” that used a thresholded image. The LabVIEW code for program “d mod fast.vi” is given in my Master’s thesis Appendix 2 [114].

2.4 Spring Constant Measurement Results

2.4.1 Experimental methods

The Brownian fluctuations of the fiber tips were recorded under a Nikon TE200 inverted microscope using either 40x magnification or 100x magnification. The video clips for each fiber were captured to PC using a digital camera connected to the microscope viewing port. Videos were decomposed into individual images using the program VideoMach at 30 frame/s time resolution. Images of the fiber” motions were analyzed using the LabVIEW program “peak.vi”. These experiments were conducted with HBSS- as the surrounding medium. This helped to damp low frequency driven oscillations better than with air as the surrounding medium. However, the low frequency oscillations were incompletely damped by the vibration isolation system. This low
frequency component could be subtracted from the original signal to give a signal that represented the thermal oscillation alone. To obtain the thermal deflections of fiber from these signals, a 50 point moving average filter (25 on either side of the point of interest) was to approximate the low frequency component in the signal. The filtered background was removed from the data to eliminate drift, and the variance of the resulting modified time sequence was computed.

Figure 2.14 shows fiber centroid displacement vs. frame number for 7, 6, 5, 4 and 3 mm-long fibers. The blue lines (values shown on right axis) represent the original microcantilever centroid displacements. The pink lines (values shown on right axis) represent the 50 point running average. The yellow lines (values shown on left) represent the final signal which is the difference between the original signal and the running average of the signal. Filtered displacements, which represent the thermal oscillation alone, were then used to compute the variance in centroid position for the fibers. Each fiber’s spring constant value was determined using these variances by the thermal fluctuation method. The standard deviation, which is the square root of the variance, and the corresponding spring constant are shown on each graph. All fibers showed some overall drift. The 7 and 6 mm-long fiber displacements had large drifts. The 5, 4, and 3 mm-long fiber displacements were less affected by outside disturbances, presumably because of their larger spring constants. The 50 point moving average filter worked well for removing the low frequency noise; however, the choice of 50 points was arbitrary and it was important to understand how the spring constant estimates changed with the size of
the smoothing window. The effect of smoothing window size on spring constant estimates was investigated using Monte Carlo simulation.
Figure 2.13: Fiber centroid displacement vs. frame number for 7, 6, 5, 4 and 3 mm-long fibers at 25°C using 40x magnification.
2.4.2 Monte Carlo simulation for moving average filter

The input-output relation for a moving average filter in the time domain is [125]:

\[ y(i) = \frac{1}{N} \sum_{j=0}^{N-1} x(i + j) = \frac{1}{N} [x(i) + x(i + 1) + \cdots + x(i + N - 1)] \tag{2.3} \]

where \( N \) is number of average points, \( x(i) \) and \( y(i) \) are input and output of moving average filter, respectively. The Fourier Transform [125] of equation 3.2 is called the transfer function on the frequency domain (also called frequency response [125]):

\[ H(\omega) = \frac{Y(\omega)}{X(\omega)} = \frac{1}{N} [1 + e^{-j\omega} + \cdots + e^{-(N-2)\omega} + e^{-(N-1)\omega}] \tag{2.4} \]

The magnitude of transfer function (also called magnitude response [125]) for a moving average filter can be derived as [126]:

\[ |H(\omega)| = \left| \frac{Y(\omega)}{X(\omega)} \right| = \frac{\sin\left(\frac{N\omega}{2}\right)}{N\sin\left(\frac{\omega}{2}\right)} \tag{2.5} \]

where \( \omega = 2\pi f / F_s \) [125].

In the case of normalized sample frequency, where \( F_s = 1 \) Hz, \( \omega = 2\pi f / F_s = 2\pi f \).

Therefore, the above transfer function is commonly expressed as [126]:

\[ |H(f)| = \frac{\sin(N\pi f)}{N\sin(\pi f)} \tag{2.6} \]

From the above transfer function, it can be seen that the magnitude response of the moving average filter varies with different numbers of average points, \( N \). Based on above transfer function, MATLAB program “movingaveragefilter.m” (attached as Appendix 6) was used to calculate the theoretical magnitude response. It first set the normalized frequency \( f = \left[ \frac{1}{4000}, \frac{1}{2} \right] \) at a step of \( \frac{1}{4000} \), and then calculated the theoretical magnitude response using the above transfer function. The theoretical magnitude response for the case of \( N=10, 20, \) and \( 50 \) is shown in Figure 2.15. It can be seen magnitude response is
close to 1 on DC and low frequencies, which means the output has no attenuation on DC and low frequencies. With the frequency increasing, the magnitude response decreases, which means the output has attenuation on higher frequencies. According to Fig.2.15, when moving average filter uses more average points, the output will attenuate more quickly on high frequencies. The purpose of moving average filter is to attenuate high frequency components, while keep the low frequency components unchanged. Because the frequency characteristics of the original data are unknown, the method should be developed to evaluate the frequency characteristics and find the optimum number of average points in the moving average filter.

Figure 2.14: The theoretical magnitude response of the average moving filter in the case of $N=10, 20, \text{ and } 50$. 
The method for finding an optimum averaging window width is given in Figure 2.15.

The original data provided the fiber positions in time domain. Those fiber position vs. time data were sampled with a certain sample frequency or frames per second (fps). A Fast Fourier Transformation (FFT) was performed. The output of FFT is the Fourier
transform of the fiber position, in the frequency domain. It can also be expressed as a
power spectrum that was estimated as the square of the absolute value of the Fourier
transform. Since the initial data is from the image processing on fiber position via
LABVIEW, the unit of the power spectrum axis is m²/Hz, given in dB format \[10 \cdot
\log_{10}(\text{power spectrum})\]. The power spectrum of fiber position for the 4 mm long fiber
sampled at a frequency of 30 Hz (section 2.4.1) is shown in Fig.2.16.

Figure 2.16: Power spectrum of fiber position in experimental data used to obtain a low
frequency sample for use in simulations in case of sample frequency of 30Hz.

Because this data was collected at 30 Hz, the frequency resolution for the FFT is
30/P Hz (where P is the number of original data points = 2450 for the 4 mm fiber). The
MATLAB program “ReadFFT.m” (Appendix 7) was used to read the original data and
perform the FFT.
To find the optimum number of averaged points, \( N \), in the moving average filter, a low frequency component, which is similar to the original data, should be provided. The low frequency components for the 4 mm fiber, which consists of direct current (DC) and 7 frequency tones, are listed in Table 2.1. The cutoff at 7 tones is arbitrary, but higher frequency tones can be neglected when compared with DC and first 7 frequency tones. The frequency resolution of the FFT is \( 30/P \) Hz (where \( P \) is the number of original data points). Thus, the discrete frequencies are DC, \( 30/P \) Hz, \( 60/P \) Hz, \( 90/P \) Hz, etc. The time interval between samples is \( 1/30 \) s.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Magnitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>345.3578</td>
</tr>
<tr>
<td>30/P</td>
<td>0.4408</td>
</tr>
<tr>
<td>60/P</td>
<td>0.1742</td>
</tr>
<tr>
<td>90/P</td>
<td>0.0945</td>
</tr>
<tr>
<td>120/P</td>
<td>0.0755</td>
</tr>
<tr>
<td>150/P</td>
<td>0.0715</td>
</tr>
<tr>
<td>180/P</td>
<td>0.0458</td>
</tr>
<tr>
<td>210/P</td>
<td>0.0703</td>
</tr>
</tbody>
</table>

Using the data from Table 2.1, one can calculate a waveform with the low frequency component found in experiments using:

\[
N(t) = DC + m_1 \sin(2\pi f_1 t) + m_2 \sin(2\pi f_2 t) + m_3 \sin(2\pi f_3 t) + m_4 \sin(2\pi f_4 t) + m_5 \sin(2\pi f_5 t) + m_6 \sin(2\pi f_6 t) + m_7 \sin(2\pi f_7 t)
\]
As long as the shape of the experimental frequency spectra remains the same, the simulated optimum number of points for the moving average filter should be applicable to other sample frequencies or video frame rates (fps).

The MATLAB program “method_verify.m” (see Appendix 8) was used to perform the simulations. A fiber can be thought of as undergoing Brownian motion in a harmonic potential. The potential energy is \( E = \frac{1}{2} k_s x^2 \). \( k_s \) is the spring constant. So the probability for the fiber being at position \( x \) could be derived as:

\[
p(x) = \left( \frac{k_s}{2\pi k_B T} \right)^{1/2} \exp \left( \frac{-k_s x^2}{2k_B T} \right)
\]

Drawing random numbers from this distribution would simulate Brownian motion data and obey the Gaussian distribution [127]. To simulate those random Brownian time series, the value of \( k_{\text{spring}} \) was first defined, and then the variance was calculated using Eq. (1.14). Finally, the random data with this variation was generated from a Gaussian distribution using a MATLAB program. In this program, \( k_{\text{spring}} \) was defined as 1, 10, and 100. Note that unlike a Langevin treatment of Brownian motion which would have a built-in time scale from viscous drag, this much simpler method has no built-in time scale. It should, however, be valid as long as the sampling frequency is less than the corner frequency where the power spectrum begins to roll off at high frequency. As shown below, the sample frequencies used here are well below the corner frequency.
Next, the program built low frequency component using magnitudes from FFT analysis in the program “Read_FFT.m”. The random Brownian motion data was combined with the low frequency component derived from an experiment, and the resulting time series was processed using a moving average filter with \( N = 10, 20, 50, \) and 100. The output of the moving average filter was removed from the combined data, and the remaining data was used for \( k_{spring} \) calculation. If \( N \) is optimum, the moving average filter should remove the low frequency component, and the calculated \( k_{spring} \) should be close to the defined \( k_{spring} \) that was used to generate the random data.

Comparing the \( k_{spring} \) value from the simulation with the \( k_{spring} \) value used to generate the data, it was possible to find the optimum average number of points, \( N \), in the smoothing filter. Tables 2.2, 2.3, 2.4 and 2.5 provide the calculated \( k_{spring} \) values for 3 mm, 5 mm and 6 mm-long simulated fibers with moving average filters of different lengths. Standard deviations were obtained from 5 iterations of Monte Carlo simulation for each moving average filter length and fiber length.

It can be seen that for the case of \( N = 50 \), the calculated \( k_{spring} \) was within 7% of the defined \( k_{spring} \). It is, thus, reasonable to use a 50 point moving average filter. It was found, however, that \( N=20 \), was even better, with the calculated \( k_{spring} \) differing by less than 5% error from the defined \( k_{spring} \). Therefore, \( N=20 \) should be the optimum number of average points in a moving average filter. The calculated \( k_{spring} \) from the data of is also included in Table 2.3-2.5. It shows that \( N=20 \) has a calculated \( k_{spring} \) which is closest to the defined \( k_{spring} \). Because the environmental noise, which is taken from the low frequency component in the for the 4 mm-long fiber measurement data, is expected to be
similar for the 3, 5 and 6 mm-long fibers, the optimum $N=20$ should be applicable to all measurement cases.

