Detection techniques for molecular interactions based on interferometry and micro-cantilever

Zhichen Zhu
Iowa State University

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Detection techniques for molecular interactions based on interferometry and micro-cantilever

by

Zhichen Zhu

A dissertation submitted to the graduate faculty

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Program of Study Committee:
Pranav Shrotriya, Major Professor
Marit Nilsen-Hamilton
Jonathan Claussen
Meng Lu
Shan Hu

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

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ABSTRACT

Biosensors based on Atomic force microscope (AFM) cantilevers have recently been showing promising potential because of its portability and high sensitivity, the gold coated cantilever surface allows functionalization on the cantilever by immobilizing biomolecules like enzyme, DNA, RNA on the cantilever surface. Biomolecular reactions between the immobilized molecules and its target molecules on the cantilever can result in change in measurable mechanical signal like deflection, resonant frequency. Researchers can detect the presence and quantity of target molecules by measuring the deflections or resonant frequency change. This property of micro cantilever allows itself to have potential application in DNA/Protein detection. In addition, the gold surface of cantilever makes it possible to apply electrochemical technology in the biosensing system.

We designed a mini optical system to monitor the deflection of cantilever when the DNA aptamer of Ebola virus glycoprotein (GP1,2) that is immobilized on the cantilever reacts with GP1,2 in the environment. The same experiment was conducted using Ebola secreted glycoprotein (sGP) and its DNA aptamer. The results show that the increase of GP1,2 concentration led to an increase in tensile surface stress, the tensile surface stress saturated at about 200nM of GP1,2. While the sGP results show that the increase of sGP concentration produced a compressive surface stress which saturates at 20nM. The linear range of experiment data has potential in the detection of Ebola virus at nanomolar concentration.

We also set up a micro optical system of shoe box size to monitor the deflection of cantilever when the immobilized aptamers bind with target molecules in the environment. A verification with human alpha thrombin and its aptamer was conducted, which shows a
promising potential of alpha thrombin detection. The portability of this new system allows a rapid detection at point-of-care.

Other future work is planned in the last chapter, which includes a molecular dynamics simulation of stress induced by the binding between glycoprotein and its aptamer, and a novel electrochemical biosensor based on micro-cantilever.
1.1 Biosensors

Biosensors are used to quantify the molecules of interest, and they are widely used in many fields, especially in disease diagnosis and drug delivery. A basic biosensor consists of six components, the target molecule, the recognition element, the transducer, the detector, the display and the end user. First, the recognition element selectively interacts with the target molecules when they are in the same environment, causing energy change that can be transduced into another detectable form of signal, such as mechanical signal change, optical signal change or electrical signal change. The detector will capture this new signal form and transport it to the display for the end user to analyze the signal.

A brief introduction about different biosensors is provided below.

Optical biosensors can be divided into label-free real-time detection (e.g. micro-ring resonator (MRR), single-particle interferometric reflectance imaging sensor (SP-IRIS)) and labelled detection (e.g. enzyme-linked immunosorbent assays). A micro-ring resonator (MRR) consists of a resonator coupled to an optical fiber, target molecule adsorbed to the surface of resonator can change the resonator’s response. It has a detection limit of nanomolar and a short response time (2 mins) [1]. Single-particle interferometric reflectance imaging sensor (SP-IRIS) uses a SiO2 surface immobilized with antibody to link nanoparticles that captures virus with antibody on the particle surface. This particle-antibody-virus-antibody complex on the SiO2 surface will be lighted with a single waveform and a high-magnification objective lens is used to image the nanoparticles on a CMOS camera. Any captured particles will appear as bright dots in the image. The labelled detection, ELISA, will be explained in the next chapter.
Nanowire biosensor is a commonly used electrical biosensors. In the detection, the semiconducting nanowire will have conduction change when target molecules bind to the surface of nanowire, the change of conduction is used as the signal to quantify the target molecule. Nanowire biosensor can achieve very a high sensitivity (low detection limit) of pico-molars levels in a short period of time. [2]