Table 2.2 $k_{spring}$ with different number of points in moving average filter for a simulated 4 mm fiber

<table>
<thead>
<tr>
<th>Number of Points (Moving Average Filter)</th>
<th>$k_{spring}=1$</th>
<th>$k_{spring}=10$</th>
<th>$k_{spring}=100$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated $k_{spring}$</td>
<td>Calculated $k_{spring}$</td>
<td>Calculated $k_{spring}$</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>1.0±0.05</td>
<td>5.2±0.1</td>
<td>40.6±1.5</td>
</tr>
<tr>
<td>200</td>
<td>1.0±0.04</td>
<td>7.4±0.2</td>
<td>62.2±1.8</td>
</tr>
<tr>
<td>100</td>
<td>1.0±0.04</td>
<td>8.7±0.3</td>
<td>84.0±3.3</td>
</tr>
<tr>
<td>50</td>
<td>1.01±0.04</td>
<td>9.6±0.2</td>
<td>93.9±3.0</td>
</tr>
<tr>
<td>20</td>
<td>1.05±0.06</td>
<td>10.3±0.2</td>
<td>101.2±1.8</td>
</tr>
<tr>
<td>10</td>
<td>1.1±0.05</td>
<td>11.1±0.2</td>
<td>108.9±1.8</td>
</tr>
</tbody>
</table>

Table 2.3 $k_{spring}$ with different number of points in moving average filter for a simulated 3 mm fiber

<table>
<thead>
<tr>
<th>Number of Points (Moving Average Filter)</th>
<th>$k_{spring}=1$</th>
<th>$k_{spring}=10$</th>
<th>$k_{spring}=100$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated $k_{spring}$</td>
<td>Calculated $k_{spring}$</td>
<td>Calculated $k_{spring}$</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>1.02±0.02</td>
<td>6.0±0.1</td>
<td>59.1±1.2</td>
</tr>
<tr>
<td>200</td>
<td>1.02±0.02</td>
<td>7.9±0.2</td>
<td>80.3±1.6</td>
</tr>
<tr>
<td>100</td>
<td>1.02±0.02</td>
<td>8.9±0.1</td>
<td>90.2±0.7</td>
</tr>
<tr>
<td>50</td>
<td>1.03±0.02</td>
<td>9.6±0.2</td>
<td>95.8±0.8</td>
</tr>
<tr>
<td>20</td>
<td>1.06±0.02</td>
<td>10.2±0.04</td>
<td>101.6±1.1</td>
</tr>
<tr>
<td>10</td>
<td>1.1±0.02</td>
<td>10.8±0.08</td>
<td>108.7±1.6</td>
</tr>
</tbody>
</table>
Table 2.4 $k_{spring}$ with different number of points in moving average filter for a simulated 5 mm fiber

<table>
<thead>
<tr>
<th>Number of Average Point (Moving Average Filter)</th>
<th>$k_{spring} = 1$</th>
<th>$k_{spring} = 10$</th>
<th>$k_{spring} = 100$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated $k_{spring}$</td>
<td>Calculated $k_{spring}$</td>
<td>Calculated $k_{spring}$</td>
</tr>
<tr>
<td>500</td>
<td>1.0±0.02</td>
<td>4.8±0.2</td>
<td>26.4±0.4</td>
</tr>
<tr>
<td>200</td>
<td>1.0±0.01</td>
<td>6.4±0.3</td>
<td>33.9±0.5</td>
</tr>
<tr>
<td>100</td>
<td>1.0±0.02</td>
<td>8.3±0.1</td>
<td>74.5±2.4</td>
</tr>
<tr>
<td>50</td>
<td>1.02±0.02</td>
<td>9.3±0.2</td>
<td>92.4±3.7</td>
</tr>
<tr>
<td>20</td>
<td>1.06±0.03</td>
<td>10.0±0.2</td>
<td>100.5±0.8</td>
</tr>
<tr>
<td>10</td>
<td>1.1±0.02</td>
<td>10.6±0.4</td>
<td>108.1±1.5</td>
</tr>
</tbody>
</table>

Table 2.5 $k_{spring}$ with different number of points in moving average filter for a simulated 6 mm fiber

<table>
<thead>
<tr>
<th>Number of Average Point (Moving Average Filter)</th>
<th>$k_{spring} = 1$</th>
<th>$k_{spring} = 10$</th>
<th>$k_{spring} = 100$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated $k_{spring}$</td>
<td>Calculated $k_{spring}$</td>
<td>Calculated $k_{spring}$</td>
</tr>
<tr>
<td>500</td>
<td>1.0±0.04</td>
<td>5.2±0.2</td>
<td>31.9±0.3</td>
</tr>
<tr>
<td>200</td>
<td>1.0±0.03</td>
<td>7.3±0.3</td>
<td>47.2±0.4</td>
</tr>
<tr>
<td>100</td>
<td>1.01±0.03</td>
<td>8.8±0.3</td>
<td>82.1±1.3</td>
</tr>
<tr>
<td>50</td>
<td>1.02±0.03</td>
<td>9.6±0.3</td>
<td>94.2±1.0</td>
</tr>
<tr>
<td>20</td>
<td>1.06±0.03</td>
<td>10.3±0.3</td>
<td>100.6±0.8</td>
</tr>
<tr>
<td>10</td>
<td>1.1±0.03</td>
<td>11.1±0.2</td>
<td>107.8±1.1</td>
</tr>
</tbody>
</table>

2.4.3 Comparison of theoretical and experimental spring constant estimates

Table 2.6 lists the spring constant values from 40x magnification thermal fluctuation experiments. The fiber diameter from optical experiment result was found to be about 4.2 µm, and the fiber nominal diameter from manufacturer is 4 µm. So there is a systematic uncertainty in the fiber diameter of 0.2 µm. There is also a random error of about 0.15 µm from the error bars in Fig. 2.10. These sources of uncertainty are added in quadrature to give the uncertainty in the calculated spring constant in table 2.6. The experimental spring constants values for 3-7 mm-long fiber lengths were used for comparison. To calculate these values, the original data for the fibers, which each had
between 3000 and 7000 frames, were divided into subgroups that had 1000 image frames. The thermal fluctuations from these 1000 frames were used compute experimental $k_{\text{spring}}$ values. The uncertainties in $k_{\text{spring}}$ were calculated from the seven groups’ standard deviation. For example, if the fiber has only 3000 images, the 1000 frames subgroups could be taken as frames 1 to 1000, 1001 to 2000, frames 2001 to 3000, 201 to 1200, 1201 to 2200, 501 to 1500, and 1501 to 2500. If the other data sets had a different number of frames, a similar method was used to divide them into subgroups. The experimental data were smoothed using a 20 point moving average filter using the MATLAB program “Read_Process.m” (see Appendix 10). This program first processed the data using the input-output relation of moving average filter (Equation 2.3). The value of output was to the average of the next 20 points (determined above to be the optimum filter length). The output of the filter was then subtracted from the original data (with an offset of 10 points to center the average within the range of averaging), and the resulting filtered data was used for $k_{\text{spring}}$ calculations.

<table>
<thead>
<tr>
<th>Fiber Length (mm)</th>
<th>Fiber Diameter (µm)</th>
<th>$k_{\text{elasticity theory}}$ (pN/µm)</th>
<th>$k_{\text{experiment}}$ (pN/µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3±0.5</td>
<td>4.00±0.25</td>
<td>10±0.77</td>
<td>13±1.3</td>
</tr>
<tr>
<td>4±0.5</td>
<td>4.00±0.25</td>
<td>4.3±0.27</td>
<td>28.5±3.1</td>
</tr>
<tr>
<td>5±0.5</td>
<td>4.00±0.25</td>
<td>22±1.2</td>
<td>9.6±0.5</td>
</tr>
<tr>
<td>6±0.5</td>
<td>4.00±0.25</td>
<td>12.9±6.4</td>
<td>13.5±2.6</td>
</tr>
<tr>
<td>7±0.5</td>
<td>4.00±0.25</td>
<td>8.1±3.8</td>
<td>11.4±0.8</td>
</tr>
</tbody>
</table>
The $\chi^2$ statistic was calculated for table 2.6 to evaluate how well the spring constant theory and experimental values agreed. The $\chi^2$ statistic was evaluated using:

$$
\chi^2 = \sum \frac{(k_{\text{theory}} - k_{\text{experiment}})^2}{\sigma_{k_{\text{theory}}}^2 + \sigma_{k_{\text{experiment}}}^2}
$$

(2.7)

where $\sigma_{k_{\text{theory}}}$ and $\sigma_{k_{\text{experiment}}}$ are the uncertainties in $k_{\text{theory}}$ and $k_{\text{experiment}}$. If the experiment and theory are in good agreement, then $\chi^2$ should be close to $n - 1$, where $n$ is the number of rows being used in the sum. For table 2.6, all 5 rows were used, so the $\chi^2$ should be approximately 4. As noted above, the error in $k_{\text{theory}}$ can be separated into systematic and random parts. Only the random errors should be included in the $\chi^2$ calculation. The fiber length errors were random errors. There was also a random uncertainty in the diameter of approximately 0.15 µm from the errors bars shown in figure 2.10. For the data shown in table 2.6, $\chi^2 = 3.4$. The reduced $\chi^2$ value (defined as $\chi^2/\nu$, where $\nu$ is number of degrees of freedom $= n - 1$) is 0.85. We must also calculate the $p$ value for the chi-squared statistic. This is the probability of getting a given $\chi^2$ value with $\nu$ degrees of freedom. The $p$ value was calculated using the Excel function CHIDIST and found to be 0.49 which suggests that the agreement observed is reasonable.

The measured spring constants of 3, 4, 5, 6, and 7 mm fibers were within the propagated error for the spring constant calculated from elasticity theory. Because these fibers were only measured with a ruler, fiber length had a large uncertainty that made the relative errors expected for the calculated spring constants even larger. For the experimental spring constants, the size of the image analysis window was not just limited to the fiber tip area, which meant that regions with different $k_{\text{theory}}$ were averaged together. This would be expected to lead to a decrease in the standard deviation of the x-
centroid and hence an increase in the systematic uncertainty of the measured spring constant. The field of view was about 30-40 µm in 40x experiments. The spring constant of the 3 mm-long fiber, however, was 108 pN/µm at the edge of the field of view, which is not substantially different from the spring constant at the tip (103 pN/µm). So this could not count as a major source of uncertainty.

Another set of fibers had their lengths measured using an inverted optical microscope using a microscope eyepiece reticle (Fryer Co., Huntley, IL) with a 10x bright field objective (Plan Fluor, Fryer Co., Huntley, IL). The spacing between each grid line in the reticle was calibrated using a stage micrometer scale slide. Each division on the eyepiece reticle was 9.8 µm. This fiber length measurement method had much lower uncertainty than using a ruler and the propagated uncertainty in the spring constant from elasticity theory was correspondingly smaller.

The Brownian motion of glass fibers of different lengths was recorded using a high frame rate PixeLink camera attached to a Nikon Eclipse TE200 inverted microscope with 100x oil immersion field objective. All videos were taken at 743 frames per second (fps) at a resolution of 360 x 40 pixels. Videos were decomposed into individual images using a Custom program “AVI decomposer.vi” (see Appendix 9). 2100 frames were captured for each fiber. Fibers with lengths of 3 – 7 mm were conducted with buffer HBSS- solution as the surrounding medium in the thin viewing chambers described in chapter 2.2.2.2. Images were analyzed using the LabVIEW programs “twomax.vi” or “twomin.vi”. The resulting thermal fluctuation data were filtered using the 20 point moving average filter, as before.
Table 2.7 lists the spring constant values from elasticity theory calculations and thermal fluctuation measurements obtained using the fast camera and the 100x objective lens. The 2100 frames for each fiber were divided into three subgroups that had 1050 image frames each. The 1050 frame subgroups used were frames 1 to 1050, 1051 to 2100, and 601 to 1650. The uncertainties in the experimental values were calculated from the three subgroups’ standard deviations. These data were compared with the elasticity theory spring constants calculated using Eq. (1.9) with $D = 4.00 \, \mu m$ and $E = 7.40 \times 10^{10}$ N/m$^2$. Comparisons between calculated and experimental spring constant values showed that these two results agreed to within error.
Table 2.7 The spring constants calculated from elasticity theory and measured from thermal fluctuations for fibers in HBSS- buffer or in air.

<table>
<thead>
<tr>
<th>Fiber Length (mm)</th>
<th>Fiber Diameter (µm)</th>
<th>$k_{\text{theory}}$ (pN/µm)</th>
<th>$k_{\text{experiment}}$ (pN/µm)</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.72±0.01</td>
<td>4.00±0.25</td>
<td>138±36</td>
<td>143.1±1.3</td>
<td>HBSS-</td>
</tr>
<tr>
<td>3.36±0.03</td>
<td>4.00±0.25</td>
<td>73±20</td>
<td>62.4±5.9</td>
<td>HBSS-</td>
</tr>
<tr>
<td>3.67±0.02</td>
<td>4.00±0.25</td>
<td>56±15</td>
<td>52.8±4.5</td>
<td>HBSS-</td>
</tr>
<tr>
<td>4.33±0.01</td>
<td>4.00±0.25</td>
<td>34.4±8.8</td>
<td>42.6±1.2</td>
<td>HBSS-</td>
</tr>
<tr>
<td>5.02±0.02</td>
<td>4.00±0.25</td>
<td>22.1±5.8</td>
<td>22.7±1.1</td>
<td>HBSS-</td>
</tr>
<tr>
<td>5.07±0.02</td>
<td>4.00±0.25</td>
<td>21.4±5.6</td>
<td>21.3±0.6</td>
<td>HBSS-</td>
</tr>
<tr>
<td>5.08±0.01</td>
<td>4.00±0.25</td>
<td>21.3±5.4</td>
<td>21.7±0.4</td>
<td>HBSS-</td>
</tr>
<tr>
<td>5.1±0.05</td>
<td>4.00±0.25</td>
<td>21.0±5.9</td>
<td>22.9±0.8</td>
<td>HBSS-</td>
</tr>
<tr>
<td>5.98±0.01</td>
<td>4.00±0.25</td>
<td>13.0±3.3</td>
<td>13.9±0.4</td>
<td>HBSS-</td>
</tr>
<tr>
<td>7.248±0.07</td>
<td>4.00±0.25</td>
<td>7.3±2.0</td>
<td>7.3±0.6</td>
<td>HBSS-</td>
</tr>
<tr>
<td>3.9±0.1</td>
<td>4.00±0.25</td>
<td>47±15</td>
<td>66.8±6.5</td>
<td>air</td>
</tr>
<tr>
<td>5.02±0.03</td>
<td>4.00±0.25</td>
<td>22.1±5.9</td>
<td>22.2±0.6</td>
<td>air</td>
</tr>
<tr>
<td>5.48±0.04</td>
<td>4.00±0.25</td>
<td>17.0±4.6</td>
<td>16.7±1.5</td>
<td>air</td>
</tr>
<tr>
<td>5.75±0.06</td>
<td>4.00±0.25</td>
<td>14.7±4.1</td>
<td>15.4±0.5</td>
<td>air</td>
</tr>
<tr>
<td>5.79±0.1</td>
<td>4.00±0.25</td>
<td>14.4±4.3</td>
<td>14.6±0.4</td>
<td>air</td>
</tr>
</tbody>
</table>

The $\chi^2$ statistic (Eq. 2.7) was also calculated for table 2.7. For the 10 experiments performed in HBSS- buffer, it was found that $\chi^2 = 3.4$ ($p = 0.94$) and the reduced $\chi^2 = 0.38$, which was considerably smaller than expected, leading to fits that were unusually
good. Given the difficulties with estimating the diameter, the random errors have probably been overestimated. The reason most likely stems from the fibers not being exactly horizontal in the optical focus measurement presented in Fig 2.8. The diameter between the troughs is, thus, likely to change from the fiber tip and the edge of the field of view. This would mean that a substantial portion of the error bars in Fig. 2.10 could be systematic and not random error. If the random error were $0.1 \mu m$ instead of $0.15 \mu m$, then the reduced $\chi^2 = 0.75$ and $p = 0.66$, a much more reasonable value. For the 6 experiments performed in air, $\chi^2 = 2.6 (p = 0.65)$ and the reduced $\chi^2$ is 0.64which is also smaller than expected but within a more reasonable range.

2.5 Results and Discussion

The expected error in the calculation of spring constant from elasticity theory was obtained from the errors of the glass fiber’s length and diameter which were propagated using the standard error propagation formulas. The percentage error of calculated spring constant is four times that of fiber diameter and three times that of fiber length. The diameter of the glass fiber was nominally $4 \mu m$ based on how it was produced. But the exact value was a major source of uncertainty. The glass fiber diameters were measured by SEM and optical images experiments. The optical measurements differed substantially from the SEM results. The fiber width was $4.20 \pm 0.15 \mu m$ from optical measurements, but on the order of $6 \mu m$ from very blurry SEM images. The optical result should closer to the real number, because the spring constant values calculated from optical measurements of width were much closer to the thermal fluctuations measurement results.
The width of the fiber should be constant and the whole fiber is supposed to be moving together, so if the left trough moves left then the right trough should also move left in exactly the same way. One would thus expect the displacements of the left and right troughs (or peaks) to be strongly correlated if the centroid estimation were working correctly. However, it was realized that the distance between the troughs from optical measurements depended on the location of the focal plane with respect to the fiber. The two edge positions were initially far apart, but came to together as focal plane moved vertically upward through the fiber. When the fibers underwent Brownian motion, they vibrated along both the horizontal and vertical axes. The trough to trough width of the fiber in images, thus, did not stay constant. This explains why the left and right minima positions in images for a fiber were not correlated in many of the images. For example, the square of correlation coefficient for the 5.02 mm fiber was $R^2 = 0.05$, which means that their motion was essentially uncorrelated. For the 5.1 mm fiber, in contrast, $R^2 = 1$. As a result, strong correlations between the two sides motion is not required for the estimates of the centroid to be valid.

The spring constants of glass fibers with length from 3 to 7 mm were measured using the thermal fluctuation method. The spring constants of glass fibers were determined by finding the variance of the cantilever’s tip position at a given temperature. The best method for finding centroids was to use the full, unthresholded image, and plot the graph of intensity along a horizontal lines passing through the fiber. The intensity peak/trough locations could determine the fiber centroid positions. In the thermal fluctuation experiments, the most noise came from slow, but large amplitude, periodic
movement of fiber x-centroid position. In data processing, the low frequency noise was
filtered out using a low pass moving average filter followed by the subtraction of filtered
signal from original signal. According the simulation results, 20 was the optimum
number of average points in a moving average filter in our experiments. The difference
was less than 6% between the calculated $k_{spring}$ and the defined $k_{spring}$ when a 20 point
moving average filter was used. The measured spring constants values were within the relative error expected for the spring constant due to the fiber length and diameter uncertainties.

According to the Langevin equation for a system in a harmonic potential [127], a random thermal force $F(t)$ acting on the fiber is balanced by a drag force (friction times velocity) and a spring force (spring constant times displacement). The resulting equation of motion for a microcantilever is:

$$\gamma \frac{dx}{dt} + kx = F(t)$$

(2.8)

Here, $x(t)$ is the microcantilever position as a function of time, $\gamma$ is the frictional drag coefficient opposing the motion of the tip of glass fiber microcantilever and $k_{spring}$ is the spring constant of glass fiber tip. The random thermal force, $F(t)$ is a rapidly varying force due to collisions, which should be zero when averaged over time. $F(t)$ should be white noise and its Power Spectrum should be constant and given by:

$$S_F(f) = |F(f)|^2 = 4\gamma k_BT$$

(2.10)

where, $F(f)$ is the Fourier Transform of $F(t)$. As derived in Gittes et al. [128], one can use Fourier Transforms to solve the Langevin Equation for a fiber in a harmonic potential and show that the power spectrum, $S_x(f)$, of $x(t)$ is given by the Lorentzian:
Here $f_c = \frac{k_{spring}}{2\pi \gamma}$ is the corner frequency [128]. When $f \ll f_c$, the power spectrum is approximately constant. For frequencies substantially above the corner frequency, the power spectrum should fall off with a slope of $-2$ on a log $S_x(f)$ vs log $f$ plot. Fitting a Lorentzian to the power spectrum can thus be used to obtain the spring constant, but the fitting is only possible if the sampling frequency range covered is substantially above the corner frequency. The question would then be to estimate what the expected corner frequency for the fiber is.

For a fiber experiencing a force perpendicular to its long axis, the drag coefficient, $\gamma$, is given by [129]:

$$\gamma = \frac{4\pi\eta R}{\ln \left( \frac{L}{R} \right) - 0.193}$$

where $R$ is the radius of the fiber (2 µm), $L$ is the length of the fiber and $\eta$ is the viscosity.

We should thus have:

$$f_c = \frac{k_{spring}}{2\pi \gamma} = \left[ \ln \left( \frac{L}{R} \right) - 0.193 \right] \frac{k_{spring}}{4\pi\eta R}$$

The logarithmic term means that the prefactor is relatively insensitive to length, but differences in length lead to large difference in $k_{spring}$. To estimate the limits on the corner frequency, we consider extremes from table 2.7: $L = 2.7$ mm ($k_{spring} = 138$ pN/ µm) gives $f_c = 6100$ Hz and $L = 7.2$ mm ($k_{spring} = 7.3$ pN/ µm) leads to $f_c = 370$ Hz. The drag coefficient used here is for a free fiber and the fiber actually has a clamped end. For a fiber with a clamped end, the form of the drag term will be similar but an effective length
would be used instead of $L$. If the effective length were $L/4$, then the corner frequency range for the limits above would be 4900 Hz and 305 Hz. Figure 2.17 shows the power spectrum of fiber position for the 5.02 mm-long microcantilever taken at 743 fps frame rate in HBSS-. With the exception of some low frequency noise, the power spectrum is flat out to 370 Hz. Since the corner frequency for this case would be 1070 Hz from the equation above, it is not surprising that there is no sign of a rolloff in the power spectrum at high frequencies.

Figure 2.17 Power spectrum of fiber position for the 5.02 mm microcantilever fiber experimental data

Because of the high corner frequencies for the fiber cantilever system, it was not possible to find the spring constant and drag factor by fitting a Lorentzian to the power spectrum given the sampling frequencies available with our video cameras. The thermal fluctuation method was the only practical method for finding the spring constant.
CHAPTER 3: HYDRODYNAMIC FORCE ON MICROCANTILEVER TIP

A novel forced unbinding system was developed for receptor-ligand binding experiments. The system applied hydrodynamic flow from one micropipette to a glass fiber microcantilever tip in order to bring the tip into contact with a bead held in another micropipette with suction pressure. The force of hydrodynamic flow applied on the fiber microcantilever tip needed to be evaluated for the forced unbinding system. This chapter presents the relationship between the forces applied on the fiber tip and the magnitude of applied pressure used to generate flow from a micropipette. The effect on the forces applied on the fiber due to relative positions of fibers and micropipettes will also be described. The effects of micropipette tip size variation on flow force magnitude will also be discussed.

3.1 Experimental Method

3.1.1 Viewing chamber

Hydrodynamic forces were applied to glass fiber microcantilever tips in the viewing chamber shown in Figure 3.1. The assembly process is described in detail as follows: glass microscope slides (Fisher Scientific, Pittsburgh, PA) were cut into four small pieces (25 x 8 x 1 mm), which formed the sides of the chamber, and one bigger piece (20 x 30 x 1 mm), which was used as the top of the viewing chamber. Strips of double-sided tape (Scotch permanent double sided tape, 3M, St. Paul, MN) were applied to two of the small pieces of glass microscope slide. The pieces were attached near the center of a tissue culture dish (Fisher Scientific, Hanover Park, IL) with a gap of approximately 10 to 13 mm between them. The gap needed to be wide enough to allow
for placement of microcantilevers. One end of an E-glass fiber section was laid on one of the small pieces of glass slide while the other end extended into the gap created between the two pieces of microscope slide. The fiber was mounted so that it was approximately perpendicular to the glass slide it was resting on. The other two small pieces of cut glass microscope slide were taped on top of the other two small pieces in order to secure the cantilevers in place and to complete the walls of the viewing chamber. The larger piece of cut glass microscope slide was secured to the top of the side walls to make a roof for the chamber. The chamber was ~13 mm wide x ~25 mm long x ~2 mm deep, (neglecting the height of double-sided tape). The approximate volume of the chamber was, thus, ~650 mm$^3$ or 0.65 ml. Openings 20 mm wide were cut in the walls of the tissue culture dish for inserting micropipettes into the open ends of the viewing chamber.

Figure 3.1: Viewing chamber assembly.
### 3.1.2 Experimental procedure

The viewing chamber was filled with HBSS+ and placed on the stage of an inverted optical microscope (Nikon Eclipse TE 300, Fryer Co., Huntley, IL) with a 20x bright field objective. A micropipette was also filled with the HBSS+ and connected to a custom made manometer with Tygon tubing. The manometer was capable of regulating water pressure differences between the inside of micropipette and the viewing chamber. The micropipette was mounted on a micromanipulation stage and inserted into the viewing chamber through the chamber’s right-hand opening. After the micropipette had been mounted and inserted into the viewing chamber, pressure differences between the micropipette inside and outside were applied.

The pressure difference between the inside and outside of the micropipette was checked frequently using polystyrene beads (1.0% w/v, 1.87 µm; SVP-15-5; Spherotech; Libertyville, IL) during the experiment. The beads were used at a 5000x dilution from the original 1% w/v bead concentration. Beads were aspirated into the micropipette. The manometer water levels were adjusted until the beads were stationary (except for Brownian motion). This adjustment ensured that the pressure difference between the micropipette and the chamber was zero.

The micropipette was manipulated so that its tip was within 2 µm to 8 µm of the glass fiber tip. The micropipette tip and the fiber tip were also brought into the same focal plane. Hydrostatic pressure differences ranging from 0 to 50 cm of water were then applied by pressurizing the manometer reservoir with compressed air using a gastight...
syringe (Hamilton Company, Reno, Nevada, 10 ml). The fiber tip was deflected from its rest position by the resulting hydrodynamic flow.

The fiber tip movements due to the resulting flow from the micropipette were recorded using a CCD video camera (Cohu, Fryer Co., Huntley, IL) which was attached the side port of microscope. Images and pressures were captured using the Labview program “Pressure Logger Ultimate.vi” (code attached as Appendix 11). The program saved pressure readings and the corresponding video images at a set time interval (usually 2s). All experiments were conducted at room temperature.