Another main group of biosensors is mechanical biosensor, which use the signals in mechanical domain like force, displacement and mass. Compared to non-mechanical biosensor, mechanical biosensors are of interest because of their advantages, such as high sensitivity and short response time. The most commonly used mechanical biosensors are suspended microchannel resonator, quartz crystal monitor, and microcantilever biosensors. A suspended microchannel resonator (SMR) consists of vacuum-packaged microcantilevers that is embedded by micro channels, the resonance frequency of microcantilever is dependent on the mass on the microcantilever. As the biomolecules pass through the microchannel, adsorption of biomolecules on the channel can displace equivalent volume, and as result increase the density of biomolecules, the change in density can results in an increase of mass on cantilever, this extra mass changes the measurable resonance frequency of microcantilever. This SMR provides an inexpensive way of measuring a 300 picomolar signal [3].

Sensors based on Atomic Force Microscope (AFM) cantilevers are functionalized by coating the cantilever surface with molecules (e.g. DNA or receptor) that can interact with target molecules, by measuring the change of deflection or natural frequency of cantilever caused by the interaction, the presence or the quantity of target molecules can be detected. This kind of sensors have been widely used since 1990s, researchers have used to detect mercury molecules with pictogram resolution [4], it was also utilized to detect the formation of alkanethiol self-
assembled monolayers (SAM) [5] and biomolecules, like single-stranded DNAs [6].

Microcantilever based biosensors have the potential in nanoscale sensing and controlling, with advantages of high sensitivity, portability and quick response, it can be applied as the essential part of controlling biological system to detect diseases. The following section will introduce the mechanics of functionalized cantilever.

1.2 Microcantilever Biosensors

Atomic force microscope (AFM) Micro-cantilevers can be applied as an important mechanical biosensor because of its high sensitivity to surface stress change, the gold coated surface of micro-cantilever also allows it to be functionalized with biomolecules using thiol-gold bond. When molecular activities such as ion adsorption/desorption, molecule binding or conformational change happens on the surface of cantilever, a surface stress change was generated, which cause the cantilever deflection. Once the deflection is measured, the surface stress change can be calculated using Stoney’s formula [7]:

\[ \sigma = \frac{Eh^2}{6R(1 - \nu)} \]

Where \( \sigma \) is the surface stress change, \( h \) is the thickness of cantilever, \( R \) is the curvature radius change of deflected cantilever, \( E \) is Young’s modulus, \( \nu \) is Poisson’s ratio of cantilever. The curvature radius change, \( R \) is dependent on \( L \) (cantilever length) and \( \delta \) (cantilever deflection):

\[ R = \frac{L^2}{2\delta} \]
By combining the above two equations, the relationship between surface stress change and deflection can be obtained:

$$\sigma = \frac{\delta E h^2}{3L^2(1-v)}$$

It can be seen that the surface stress change is proportional to deflection of cantilever. By using a Young’s modulus of silicon as 170 GPa, Poisson’s ratio of 0.22, cantilever thickness of 10E-6 m, cantilever length of 5E-4 m, the factor, $\frac{E h^2}{3L^2(1-v)}$ for cantilever can be calculated as $290598 \frac{N}{m^2}$, which means each nanometer of deflection is produced by $0.290598 \frac{mN}{m}$ of surface stress change.

Because the sensor surface is coated with receptor (the recognition element) molecules that have affinity to the target molecule, when the receptor coated surface is exposed to target molecules in solution, the fraction of receptor molecules binding to target molecules may be estimated by Langmuir equation:

$$\varphi = \frac{K_d[B]}{K_d[B]+1} \Rightarrow \varphi \approx K_d[B]forK_d[B] << 1$$