3.2 Fiber Tip Displacement with Applied Pressure

Figure 3.2 shows an example of the procedure. Here an 8.28 mm-long fiber tip was displaced by fluid flow from a 9.06 µm ID micropipette. The pictures are before (a) and after (b) the application of a 50 cm of water pressure difference across the micropipette tip. The distance between the fiber tip and the micropipette opening was initially 2.56 µm before application any hydrodynamic forces (Figure 3.2 (a)). The distance between the fiber tip and micropipette opening increased following application of pressure (Figure 3.2 (b)).
Pressure differences of 0, 5, 10, 20, 30, 40 and 50 cm of water were used to produce flow from the micropipette. Each pressure was held for around 30 seconds during the force application process. The distances between the fiber tip and micropipette opening were measured using a LABVIEW program called “Manual Particle Tracker from File Stack”. The initial distance between fiber and micropipette was nominally set at 10, 20, 30, 40 or 50 pixels. The displacement of fiber tip could be determined by subtracting the zero pressure distance between fiber tip and micropipette from the distances between the fiber tip and micropipette opening for some other pressure. The force applied on the fiber tip could be calculated from Elasticity theory (equation 1.9) with $E = 7.40 \times 10^{10} \text{ N/m}^2$. Note that because the actual initial distance was not always exactly equal to even multiples 10 pixels, the nominal displacements based on an assumed initial displacement sometimes come out as less than zero for reasons that are an artifact of the data processing. In the end, the initial value for displacement is not crucial.
Only the slope of the curve matters. The forces applied on fiber tip are shown plotted versus applied pressure in Figure 3.3. The fiber length in this figure was 8.28 mm and the fiber diameter was taken to be 4.0 μm. The Force applied to the fiber tip increased linearly as the pressure difference increased. Linear fits were obtained to these curves. The $\chi^2$ values were calculated using equation 3.1.

$$\chi^2 = \sum \frac{(k_{\text{experiment}} - k_{\text{fitting}})^2}{\sigma^2_{k_{\text{experiment}}}} \quad (3.1)$$

Table 3.1 gives the fitted slope, reduced $\chi^2$ and $p$ value for the linear fits. There are seven data points and two free parameters here so the degrees of freedom could be calculated as the the number of data point minus the number of fitted parameters. The $p$ values were calculated using the function CHIDIST in Excel.

![Figure 3.3: Force applied on fiber tip vs. applied pressure difference $\Delta P$ for flow from a 9.06 μm ID micropipette. $\Delta P$ is Hydrostatic pressure coming from micropipette. The initial gap distance was nominally 10 pixels (~2.56 μm). The fiber length was 8.28 mm.](image-url)
Table 3.1 The fitting parameters

<table>
<thead>
<tr>
<th>slope (pN/cm of water)</th>
<th>Reduced $\chi^2$</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5± 0.07</td>
<td>7.5</td>
<td>4.8×10^{-7}</td>
</tr>
</tbody>
</table>

It is clear that while the fit in Fig. 3.3 is decent by eye, the first two points in the curve led to $\chi^2$ values that were excessively large. It is probable that the uncertainty in these points (which had the smallest displacements and were, thus, the most susceptible to error) was underestimated.

The flow rate $Q$, from the micropipette is a function of micropipette diameter and the pressure difference [130]:

$$Q = \frac{\pi D^4 \Delta P}{128 \mu L}$$  \hspace{1cm} (3.2)

where $D$ is the diameter of micropipette, $\Delta P$ is the pressure difference, $\mu$ is the viscosity of the fluid ($\mu = 0.001 \text{ pN}\cdot\text{s/}\mu\text{m}^2$), and $L$ is the equivalent length over which the pressure difference happens. The value of $L$ is typically about 500-700 $\mu$m micrometers from the work of Dr. Young Eun Choi [131], a previous graduate student in the Tees lab. 1 cm of water is equivalent to 98 Pa. Applying a hydrostatic pressure of 50 cm of water from the 9.06 $\mu$m ID micropipette, the flow rate from the micropipette should be about $1.35 \times 10^6 \mu m^3/s$ ($L = 600 \mu m$). The flow rate, $Q$, can also be defined as

$$Q = Av$$  \hspace{1cm} (3.3)

where $A$ is the cross-sectional area of the micropipette and $v$ is the average velocity. The centerline flow velocity is twice the average velocity for flow in a circular tube.

Combining Equations 3.2 and 3.3, gives the relationship between centerline flow velocity and micropipette diameter:
A typical centerline velocity for a pressure of 50 cm of water would thus be $1.05 \times 10^4$ µm/s ($L = 600$ µm).

3.3 Effect on Fiber Tip Displacement Due To Different Relative Positions

3.3.1 Variation with relative vertical positions

Different relative vertical positions of the fiber tip and the micropipette opening were investigated to determine the proper relative vertical positions for maximizing the hydrodynamic force on the fiber. The micropipette opening was placed next to the fiber tip using a water hydraulic micromanipulator and moved vertically using a control knob where one full turn is 50 µm and its minimum graduation is 0.2 µm. Hydrodynamic flow from a 7 µm ID micropipette was applied to a 5.4 mm-long fiber tip. The initial distance between the fiber tip and micropipette end was set to 10 pixels (~2.6 µm). The focal plane where both micropipette and fiber seemed to be in focus was set as 0 for relative vertical position of the fiber and micropipette. The micropipette was moved up and down in 1 µm steps using the vertical control knob. To ensure that deflection would be obvious, a pressure difference of $73 \pm 3$ cm of water was applied for the relative vertical positions experiments. The pressure was held for 30 seconds during the force application process. The fiber tip displacements were measured at different initial relative vertical positions when hydrodynamic flow was applied. Force applied on fiber tip were calculated by Elasticity theory (equation 1.9) with $E = 7.40 \times 10^{10}$ N/m$^2$, the fiber length is 5.4 mm and the fiber diameter is 4 µm.
Figure 3.4 shows that the force applied on fiber tip varies when the micropipette was moved up and down after hydrodynamic flow was applied. From -4 µm to +2 µm initial relative vertical position, the fiber tip was moved by hydrodynamic flow. The fiber tip was barely displaced for relative vertical positions below -4 µm or above 2 µm. The influence area for hydrodynamic flow is, thus, slightly smaller than the inner diameter of the micropipette. The maximum force applied on fiber tip happened at -1 µm initial relative vertical position where the micropipette end was slightly below the apparent position of the fiber tip. One possible reason is that the micropipette was angled downward by a couple of degrees. Another possibility is that when the micropipette appears to be in focus, it is actually not in focus due to distortion from the curvature of the micropipette cross section.

Figure 3.4: Force applied on fiber tip vs. initial vertical relative position of fiber tip and micropipette. A pressure difference of 73 ± 3 cm of water was applied to a 5.4 mm-long fiber tip that was initially 2.56 µm from a 7 µm ID micropipette.
The same procedure was applied for two additional cases. Flow from 7.4 or 12 µm ID micropipettes was applied to a 5.3 mm-long fiber tip. The zero flow distance between the fiber tip and micropipette opening was set to either 2.56 µm or 7.68 µm and the tip and opening were set to be in the same apparent focal plane (0 for relative vertical position). The pressure difference was increased to 97 ± 1 cm of water and held for 30 seconds as in the previous experiments. Figure 3.5 shows how the force applied on fiber tip varies when the initial relative vertical position varies after the hydrodynamic flow were applied from the two different inner diameter micropipettes.

Figure 3.5: Force applied to a fiber tip vs. vertical position for a 5.3 mm-long fiber exposed to a pressure difference of 97 ± 1 cm of water from a 7.4 µm ID micropipette or a 12 µm ID micropipette. The micropipette opening was initially either 10 pixels (~2.56 µm) or 30 pixels (~7.68 µm) away from the fiber.
The maximum force applied on fiber tip for the case where the fiber tip and micropipette opening were initially 10 pixels apart was found to be at 0 µm relative vertical position for the 7.4 µm ID micropipette and at +1 µm relative vertical position for the 12 µm ID micropipette. The maximum force applied on fiber tip for the fiber that was initially 30 pixels (~7.7 µm) from the micropipette opening happened at -1 µm relative vertical position for both size micropipettes, although the forces applied on fiber tip were similar from -2 µm to 1 µm initial relative vertical position for the 7.4 µm ID micropipette. It was found that the forces applied on fiber tip were much larger when the fiber was initially 10 pixels from the micropipette opening than when the initial separation was 30 pixels. This was consistent with the expectation that the force would be larger the closer the fiber was to the micropipette opening. The drag effectiveness of fluid flow was broader and smaller under the same other conditions from 30 pixels than 10 pixels. Comparing the two sizes of micropipette, the larger forces applied on fiber tip were produced by for the 12 µm ID micropipette when the initial horizontal distance was 10 pixels. Equation 3.4 shows that it would be expected that larger micropipettes would produce larger centerline velocities for the same pressure differences, which would lead in turn to greater hydrodynamic forces on the fiber. Applying a hydrostatic pressure of 97 ± 1 cm of water to 7.4 µm and 12 µm ID micropipettes, should produce the flow rates of 2.9×10⁶ µm³/s and 8.05×10⁷ µm³/s (L = 600 µm) respectively. The centerline velocity to 7.4 µm and 12 µm ID micropipettes were 1.4×10⁴ µm/s and 3.6×10⁴ µm/s (L = 600 µm). The expected velocity difference became smaller for larger separation distances. 2.9×10⁶ µm³/s and 8.05×10⁷ µm³/s (L = 600 µm) respectively.
3.3.2 Varying the fiber tip-micropipette initial relative position

Based on the results of the previous section, the effect of initial relative horizontal distances between the micropipette opening and the fiber tip on tip displacements was thoroughly investigated in hydrodynamic flow force experiments. Pressure differences of 0, 5, 10, 20, 30, 40 and 50 cm of water from 7.0 µm, 7.4 µm and 12 µm ID micropipettes were applied to a 5.4 mm-long and 5.3 mm-long fiber tip. Each pressure was held for around 1 minute during the hydrodynamic flow application process.

Using the results from the vertical relative position experiments, the -1 µm focal plane was chosen for the 7 µm and 12 µm ID micropipettes, and the 0 µm focal plane was chosen for the 7.4 µm ID micropipette as the relative vertical positions which maximized the forces applied on fiber tip when hydrodynamic flow was applied. The initial distances between the fiber tip and micropipette opening were nominally 10, 20, 30, 40 and 50 pixels (corresponding to approximately 2.56, 5.12, 7.68, 10.24, and 12.8 µm). The fiber tip displacements after flow force application were measured for different pressure values and corresponding forces applied on fiber tip were calculated. Final forces applied on fiber tip vs. applied pressure was plotted for these five initial tip-micropipette distances in Figure 3.6 (7.0 µm ID micropipette), Figure 3.7 (7.4 µm ID micropipette) and Figure 3.8 (12 µm ID micropipette). Forces applied on fiber tip vs. applied pressure curves were linearly fitted for each initial tip-micropipette distance. The forces applied on the fiber tips mostly increased linearly as the pressure difference increased for all three micropipette sizes. The exceptions were all for large initial
separation distances where there was no change in displacement as a function of pressure and hence there was evidently very little applied force on the fiber (perhaps because the flow was missing the fiber at this large separation distance). As noted above, the negative forces represent an artifact from the way that initial distance was subtracted to generate the displacements used to calculate the forces. The only parameter that matters for the following analysis is the slope.

Figure 3.6: Forces applied on fiber tip vs. applied pressure difference for flow from a 7.0 µm ID micropipette for a range of initial gap distances. The fiber length was 5.4 mm.
Figure 3.7: Forces applied on fiber tip vs. applied pressure difference for flow from a 7.4 µm ID micropipette for a range of initial gap distances. The fiber length was 5.3 mm.

Figure 3.8: Forces applied on fiber tip vs. applied pressure difference for flow from a 12 µm ID micropipette for a range of initial gap distances. The fiber length was 5.3 mm.
The slopes of the fitting lines were calculated for each initial relative horizontal distance. Table 3.2 gives the best fit slopes with errors and reduced $\chi^2$ values for each initial relative horizontal distance of the 7.0 µm, 7.4 µm and 12 µm ID micropipettes. Most of the $\chi^2$ values in table 3.2 are well below 1, which suggests that the typical error is overestimated. The uncertainty arises entirely from the uncertainty in the position, which was relatively large (typically 10%). Brownian motion of the fibers in the chamber is negligibly small at this magnification, so that was not a factor in the uncertainty, but the fiber did move visibly (although the micropipette did not). This was thought to be due to air currents in the room that acted unpredictably on the fluid-air interfaces where the micropipettes entered the chamber. There was also a substantial uncertainty from the fact that the distances were measured by eye and had to be an integer number of pixels. This would be expected to introduce a systematic uncertain number of at least half a pixel.