Where $K_d$ is the binding affinity between receptor and target molecules, $[B]$ is the concentration of target molecules. At low concentrations of target molecules, the surface coverage becomes almost proportional to $[B]$. Thus, the sensor response is proportional to the number of target molecules bound to the functionalized surface. As a result, the sensor’s detection limit is limited by two factors: 1) the $K_d$ between the target molecules and the receptor molecules; 2) the recognition element’s sensitivity for target/receptor complexes.
Antibodies and aptamers are commonly used as the receptor. Antibodies, they proteins that have high affinity with specific epitopes of antigens, can be produced in vivo, which makes them expensive to make. Aptamers are DNA or RNA molecules with affinity towards target molecules, they are produced by systematic evolution of ligands by exponential enrichment (SELEX), in which the aptamer was selected by repeating the SELEX cycle including binding, partition, elution, amplification, and conditioning. Compared to antibodies, aptamers have some characteristics: 1) easier and cheaper to make; 2) can be modified with different functional groups; 3) easier to store. These advantages make aptamers competitive in biosensing research.

1.3 Ebola Virus and the glycoproteins of Ebola virus

Ebola Virus Disease (EVD) is a highly infectious disease caused by Ebola virus and causes serious hemorrhagic fever. It was first found in 1976 and quickly became one of the deadliest diseases. The latest Ebola outbreak was found in West Africa in December 2013 and more than 24600 cases have been reported by March 2015 [8, 9]. Three of four know Ebola virus species can cause hemorrhagic disease in human body, and they are Zaire, Sudan, and Ivory Coast. Ebola virus can be easily transmitted between humans and animals through contact with infected body fluids [8, 10-12]. Due to the short time from infection to death, a rapid, sensitive and cost-effective biosensing method is worth to develop to allow early diagnostics before therapeutics.

The envelope glycoprotein GP1,2, which can be found on the surface of Ebola virus, composed of disulfide-linked fragments GP1 and GP2 [13], is one minor product of the GP gene 4 of EBOV genome. GP1,2 is able to activate the endothelial cells and induce a decrease in barrier function of cell membrane, as a result, allows the Ebola virus to enter the cell. While the
secretory glycoprotein (sGP), the primary product of the GP gene 4 of EBOV, sGP plays a protective role in endothelial layer integrity when endothelial cells upon infection, thus showing an anti-inflammatory function during EBOV hemorrhagic fever [14].

The transcription encoding of GP gene 4 of EBOV genome consists of 70% of sGP and 25% GP1,2, [15] thus the sGP has higher expression level. The sGP has also been detected in the infected patients [16], which make it a good target molecule in Ebola detection.

1.4 Ebola virus Aptamer

Both Ebola GP1,2 and sGP have good affinity to their DNA aptamer (oligo 6011 from Prof. Marit Nilsen-Hamilton’s lab), a single strand DNA molecule, which enables us to use its binding process to develop medical application. Compare to antibody and its ligand, protein and its aptamer have advantages in biosensing and controlling experiments because aptamer has high affinity to its protein at low concentration even though the sequence of aptamer is short. This Ebola DNA aptamer was synthesized in Systematic evolution of ligands by exponential enrichment (SELEX) by my colleague, Dr. Soma Banerjee. In one SELEX cycle, the GP1,2 and sGP were incubated in a large single-strand DNA (ssDNA) library, where the protein bound with ssDNA will be collected on a nitrocellulose membrane, then the collected ssDNA will be extracted with 7M urea and purified, then the extracted ssDNA will be amplified through polymerase chain reaction (PCR) with reverse primer biotinylated, finally the non-biotinylated ssDNA will be separated by streptavidin coated magnetic beads (MB) and purified. Several SELEX cycles will be conducted until the target-specific aptamers dominate within the selected oligonucleotide pool.
1.5 Ebola biosensors

A literature review about Ebola virus biosensor was conducted and we found that the existing Ebola biosensors can also be classified into optical methods, electrical methods, mechanical methods and hybrid methods.

Optical Ebola biosensors transduce the biomolecular interaction into the change in light intensity or colors, they include enzyme-linked immunosorbent assay (ELISA), Lateral Flow Immunoassays (LFI), Reverse transcriptase polymerase chain reaction (RT-PCR).