<table>
<thead>
<tr>
<th>Initial distances (pixels)</th>
<th>7.0 µm ID micropipette</th>
<th>7.4 µm ID micropipette</th>
<th>12 µm ID micropipette</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>slope</td>
<td>reduced $\chi^2$</td>
<td>slope</td>
</tr>
<tr>
<td>10</td>
<td>0.54±0.06</td>
<td>0.53</td>
<td>0.69±0.10</td>
</tr>
<tr>
<td>20</td>
<td>0.42±0.03</td>
<td>0.10</td>
<td>0.33±0.5</td>
</tr>
<tr>
<td>30</td>
<td>0.16±0.04</td>
<td>0.14</td>
<td>0.30±0.07</td>
</tr>
<tr>
<td>40</td>
<td>-0.06±0.07</td>
<td>0.91</td>
<td>0.28±0.07</td>
</tr>
<tr>
<td>50</td>
<td>0.03±0.03</td>
<td>0.13</td>
<td>0.27±0.05</td>
</tr>
</tbody>
</table>
Figure 3.9 shows the slope of the forces applied on the fiber tip versus applied pressure plotted against the initial relative horizontal distance between the micropipette opening and the fiber tip for the 7.0 µm, 7.4 µm and 12 µm ID micropipettes. The slope of the force applied on fiber tip vs. applied pressure decreased linearly as the initial relative horizontal distance between the micropipette end and the fiber tip increased until it reached zero between 10 and 12 µm. It then stayed zero for all distances greater than this. This result demonstrates that for this method to work, the micropipette tip is best positioned within 10 µm of the fiber tip and the closer it is placed, the larger the force applied. The fitting line for 12 µm ID micropipette is a little steeper than 7.4 µm ID micropipette one. The slopes of deflection vs. hydrostatic pressure graph of 12 µm ID micropipette and 7.4 µm ID micropipette are not significantly different as determined by Student’s t-test ($p = 0.46$). The slopes of 7 and 7.4 µm ID micropipettes are not statistically significantly different ($p = 0.30$). The slopes of 7 and 12 µm ID micropipettes are not statistically significantly different ($p = 0.17$). This is what would be expected since plotting force vs applied pressure should universalize the curves giving the same Force vs Pressure slope for all fiber lengths. These different lines of two different size ID micropipettes were expected have different slope values, because the water flow came from the bigger size of micropipettes produced larger fiber displacement under the same other conditions. The expected spring constant of 5.3 or 5.4 mm fiber was about 18 pN/µm. The generated force was up to 54 pN when the displacement was 3 µm for a pressure of 50 cm of water. The longer fiber would show more obvious deflection when the same pressure was applied.
Figure 3.9: Slope of force vs. hydrostatic pressure graph vs. the initial fiber tip-micropipette distance for micropipettes with 7, 7.4 and 12 µm ID micropipettes acting on a 5.33 mm-long fiber.

### 3.4 Effects of Micropipette Size on Flow Force

The effect of micropipette ID on fiber displacement was further investigated in hydrodynamic flow experiments. Pressure differences of 0, 5, 10, 20, 30, 40 and 50 cm of water were used to generate flow from micropipettes to deflect 6.56 and 8.28 mm-long fibers. The initial distance between the fiber and micropipette was either 10 pixels (2.56 µm) or 30 pixels (7.68 µm). The final fiber displacements were measured under different applied pressures. As shown in Figure 3.10, the slope of graphs of the force applied to the fiber tip vs. hydrostatic pressure increased linearly with increasing micropipette size for both 2.56 µm and 7.68 µm initial distances. The fitting lines for the 2.56 µm gap are
steeper than the lines for the 7.68 µm gap for the longer fiber; however the slopes are very marginally different for the shorter fiber. This is consistent with the varying horizontal relative position experiments. Comparing the different lengths of fibers, because we are measuring force vs pressure and hence the length should not be a factor in the measurement (since it is already included in the calculation of the force), the 8.28 mm long fiber was expected to have the same force vs pressure slope as the 6.56 mm long fiber according to the equation (1.9). The slope of the force vs pressure fitting line for the 8.28 mm-long was insignificantly different from the 6.56 mm-long fitting line.

Figure 3.10: Slope of force vs. hydrostatic pressure graph vs. inner diameter of micropipette for 8.28 mm and 6.56 mm length fibers.
3.5 Discussion

The strength of hydrodynamic flow forces applied to the glass fiber microcantilever tip was quantified as a function of applied pressure used to produce flow from the micropipette, for a range of relative positions of fibers and micropipettes and for different micropipette sizes.

The optimal relative positions between micropipettes and fibers were that they should be within 1 \( \mu m \) of the same focal place vertically and close enough horizontally to maximize the hydrodynamic force on the fiber tip. 10 pixels (2.56 \( \mu m \)) was the closest rest distance between the edge of the fiber tip and micropipette opening that could still be seen clearly under the 20x objective. The diffraction pattern at the edge of the micropipette and fiber and the fiber vibration were limiting factors for the minimum gap.

The micropipette ID and fiber length were optimized based on deflection of the fiber tip under the different water pressure results using various micropipette ID sizes and fiber lengths. A larger micropipette size led to a larger hydrodynamic flow force, as expected from Equation 3.4. The influence area is related to the diameter of the micropipette. The influence area for hydrodynamic flow is expected to be confined to the area of the fiber tip, so the optimal micropipette ID should be 10 to 12 \( \mu m \) in further bonding experiments. The longer length fibers presented the more obvious deflection of fiber tip under the same water pressure. A 14 mm long fiber, however, was observed to be stuck onto the glass cover slip that forms the floor of the viewing chamber as discussed in my Master’s thesis[114]. Static electricity often caused long fibers to stick to the bottom of the chamber. An optimal maximum fiber length would thus be 9 to 10 mm.
The displacement of the fiber tip is proportional to the water flow pressure for fixed micropipette ID size and fiber length. As a result, a desired hydrodynamic force applied to the glass fiber microcantilever tip could be obtained by using various water pressures. The displacement of the fiber tip would be determined by the water pressure value for the fixed micropipettes ID size and fiber length at the optimal relative positions of micropipettes and fibers. This method was used to apply forces to receptor-ligand bonds in the receptor-ligand bonding experiments described in the next chapter.
CHAPTER 4: ADHESION EXPERIMENTS

The interaction of protein A and antibody was measured using a novel microcantilever forced unbinding system. The interaction of protein A and BSA-coated glass fiber surfaces was investigated in nonspecific adhesion experiments as a negative control. The protein A and human IgG bonding interaction was investigated for specific adhesion experiments.

4.1 Materials and Methods

4.1.1 Human IgG and protein A

The protein A – Immunoglobulin G (IgG) bonding interaction was investigated in this dissertation. Protein A and IgG form a receptor- ligand pair whose molecular interactions and binding have been studied using a variety of methods [132-134]. Protein A is a constituent of the cell wall of the bacterium *Staphylococcus aureus*. Protein A is a highly stable surface receptor with a molecular weight of 42 kDa that binds to immunoglobulins. IgG is an antibody isotype that was described in the Introduction. IgG is found in blood and extracellular fluid, allowing it to control infection. Protein A has high affinity for the Fc region of IgG, presumably as a defense mechanism. Protein A has been used technologically to act as an orienting agent for antibodies. If a surface is first coated with protein A, the antibody will be oriented with the antigen binding arms oriented out into the solution [135].

Protein A-coated microspheres (9.77 µm in diameter) were purchased from Bangs Labs (Catalog Code: CP02N; Manufacturer Lot Number: 10279; Fishers, IN). Protein A has four antibody binding sites, while only two of these can be used at one time which are
located in the Fc region of the antibody. The original concentration of protein A-coated bead solution is 1% by weight. The beads were diluted 10,000 fold in two stages with 1% (wt/wt) BSA (bovine serum albumin, minimum 98%, A7030, Sigma) in HBSS+ (pH 7.5, Hank’s Balanced Salt Solution with added Ca\(^{2+}\) and mg\(^{2+}\), Cambrex, Walkersvile, MD). Before addition, the 1% BSA in HBSS+ solution was heat treated by placing it in a water bath maintained at 50\(^{\circ}\)C for 30 min. Experience has shown that this treatment reduces non-specific adhesion, most likely by partially denaturing the BSA. The 10000-fold dilution was adequately concentrated for micropipette aspiration experiments, but not so concentrated that the optics were compromised. The diluted protein A-coated bead suspension was used in non-specific binding and specific binding experiments. Human IgG (Product No. F9636) conjugated with the fluorescent dye fluorescein isothiocyanate (FITC) was purchased from Sigma. The original concentration of human IgG solution was 20 mg/ml. Concentrations of 4 mg/ml and 1 mg/ml human IgG conjugate FITC solution diluted in HBSS+ were used in specific adhesion binding experiments. To avoid contamination of the original bead suspensions, all dilutions were performed in a bio-safety cabinet. The UV light and air blower of the bio-safety hood were turned on at least 15 minutes before the dilution procedures were performed.

4.1.2 Viewing chamber

A novel forced unbinding system was designed for bonding experiments. It used hydrodynamic flow from one micropipette to “blow” a microcantilever tip into contact with a bead held in another micropipette. The viewing chamber assembly process was the
same as that described in section 3.1.1. The schematic diagram of viewing chamber is shown in figure 4.1.

Figure 4.1: Binding viewing chamber design and side view.

4.1.3 Mounting viewing chamber on stage

The viewing chambers were filled with corresponding experimental solutions for non-specific and specific adhesion experiments. The two micropipettes were filled with the same buffer used in the chamber. One filled micropipette with tip ID around 10 μm was connected to a custom made manometer by a length of Tygon tubing. The micropipette was mounted on a micromanipulation stage (Narishige, Fryer Corp, Huntley, IL) and inserted into the viewing chamber through the right-hand opening. The manometer was initially adjusted to equalize the hydrostatic pressures between of the
micropipette and the viewing chamber by adjusting the height of the manometer reservoirs. The manometer produced hydrostatic pressure through the micropipette to apply a force on the coated glass fiber tip as described in Chapter 3. The other filled micropipette with tip ID a little smaller than 10 μm was mounted on a second micromanipulation system and inserted into the viewing chamber through the left hand opening. The left micropipette was connected via a length of Tygon tubing to a syringe (BD, Florence KY) that had the plunger removed so that the barrel could be used as a head tube to apply hydrostatic pressure to the micropipette. The head tube syringe is shown in Figure 4.3. The head tube syringe was partially filled with water and attached to a jig that allowed it to be repositioned vertically. The height of the water surface in the head tube was maintained at a level slightly below the microscope stage. The resulting hydrostatic pressure difference was used to aspirate protein A-coated microspheres. This suction pressure was constant and strong enough to hold the bead firmly onto the micropipette tip throughout the experiments. Figure 4.2 shows the viewing chamber filled with experimental solution on the stage of an inverted optical microscope (Nikon Eclipse TE 300, Fryer Co., Huntley, IL).
4.1.4 Bonding experiment setup

The micropipettes and fiber needed to be exactly aligned for binding experiments. The right-hand micropipette was manipulated so that its tip was 10 pixels (~2.56 µm) from the glass fiber tip. The left-hand micropipette with its aspirated protein A-coated bead was also adjusted so that it was in the same focal plane as the right-hand micropipette and the glass fiber tip.

Applying a positive hydrostatic pressure to the right-hand micropipette produced a hydrodynamic flow from the micropipette tip. A gastight syringe (10ml Hamilton Company, Reno, NV) was used to apply the pressure whose value was shown on a
pressure display. The fiber tip was deflected from its rest position by the hydrodynamic flow and pushed into contact with the bead held in the left-hand micropipette. Higher pressures than necessary were applied to ensure that the fiber tip and bead were fully in contact. Following a brief period to allow for the formation of bonds, the positive hydrostatic pressure was rapidly reduced to zero by releasing the compressed air that had been used to create the overpressure using a computer controlled valve (3-way solenoid pinch valve, S-98302-46; 12 VDC, 1/8" ID x 1/4" OD tubing, Cole Parmer, Vernon Hills, IL, with a custom-made power supply and switch box). The flow that held the fiber in contact with the bead was consequently eliminated. Any bonds that had formed during the contact period were then exposed to a force that depended on the deflection of the glass fiber cantilever. Subsequent bond dissociation allowed the fiber to relax back to its rest position. Bond lifetimes were measured from the moment that the syringe pressure returned to zero, until the moment when the fiber left the bead. Hundreds of contacts were performed and observed under an inverted optical microscope (Nikon Eclipse TE 300, Fryer Co., Huntley, IL) with a 20x bright field objective (Plan Fluor, NA 0.45, Nikon, Japan). All experiments were conducted at room temperature. Figure 4.3 shows the complete experimental setup. The entire assembly was mounted on a vibration isolation workstation (Isostation, Newport Corp., Irvine, CA) to minimize the effects of vibration.
Figure 4.3: Bonding experimental setup.
4.1.5 *Image capture*

The fiber movements inside the viewing chamber were recorded using a CCD video camera (Cohu, Fryer Co., Huntley, IL) which was attached the side port of the microscope. The video camera sent images to a video monitor (Sony Trinitron, Edmunds Optics, Barrington, NJ) through a Video Cassette Recorder (Sony SVO-9500MD, Fryer Co., Huntley, IL). All images are captured in real time (30 frames per second) using a custom-made LabVIEW image acquisition program “fast pressure logger with valve.vi” (see Appendix 12) which also logged the manometer’s hydrostatic pressure and controlled the valve that vented the manometer overpressure to end the flow from the micropipette. The adhesive events were displayed on a TV monitor and computer monitor screen during the experiment. The initial distance between the micropipette and fiber before each bonding event was measured using LABVIEW program “Manual Particle Tracker from File Stack”. Figure 4.4 shows the fiber’s movement inside the viewing chamber. The upper picture shows the bead and the fiber in their original rest position. The bottom picture shows the fiber touching the bead when elevated hydrostatic pressure induced fluid flow from the bigger ID micropipette.
Figure 4.4: The fiber’s movement inside the viewing chamber. (Upper) the bead and fiber in their original rest position. (Bottom) fiber touched bead when hydrostatic pressure applied.

4.2 Nonspecific Adhesion Experiments

Non-specific adhesion can be caused by weak van der Waals or electrostatic interactions between surfaces, or by unexpected specific interactions between the target molecule and contaminants on the other surface. Detection of non-specific interactions between proteins and glass surfaces is common and it is extremely difficult to make it totally disappear. It is necessary to investigate the significance level of nonspecific adhesion background in order to be sure that most of the adhesive events seen are actually the specific adhesive interaction that one wishes to probe. BSA was used to
block nonspecific interactions between protein A-coated beads and glass fiber surfaces. BSA was used because it is inexpensive, readily available, and is frequently used as a blocker of non-specific adhesion. BSA as a component of coating, blocking and wash buffers helps prevent non-specific adhesion [136].

To determine the level of non-specific adhesion, three sets of nonspecific adhesion experiments were performed as shown in Figure 4.5. For undenatured BSA experiments, the undenatured BSA was observed to support a relatively large amount of nonspecific adhesion. The beads and fibers were brought together by hydrodynamic flow forces 669 times, and 18 adhesive events were observed. Heat treated (or denatured) BSA was also tested as an adhesion blocker. About 200 µl of 1% BSA in HBSS+ was introduced into a 50 ml centrifuge tube and placed in water bath for 30 minutes or two hour at 50°C. For the 30 min denaturation time, there were only 3 adhesive events out of total of 626 contacts (an event rate of 0.48%). When the BSA was denatured for 2 hr, there were 4 adhesive events out of 605 contacts (an event rate of 0.66%). Given that the uncertainty in a count is the square root of the value, the results for these two sets of denatured BSA experiments, results were identical within error, suggesting that the longer heating time did not further reduce adhesive events compared to the 30 min time. The two conditions were combined in the denatured BSA point shown in Figure 4.5. Accordingly, a 30 min denaturation time interval in water bath was used for all later experiments.
The percentage of adhesive events decreased from 2.69% to 0.57% after the BSA was denatured. This means that heat treatment of the BSA is necessary to reduce the frequency of nonspecific adhesion. Denatured BSA is a better negative control compared to undenatured BSA. Undenatured BSA is actually quite sticky. Denaturing the BSA most likely partially unfolds the BSA which makes it spread out more on the glass and block non-specific adhesion better. The “heat treatment” of the BSA was done routinely when coating glass fiber microcantilever with adhesion molecules in all later specific adhesion experiments.

The bonding lifetime was determined from the interval between the moment when the pressure monitor showed that the hydrodynamic pressure returned to zero and the moment the fiber detached from the bead and started to relax back to its rest position. It
was found that about three video frames were required for the hydrodynamic flow to return to zero, with an uncertainty of around 1 frame. The minimum break up time below which break-up would be indistinguishable from no bond formation would therefore be ~0.1 s and the uncertainty in the starting point would be ~30 ms (since the frame rate is 30 frames per second).

Figure 4.6 shows the cumulative probability distribution of bonding lifetime for denatured and undenatured BSA in nonspecific adhesion experiments. The red line represents the cumulative probability distributions of bonding lifetime for undenatured BSA. The blue dashed line represents the cumulative probability distributions of bonding lifetime for denatured BSA. The cumulative probability distributions of of bonding lifetime for denatured BSA were broader than that for undenatured BSA and the bonding lifetime for denatured BSA is longer than that for undenatured BSA. The difference in bonding lifetime for denatured and undenatured BSA was small enough, however, that Student t-tests demonstrate that the difference was not significant ($p = 0.22$).
4.3 Specific Adhesion Experiments

4.3.1 Bonding test using fluorescence microscopy

As described in the introduction, IgG is the major class of immunoglobulin in blood. Protein A is a component of the cell wall of the bacterium *Staphylococcus aureus*. The protein A binds to the Fc or tail region of IgG molecule. Protein A has four antibody binding sites. There are at least two Protein A binding sites on any antibody, and these are located in the Fc region of the antibody. The binding ratio between Protein A and IgG is 1:2 at equilibrium state [134]. The Fc-directed binding in human IgG is desirable for maximizing its biological activity. As described below, the receptor-ligand molecular binding lifetime of protein A and immunoglobulin G (IgG) was studied. Protein A was pre-conjugated to microspheres that were 9.77 µm in diameter. Human IgG was
conjugated with the fluorescent dye Fluorescein Isothiocyanate. The presence of human IgG on microcantilevers was detected using the fluorescence signal.

The following parameters were investigated during the optimization of bonding experiments: protein A bead concentration, antibody concentration, solution pH and the presence or absence of Ca$^{2+}$ or Mg$^{2+}$. The original concentration of protein A solution was 1.06 g/ml. The original concentration of human IgG in solution was 20 mg/ml. According to the certificate of analysis from Bangs Labs, the binding capacity of the protein A beads is 15.83 µg human IgG-FITC/mg particles. The antibody solution was diluted to 4 µg/ml and the bead solution was diluted to 0.256 mg/ml. Assuming 100% adsorption, the theoretical estimate of the concentration ratio is 15.6 µg human IgG-FITC/mg particles, which is quite close to the bonding capacity of the beads. A pH of 7.4 was used in previous experiments. For coating cantilevers, however, the protocols often say to do the coating at a different (usually more acidic) pH [112]. The pH value was varied to optimize bonding. The literature generally does not recognize that divalent cations are required for binding. For example, the study of binding between protein A and Human IgG used only phosphate buffered saline without any Calcium or Magnesium [134]. There is anecdotal evidence from the manufacturer's data sheet, however, that in some cases it has helped (Bangs Labs product data sheet No. 722 [137]). Accordingly, the effect of the presence or absence of the divalent cations Ca$^{2+}$ or Mg$^{2+}$ was also investigated.

Protein A and FITC-conjugated human IgG bonding tests were done using fluorescence microscopy [138]. The bonding test procedure is as follows. A preliminary
wash of the original protein A-coated bead solution was needed to remove additives like antimicrobials and surfactants. 975 µl of each the buffers in table 4.1 was put into six Eppendorf tube (1.5 ml) which were filled with 25 µl of original bead solution. The Eppendorf tubes were shaken on a vortex mixer for 5 seconds to mix the protein A-coated bead in corresponding buffer evenly. The bead suspension was spun in a microcentrifuge at 2000 rpm for 5 min. 900 µl of supematant was removed. An equal amount of fresh buffer was added to each tube before repeating the vortexing and microcentrifugation one more time. The bead solution was washed twice before bonding. 890 µl of buffer was added into the six tubes. The original concentration of human IgG solution was 20 mg/ml. The first dilution of antibody was done by adding 980 µl of each of the buffers in table 4.1 to the 20 µl of original antibody solution. 10 µl of antibody first dilution in HBSS+ was put in HBSS+ tubes, and 10 µl of antibody first dilution in HBSS- was put in HBSS- tubes. The beads were incubated in antibody solution on a 12 rpm rotary mixer (Hematology/Chemistry Mixer, Fisher Scientific, Hanover Park, IL) for 90 min in the dark at room temperature. After incubation, the tubes were spun and supernatant was removed. The beads in each tube were washed two times with the corresponding buffer. One drop from each tube was put on a glass slide, and a cover slip was laid on the top of the drop. The slide with liquid was placed on the stage of Nikon Eclipse TE 300 microscope. Fluorescent images were captured using an Andor iXon cooled CCD camera.
Table 4.1: Buffer and pH value for bonding test using fluorescence microscopy

<table>
<thead>
<tr>
<th>Identifier</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>HBSS-</td>
<td>HBSS-</td>
<td>HBSS-</td>
<td>HBSS+</td>
<td>HBSS+</td>
<td>HBSS+</td>
</tr>
<tr>
<td>pH</td>
<td>6.5</td>
<td>7.55</td>
<td>8.41</td>
<td>6.38</td>
<td>7.52</td>
<td>8.31</td>
</tr>
</tbody>
</table>

Glowing beads were found in Case A (HBSS-, pH is 6.5). The beads in Case B (HBSS-, pH is 7.55) were not as bright as in Case A. The beads in C (HBSS-, pH is 8.41) were less bright than in B. The beads in Case D were faint compared to those in Cases A, B and C. The beads in Case E and F were even fainter than Case D, however, the signal still could be seen clearly and many beads were detected in view, possibly because the presence of Ca$^{2+}$ or Mg$^{2+}$ can increase bonding percentage. The fluorescence signals in order of decreasing brightness were A > B > C > D > E > F. One possible reason was that the higher pH value made the beads photo bleach faster than the lower pH buffer. The cases A, B, C, D, E and F were made up at the same time, and then were processed in that order. So another possible reason for the drop in fluorescence could be that the fluorescence progressively bleached (even though kept in the dark) as time elapsed.

4.3.2 Bonding experiments of human IgG and protein A

The protein A and human IgG bonding interaction was investigated using the novel forced unbinding technique described above. Viewing chambers with E-glass fibers mounted in the side wall were filled with 4 mg/ml and 1 mg/ml human IgG conjugate FITC in HBSS+ or HBSS- for coating the glass fibers with human IgG. The E-glass fibers were incubated with antibody for 2 hours in the dark at room temperature to allow the antibody to adsorb to the E-glass fibers. The human IgG solution was then removed
and the viewing chambers were washed three times with fresh HBSS+ or HBSS- buffer, to wash out any unabsorbed antibody. Viewing chambers were refilled with protein A-coated microsphere solution. The protein A-coated microsphere solution was diluted 10000x in the buffers used in the antibody solution. The 1% BSA solution was heat treated in a water bath maintained at 50°C for 30 min. The viewing chamber filled with bead suspension was mounted on the stage of a Nikon TE 300 microscope with a 20x bright field objective. A pressure-induced flow from a micropipette was used to bring the fiber tip and bead into contact. Hundreds of fiber/bead apposition tests were recorded using a Labview program “fast pressure logger with valve.vi”. Images of each test was captured for data analysis and saved if an adhesive event was observed. The adhesive events were counted and the bond lifetime was measured.

Three sets of protein A and human IgG bonding interaction experiments were performed. Table 4.2 gives the IgG concentrations, fiber lengths, spring constant of glass fibers, suspension buffer type, and solution pH value used in specific bonding experiments. The fiber spring constant in table 4.2 were calculated from Elasticity theory using equation (1.9) with fiber diameter, $D = 4.2 \, \mu m$. As in the previous experiments, hydrodynamic flow was applied to the microcantilever tip which was moved from its initial rest position into contact with the bead. It was assumed that bonds had been formed if the microcantilever tip was still attached to the bead after the flow was eliminated. The forces applied on the bonds were calculated from the fiber tip displacement multiplied by the fiber spring constant (calculated from elasticity theory).
The initial distance between the micropipette and fiber before each bonding event was measured using LABVIEW program “Manual Particle Tracker from File Stack”.

<table>
<thead>
<tr>
<th>IgG concentration</th>
<th>Fiber length (mm)</th>
<th>Theory k (pN/um)</th>
<th>medium</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mg/ml</td>
<td>10.29</td>
<td>2.56</td>
<td>HBSS+</td>
<td>7.5</td>
</tr>
<tr>
<td>4 mg/ml</td>
<td>10.78</td>
<td>2.23</td>
<td>HBSS-</td>
<td>7.5</td>
</tr>
<tr>
<td>1 mg/ml</td>
<td>9.44</td>
<td>3.32</td>
<td>HBSS+</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Figure 4.5 shows the total contact test number and the percentage of contact events that led to adhesive events for the three sets of specific experiments. The percentage of adhesive events decreased from 1.2 % to 0.48 % after the buffer was changed from HBSS+ to HBSS- with 4 mg/ml human IgG and pH 7.5. This would suggest that Calcium or Magnesium can improve the binding frequency of Protein A and antibody, but there is no significant difference between the lifetimes for HBSS- and HBSS+. We conclude that that Calcium and Magnesium does not change the bonding lifetime (an observation that would be consistent with Calcium and Magnesium being unnecessary for the binding reaction itself between Protein A and IgG, as all the literature agrees). It may, however, change the binding frequency and that could mean that with Calcium and Magnesium present, the protein A or IgG is more available for binding. Another possibility is that changes in ionic strength due to the presence of divalent cations in the buffer produced changes in the non-specific environment that led to improved availability for the Protein A. With 1 mg/ml human IgG conjugate FITC in
HBSS+, the percentage of adhesive events was only 0.49%. This might be because pH 8.31 is a little high for IgG bonding; however, a lower pH value is needed for coating cantilever. It might also be because the concentration of human IgG used in this set experiments is too low for efficient bond formation.

Figure 4.7 shows the cumulative probability distributions of the bonding life time for nonspecific (red, solid line) and specific (blue, dashed line) adhesion experiments. The cumulative probability distributions of bonding lifetime for nonspecific and specific bonding experiments are not significantly different ($p = 0.098$). The bonds between protein A and human IgG are, thus, stronger than the nonspecific bonding.
4.3.3 Catch-slip transition fitting

Depending on how the lifetimes of individual receptor-ligand bond respond to applied force, bonds can be classified as catch bonds and slip bonds. The catch bond lifetime increases exponentially with force, whereas the slip bond lifetime decreases exponentially with force. Many of the bonds examined to date have shown catch behavior at lower forces and slip behavior at higher forces. A critical force corresponds to the transition point for which the interaction is transformed from a catch-bond at moderate force to a slip-bond at large force. At the critical force the catch-slip bonds reach their maximum lifetime.

For the catch-slip transition model [139], the unbinding rates rate, \( k \) is the sum of the catch bond rate \( k_c \) and the slip bond rate \( k_s \) in Eq. (4.1). The indices “c” and “s” refer to catch and slip bonds.

\[
k = k_c + k_s. \tag{4.1}
\]

The catch bond dissociation rate \( k_c \) and the slip bonds dissociation rate \( k_s \) can be modeled as exponential functions of applied force in Eq. (4.2) and Eq. (4.3).

\[
k_c = k_c^0 e^{x_c f / k_B T} \tag{4.2}
\]
\[
k_s = k_s^0 e^{x_s f / k_B T}, \tag{4.3}
\]

where \( k_c^0 \) and \( k_s^0 \) are the unbinding rates in the absence of force, \( x_c \) and \( x_s \) are the distances projected onto the directions of applied force, \( x_c \), will be negative, and \( x_s \) will be positive. The lifetime of receptor-ligand bond failure, \( \tau \), is a function of applied force, and can be calculated by the reciprocal of the reverse reaction rate leading to:

\[
\frac{1}{\tau(f)} = k_c^0 e^{x_c f / k_B T} + k_s^0 e^{x_s f / k_B T} \tag{4.4}
\]
Recall that at room temperature, $k_B T$ is around 4.1 pN·nm.

Figure 4.8 shows a plot of bonding lifetime as a function of applied force for protein A-human IgG bonds for three different fibers with lengths 10.29 mm (green, diamonds), 10.78 mm (yellow, squares)) and 9.44 mm (blue, triangles). The protein A-human IgG bonds have a biphasic response to force with longest lifetime at an intermediate level of force. The lifetime of bonds was short for the single data point at 4 pN then increased with increasing force up to a certain level like a catch bond, after which the lifetime of bonds decreased with increasing force like slip bonds. Bonds lasted several tens of seconds for forces of around 7 pN. The response of protein A-human IgG bonds interaction to force agreed with catch-slip transition mechanics.

![Figure 4.8](image.png)

Figure 4.8: Plot of bonding lifetime as a function of applied force for protein A-human IgG bonds.
4.3.4 Catch-slip transition fitting method

The experimental data of the lifetime vs. force was fitted to catch-slip transition using MATLAB. Figure 4.9 shows the flow chart of the curve-fitting process. The curve-fitting with Catch-slip transition model consists of three MATLAB programs. The main program “curvefitting_main.m” (attached as Appendix 13) was responsible for setting the initial value of variables which to be optimized and then performing the optimization using the function “fmincon”. The program “curve_fit.m” (attached as Appendix 14) was defined as a function, which was called by main optimization program. It calculated the optimization target and returned it to main program. The program “fit_equation.m” (attached as Appendix 15) converted the optimized results into catch-slip transition model parameters and performed Monte-Carlo simulation with this model. This program also checked the validity of the optimized variants with the condition: $\frac{k_c^0 \cdot \Delta_c}{k_s^0 \cdot \Delta_s} > 1$. 
To process the curve-fitting, the measurement data were read into the MATLAB program and the catch-slip transition model, from Eq. 4.4 was fitted.

In this model, there are four parameters to be optimized: $k_c^0$, $x_c$, $k_s^0$ and $x_s$. For each value of $f$, the difference between measured data and model result is calculated and squared:

$$\text{Difference}(i) = (\text{Measurement Data at } f_i - \tau(f_i))^2$$

The differences are then summed:

$$\text{Sum} = \sum_{i=1}^{N} \text{Difference}(i)$$
where $N$ is number of measurement points. The MATLAB program “curve_fit.m” performed the above operations, used the sum of squared differences as the optimization target and returned it to main program (curvefitting_main.m).

The program „fit_equation.m” printed the curve of Catch-slip mode, using the optimized parameters. Table 4.3 showed the initial values, optimization limit and best fit parameters. Those parameters should satisfy the condition:

\[
- \frac{k^0_c x_c}{k^0_s x_s} > 1
\]

<table>
<thead>
<tr>
<th>Table 4.3 Parameters in Catch-slip transition model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catch</td>
</tr>
<tr>
<td>$k^0_c$ (s$^{-1}$)</td>
</tr>
<tr>
<td>Initial value</td>
</tr>
<tr>
<td>Limit</td>
</tr>
<tr>
<td>Optimized value</td>
</tr>
</tbody>
</table>

Fig 4.8 showed the optimization points with red circular symbols. The red solid line represented the Monte-Carlo simulation, using the catch-slip model with optimized parameters. Random values of $f$ in the range of 0-11 pN were generated, and this simulation demonstrated that the Catch-slip transition model with optimized parameters fit the measurement data well. It also can be seen that the bond lifetime increased exponentially with force in simulation data; the bond lifetime increased with force up to
27.3 s at a critical force of 7.3 pN. Above this force, the trend reversed and the mean lifetime decreased with force just like slip bonds.

To estimate the probability of lifetime at 4 pN of small lifetime with catch-slip model, the probability density of lifetime of a single bond [33] is used:

$$p_r dt = k_r(f) \exp[-k_r(f)t] dt$$

(4.5)

Matlab program “Probability_catchslip” (attached as Appendix 16) was used to generate the probability of lifetime at 4 pN in range of 0 to 5 seconds. In this program, the probability density of lifetime of single bond was first calculated with the parameters of catch-slip model derived from curve fitting with average lifetime (Table 4.3). Then, the probability in range of 0 to 5 seconds is calculated by MATLAB integrator function. This probability is calculated as 0.99 which means that the probability of a short lifetime at 4pN is really high.

4.3.5 Bell model fitting

For Bell model, the unbinding rates rate $k$ is the slip bond rate $k_s$. The slip bonds dissociation rate $k_s$ can be modeled as exponential functions of applied force in Eq (4.6).

$$k_s = k_s^0 e^x_s f / k_B T$$

(4.6)

where $k_s^0$ are the unbinding rates in the absence of force, $x_s$ are the distances projected onto the directions of applied force. The lifetime of receptor-ligand bond failure, $\tau$, is a function of applied force, and can be calculated by the reciprocal of the reverse reaction rate leading to:

$$\frac{1}{\tau(f)} = k_s^0 e^{x_s f / k_B T}$$

(4.7)
The experimental data of the lifetime vs. force was fitted to Bell transition using MATLAB. The flow of curve-fitting is same as catch-slip curve-fitting process (Figure 4.9) with exception replacing the catch-slip model with the Bell model. The curve-fitting with Bell transition model consists of three MATLAB programs. The main program “curvefitting_main_ks.m” (attached as Appendix 17) was responsible for setting the initial value of variables which were to be optimized and then performed the optimization. The program “curve_fit_ks.m” (attached as Appendix 18) was defined as a function, which was called by main optimization program. It calculated the optimization target and returned it to main program. The program “fit_equation_ks.m” (attached as Appendix 19) converted the optimized results into Bell transition model parameters and performed Monte-Carlo simulation with this model. Table 4.4 showed the initial values, optimization limit and best fit parameters.

<table>
<thead>
<tr>
<th>Bell model parameters</th>
<th>(k_s^0) (s(^{-1}))</th>
<th>(x_s) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial value</td>
<td>0.001</td>
<td>5</td>
</tr>
<tr>
<td>Limit</td>
<td>[0,100]</td>
<td>[3,100]</td>
</tr>
<tr>
<td>Optimized value</td>
<td>0.0042</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig 4.8 showed the optimization points in pink diamond symbols. The pink solid line represented Monte-Carlo simulation, using the Bell model with optimized parameters. Random values of \(f\) in the range of 0-11 pN were generated, and this
simulation demonstrated that the Bell model with optimized parameter doesn’t fit the measurement data well. The high lifetime events at small forces that are expected from the Bell model were lacking. On the other hand, the main reason for the poor fit is the single data point at 4 pN. To assess whether the Bell model can be excluded, the likelihood of obtaining a lifetime that was that short, given the parameters from the Bell model, needed to be determined.

To calculate the probability of lifetime at 4pN of short life time with Bell model, the probability density of the lifetime of a single bond, Equation 4.5 is used again. In this case, the probability density of lifetime of single bond is calculated with the parameters of Bell model derived from curve fitting with average lifetime (Table 4.4). Matlab program “Probability_Bell” (attached as Appendix 20) was used to generate the probability of lifetime at 4pN in range of 0 to 5 seconds. This program is similar to the MATLAB program “Probability_catchslip” (Appendix 16). In this program, the probability density of lifetime of single bond was first calculated with the parameters of Bell model derived from curve fitting with average lifetime (Table 4.4). Then, the probability in range of 0 to 5 seconds is calculated by MATLAB integrator function. The probability with Bell model is calculated as 0.3061 which means that the probability of obtaining a short lifetime for 4pN is not especially small given the parameters calculated for the Bell model. It is plausible to say that the catch-slip model is a better fit to the data, but the Bell model could not be excluded. More data would be required to be certain.
4.4 Results and Discussion

A novel microcantilever system was built to apply forces to receptor-ligand bonds. In this experiment, a bead coated with protein A was held using a micropipette, and a glass fiber microcantilever coated with human IgG was pushed into contact with the bead using pressure-induced flows from a second micropipette. If a bond formed, then when the flow stopped, the force would be applied to the protein A-human IgG bond rapidly. The hydrodynamic flow changed from the maximum value back to zero within 0.1 second. Applying forces on receptor-ligand bonds was very quick and could be regarded as quasi-instantaneous using this novel microcantilever system.

In other spring-based techniques, like AFM, biomembrane force probe or the previous microcantilever method, it takes a significant amount of time to establish the desired force on the bonds since the elastic element must be stretched. The forces with some constant loading rate were applied to receptor-ligand bonds. The typical scale of these loading rates was that a 100 pN force needed 0.01-1 seconds time period to load. The rupture forces were the function the loading rates. The force increased linearly as a logarithm of the loading rate in the force spectroscopy model. The Bell model parameters were calculated from the average force at the moment of bond dissociation and force loading rate. The advantage of the current technique compared to these other methods is that the bond lifetimes as a function of forces can be measured directly in the novel microcantilever system. The measurement of the force dependence of bond lifetime is model-independent process in this experiment.
Bond lifetimes will increase greatly when multiple bonds are present in receptor-ligand adhesions. The probability adhesive events can be used to determine the single and multiple bonds presence ratio. The probability of having multiple bonds obeys a Poisson distribution [140]. The adhesive events occur in only 15-20% of tests to ensure that most of the events will involve single bonds. The percentages of adhesive events stayed from 1% to 2%, so the bond lifetime distributions should represent single bonds dissociation rates in these experiments. Nonspecific adhesion still existed in adhesive events. The bond dissociation rate’s dependence on force represented a combination of nonspecific adhesion and specific adhesion.

The slip bond dissociation parameters in catch-slip transition model and the critical force value obtained in this Ph.D dissertation work are reasonably consistent with measurements of protein A - IgG binding experiments results reported in the literature [141]. Nguyen-Duong et al. used the biomembrane force probe method to measure the dissociation rate of single specific bond between protein A and IgG. They found a characteristic force of \( f_0 = 4.5 \pm 0.5 \, pN \) by simulation fitting. The characteristic force is related to the \( r_0 \) in following equation:

\[
f_0 = \frac{k_B T}{r_0}
\]

Then the Bell model parameter could be calculated as \( r_0 \approx 1.1 \, nm \) which is at least the same order of magnitude as the 3.1 nm from the results of the present work. They also found a value of the dissociation rate of the molecular bond is 0.001s\(^{-1}\) which is 10 times the slip bond dissociation parameters in catch-slip transition model in this work, but in complete agreement with the Bell model fit value. Because of the force
spectroscopy method limitation, they only fitted the experimental data to the Bell model.

The catch bond was also not generally recognized at that time.
CHAPTER 5: CONCLUSION AND SUGGESTIONS FOR FUTURE WORK

5.1 Concluding Remarks

In this dissertation, a novel microcantilever unbinding technique system was built and proved useful in a preliminary receptor-ligand forced unbinding adhesion study. Microcantilevers made from E-glass fiber were used as spring-based force sensors. The spring constants of microcantilevers were calibrated using the thermal fluctuation method (described in Chapter 2). The novel microcantilever unbinding system was built to apply force to receptor-ligand bonds rapidly, which should allow the force dependence of bond lifetime to be determined without assuming a particular model. The relative positions of fibers and micropipettes in the horizontal and vertical directions were investigated to optimize the strength and positioning of the hydrodynamic flow applied on glass fiber tips. The relationship between the fiber tip displacement vs applied pressure at a constant micropipette tip ID and between fiber tip displacement and the micropipette tip ID for a constant pressure were (described in Chapter 3). The Protein A and human IgG bonding interaction was investigated using the novel microcantilever unbinding technique system, and the lifetime as a function of force was directly observed without any model limitation (described in Chapter 4). The results and conclusions were summarized as follows:

1. The spring constant of glass fibers with different lengths measured by the thermal fluctuation method were consistent with the calculations from Elasticity theory using of 40x and 100x microscope objectives.

2. The moving average filter with 20 points used for image analysis of microcantilever motion during original data processing was shown to be the
optimal length filter choice by Monte Carlo simulation in our experimental conditions.

3. For this novel microcantilever unbinding technique system made from E-glass fiber, the fiber displacement increased linearly with pressure and with increasing micropipette inner diameter. The fiber tip deflection was easy to control and was able to apply 1-50 pN range forces on receptor-ligand bond in 0.1 second.

4. The presence Ca$^{2+}$ and/or Mg$^{2+}$ was necessary for protein A and human IgG bonding.

5. The catch-slip is a better fitting model for the lifetime vs. force of protein A-human IgG bonding, but the Bell model could not with confidence be excluded without additional data.

5.2 Suggestions for Future Studies

A novel forced unbinding system was developed to measure the force dependence of receptor-ligand bonding dissociation rates by applying hydrodynamic forces on coated microcantilever and beads with adhesion molecules in the work. However, more work needs to be done in order to evaluate the validity of the novel microcantilever unbinding system.

1. The parameters in optimization of protein binding still need more investigation. The antibody concentration needs to be adjusted to increase the percentage of adhesive events to a proper number when the adhesive events occur in only 15-20% of tests to ensure that most of the events will involve single bonds. The percentage of adhesive events is still too low compared to 15-20%.
2. Other receptor-ligand bond pairs could be used to test the validity of the novel microcantilever unbinding system. The receptor E-selectin and corresponding ligands could be chosen as adhesion molecules. Two ligands that E-selectin binds to are P-selectin glycoprotein ligand-1 (PSGL-1 or CD162; a recombinant chimeric version is available from R&D Systems) and various forms of the adhesive protein CD44 that are decorated with different sets of sugars.

3. A combined optical tweezer / microcantilever system still needs to be built for applying force to bonds quasi-instantaneously. The optical trap will need to be strong enough to trap and drag the microcantilever tip, however, which prevented the use of our optical tweezer system for this purpose.
REFERENCES


137. Proactive protein A coated microshperes. 2013; Available from:


APPENDIX 1 LABVIEW CODE FOR PROGRAM “VERTICAL AVERAGE X PIXELS VALUE.VI”

Read Images Path

Y Start | X Start
-------|-------
180    | 280   

Y end | X end
------|------
450   | 480   

file path

```text
C:\work\PHD research\Fiber sections\Fiber 1\focus 36.bmp
```

```text
C:\work\PHD research\Fiber sections\oneframe.txt
```
APPENDIX 2 LABVIEW CODE FOR PROGRAM “TWOMIND.VI”
APPENDIX 3 LABVIEW CODE FOR PROGRAM “PEAK.VI”
APPENDIX 4 THE LABVIEW CODE FOR “TWOMAX.VI”
APPENDIX 5 THE LABVIEW CODE FOR “TWOMIN.VI”
APPENDIX 6 THE MATLAB CODE FOR “MOVINGAVERAGEFILTER.M”

clear;
cle;

P=4000;
N=10;

for i=1:2000
    freq(i)=i/P;  f=freq(i);
    A=sin(pi*f*N);
    B=N*sin(pi*f);
    H_10(i)=abs(A/B);
end

N=20;

for i=1:2000
    freq(i)=i/P;  f=freq(i);
    A=sin(pi*f*N);
    B=N*sin(pi*f);
    H_20(i)=abs(A/B);
end

N=50;

for i=1:2000
    freq(i)=i/P;  f=freq(i);
    A=sin(pi*f*N);
    B=N*sin(pi*f);
end
H_{50}(i)=\text{abs}(A/B);

end

plot(freq,H_{10},freq,H_{20},freq,H_{50})
APPENDIX 7 THE MATLAB CODE FOR “READ_FFT.M”

clear;

%open txt file and read into data array

fid=fopen('sample.txt');
D=fscanf(fid,'%f');
P=length(D);

Fs=1;

freqres = Fs/P;
x_fft= fft(D);
xdft = x_fft(1:P/2+1);
xdft = 1/P*xdft;
xdft(2:end-1) = 2*xdft(2:end-1);

Data_Mag=abs(xdft)
Data_dB=20*log10(abs(xdft));

freq = 0:Fs/P:Fs/2;
plot(freq,Data_dB),axis([0,0.5,-80,65])
APPENDIX 8 THE MATLAB CODE FOR “METHOD_VERIFY.M”

clear;
cle;

KBT=13.806503*(273.15+25)*10^(-6);
P=2425; %set the number of random data,

for m=1:3

K(m)=10^(m-1)     %unit is pN/um
S_D=sqrt(KBT/K(m))/0.13;

%Generate random data, which has standard deviation of S_D and average of
%zero. It satisfy Gaussian distribution.
R=normrnd(0,S_D,P,1);

for n=1:P
    Data(n)=R(n,1);
end

%calculate the k_spring of known data
std_pixel=std(Data);
k_spring_known(m)=KBT/(std_pixel*0.13)^2;

%Generate low freq. noises and added to high freq. data
DC=345.3578; M1=0.4408; M2=0.1742; M3=0.0945; M4=0.0755; M5=0.0715;
M6=0.0458; M7=0.0703;

f1=1/P; f2=2/P; f3=3/P; f4=4/P; f5=5/P; f6=6/P; f7=7/P;

for n=1:P

    Low_Noise(n)=M1*sin(2*pi*n*f1)+M2*sin(2*pi*n*f2)+M3*sin(2*pi*n*f3)+M4*sin(2*pi*n*f4)+M5*sin(2*pi*n*f5)+M6*sin(2*pi*n*f6)+M7*sin(2*pi*n*f7)+DC;
    Data(n)=Data(n)+Low_Noise(n);
end
Data_Ave=0;

%calculate the average of N=500
N=500; a=0;

for i=1: P-N+1
    for j=i:i+N-1
        a=a+Data(j);
    end
    a=a/N;
    Data_Ave(i)=a;
    a=0;
end
APPENDIX 9 THE LABVIEW CODE FOR “AVI DECOMPOSER.VI”

The output images will be placed in a subdirectory of the base path with the same filename as the video. If that subdirectory does not exist, it will be created.
APPENDIX 10 THE MATLAB CODE FOR “READ_PROCESS.M”

clc;
clear;

%open txt file and read into data array

fid=fopen('3peak.txt');
D=fscanf(fid,'%f');

KBT=13.806503*(273.15+25)*10^(-6);
P=length(D);

Fs=1;

freqres = Fs/P;
xdft= fft(D);
xdft = xdft(1:P/2+1);
xdft = 1/P*xdft;
xdft(2:end-1) = 2*xdft(2:end-1);

xdft_f_amplitude=abs(xdft)
xdft_f_dB=20*log10(abs(xdft));

freq = 0:Fs/P:Fs/2;
plot(freq,xdft_f_dB);

%calculate the average of N=20
N=20; a=0;

for i=1: P-N+1
    for j=i:i+N-1
        a=a+D(j);
    end
    a=a/N;
    Data_Ave(i)=a;
a=0;
end

Data_N=size(Data_Ave);

% The result average Data_Ave(i) match to D(i+N/2)
% calculate the difference

for i=1:Data_N(2)
    diff_var(i)=D(i+N/2)-Data_Ave(i);
end

std_pixel=std(diff_var);
k_spring_20=KBT/(std_pixel*0.059)^2
APPENDIX 11 THE LABVIEW CODE FOR “PRESSURE LOGGER ULTIMATE.VI”
APPENDIX 12 LABVIEW CODE FOR PROGRAM “FAST PRESSURE LOGGER WITH VALVE.VI”
APPENDIX 13 THE MATLAB CODE FOR “CURVEFITTING_MAIN.M”

curvefitting_main.m

clc;
clear;

x=[50,-7.5,0.1,0.6];
lb=[0,-100,0.0001,0];
ub=[100,0,100,100];

options=optimset('LargeScale','off');

[x,fval]=fmincon(@curve_fit,x,[],[],[],[],lb,ub,[],options);

x
APPENDIX 14 THE MATLAB CODE FOR “CURVE_FIT.M”

curve_fit.m

function ripple=curve_fit(x);

%open txt file and read into data array
fid=fopen('F:\binding\X.txt');
xdata=fscanf(fid,'%f');
close(fid);

fid=fopen('F:\binding\Y.txt');
ydata=fscanf(fid,'%f');
close(fid);
P=length(ydata);

kc1=x(1);      xc1=x(2); ks1=x(3);      xs1=x(4);

for i=1:P
Result(i)=kc1*exp(xc1*xdata(i)/4.1)+ks1*exp(xs1*xdata(i)/4.1);
DI(i)=(1/Result(i)-ydata(i))^2;
end
a=0;
for i=1:P
a=DI(i)+a;
end
ripple=a;
APPENDIX 15 THE MATLAB CODE FOR “FIT_EQUATION.M”

fit_equation.m

kc1=x(1); xc1=x(2); ks1=x(3); xs1=x(4);
condition_f=-kc1*xc1/ks1/xs1;
if (condition_f>1)
    T=1
end

fid=fopen('F:\binding\X.txt');
xdata=fscanf(fid,'%f');
close(fid);

fid=fopen('F:\binding\Y.txt');
ydata=fscanf(fid,'%f');
close(fid);

P=length(ydata);
for i=1:P
    result(i)=kc1*exp(xc1*xdata(i)/4.1)+ks1*exp(xs1*xdata(i)/4.1);
    result(i)=1/result(i);
end

for i=1:1100
    m=i/100; x_axis(i)=m;
    result_p1(i)=kc1*exp(xc1*m/4.1);
    result_p2(i)=ks1*exp(xs1*m/4.1);
    result_p(i)=1/(result_p1(i)+result_p2(i));
end

plot(xdata,ydata,'o',xdata,result,'*',x_axis, result_p)
clear;
cle;

fid=fopen('X.txt');
xdata=fscanf(fid,'%f');
fclose(fid);

KBT=4.1; %unit is pN*nm
kc0=100; ks0=0.0001; xc=-4.9931; xs=3.0525;

f=xdata(2);
kr=kc0*exp(xc*f/KBT)+ks0*exp(xs*f/KBT);

% Exponential Probability model
% Probability density=kr*exp(-kr*t)

P_D = @(t) kr*exp(-kr*t);
P=quad(P_D,0,5)
APPENDIX 17 THE MATLAB CODE FOR “CURVEFITTING_MAIN_KS.M”

curvefitting_main_ks.m

cle;
clear;

x=[0.001,5];
lb=[0,3];
ub=[100,100];

options=optimset('LargeScale','off');

[x,fval]=fmincon(@curve_fit_ks,x,[],[],[],[],lb,ub,[],options);
function ripple=curve_fit_ks(x);

%open txt file and read into data array

fid=fopen('X.txt');
xdata=fscanf(fid,'%f');
fclose(fid);

fid=fopen('Y.txt');
ydata=fscanf(fid,'%f');
fclose(fid);

P=length(ydata);

ks1=x(1);     xs1=x(2);

for i=1:P
    Result(i)=ks1*exp(xs1*xdata(i)/4.1);
    DI(i)=(1/Result(i)-ydata(i))^2;
end

a=0;
a=DI(1)+DI(3)+DI(4)+DI(5)+DI(6)+DI(7)+DI(8)+DI(9)+DI(10)+DI(11)+DI(12)+DI(13);

ripple=a;
APPENDIX 19 THE MATLAB CODE FOR “FIT_EQUATION_KS.M”

```
fit_equation_ks.m

%clc;
%clear;

ks1=x(1);    xs1=x(2);

max_x_axis=0;        max_y_axis=0;

fid=fopen('X.txt');
xdata=fscanf(fid,'%f');
close(fid);

fid=fopen('Y.txt');
ydata=fscanf(fid,'%f');
close(fid);

P=length(ydata);

for i=1:P
    result(i)=ks1*exp(xs1*xdata(i)/4.1);
    result(i)=1/result(i);
end
```
appendix 20 the matlab code for “probability_bell.m”

probability_bell.m

clear;
clc; th

fid=fopen('x.txt');
xdata=fscanf(fid,'%f');
fclose(fid);

KBT=4.1; %unit is pN*nm
ks0=0.0042; xs=3;

f=xdata(2);
kr=ks0*exp(xs*f/KBT);

%Exponential Probability model
%Probability density=kr*exp(-kr*t)

P_Bell_D = @(t) kr*exp(-kr*t);
P_Bell=quad(P_Bell_D,0,5)