Enzyme-linked immunosorbent assay (ELISA), a typical labelled detection, uses an antibody-antigen-antibody-enzyme structure (a sandwich structure), the enzyme on the top of sandwich catalyze added substrate to produce measurable optical signal. This structure amplifies the signal from target molecule and gives ELISA a detection limit of pico-molar. However, it requires different kinds of biomolecules and a period of time for biosensing. [2]. Jae-Sung Yu et al. developed an ELISA based on one antibody of envelope glycoprotein (GP1,2) and achieved a detection limit of 20ng [17].

Lateral Flow Immunoassays (LFI) is a commonly used biosensor, a typical LFI is a pregnancy test bar. In a LFI, sample of antigen will be dropped onto a nitrocellulose membrane, binding with detector antibody (colored or fluorescent), this antibody-antigen complex will pass to an indicating region of membrane where the capture antibody is immobilized, the capture antibody will bind with the antibody-antigen complex and delivers a color or fluorescent on the indicating region as a signal of detection. Chun-Wan Yen et al. [18] and Jill C. Phan et al. [19] applied LFI based techniques to achieve detection limit of 150ng/ml of GP1,2 and 50000 plaque-forming-units/test respectively.
Electrical methods transduce the biomolecular interaction into the change in electrical signal (like, voltage, current or impedance). A typical electrical Ebola biosensor is field-effect transistor (FET) method. In a Field-Effect Transistor Biosensor, a fixed drain-source voltage was applied between the drain and source electrodes of a reduced graphene oxide (rGO) FET, the binding between the antibody on the gold nanoparticle and the negatively charged glycoprotein can induce a positive potential on the gold NPs through the dielectric antibody. The positive potential of the gold nanoparticles then leads to a decreased hole concentration in the p-type rGO through Al2O3 gate oxide, leading to a decrease in the electrical conductance of the rGO, which results in a change in drain-source current. Yantao Chen et al. applied this rGO FET as a GP1,2 biosensor to achieved a detection limit of 1ng/ml of GP1,2.[20]

Mechanical methods transduce the biomolecular interaction into the change in mechanical signals (like, deformation, natural frequency). Typical mechanical Ebola biosensors includes Surface acoustic wave (SAW) and quartz crystal microbalance (QCM).

Surface acoustic wave (SAW) applies an alternating voltage on an inter-digital transducer made of piezoelectric substrate to produce a horizontally polarized surface shear waves. The resonant frequency of this shear wave is sensitive to changes on the sensor surface immobilized with antibody bound with Ebola virus. This method was done by Justin T. Baca [21] with detection limit of 19000FPU/ml.

A quartz crystal monitor (QCM) uses a quartz crystal resonator with a resonance frequency that is highly sensitive to addition and removal of mass on the surface of resonator, when target molecules bind to the surface of resonator, a measurable change in the resonance frequency of the resonator can be used to estimate the quantity of target molecules. The label-free QCM has detection limit of 1 nM [22], but labeled QCM can achieve femtomolar level
detection limit [23]. Jae-Sung Yu et al. also applied one GP1,2 antibody on the quartz crystal monitor (QCM) to obtain a GP1,2 detection limit of 14nM [17].

Hybrid methods combines two or more above methods to amplify the transduced signal, thus achieves high sensitivity or specificity. Hybrid Ebola biosensors includes reverse transcription polymerase chain reaction (RT-PCR), electroluminescent nanosphere method, nanoporous membrane system.

Reverse transcriptase polymerase chain reaction (RT-PCR) followed by gel electrophoresis, is usually used to detect RNA molecule, thus can detect RNA virus. This method used reverse transcriptase to synthesize complementary DNA (cDNA) based on a template RNA, and then use polymerase to amplify the amount of cDNA. The product of RT-PCR (the DNA) will be analyzed with gel electrophoresis to determine its affinity to a certain protein in order to identify the DNA. Tammy R. Gibb et al. applied RT-PCR for the detection of Ebola GP1,2 gene with a detection limit of 100pg of GP1,2 gene [24].

Electroluminescent nanosphere method basically immobilize magnetic nanobeads (MBs) with antibody, when the immobilized antibody capture Ebola virus, electroluminescent nanospheres (ENs) with antibody on them also binding with Ebola virus to form a sandwich structure. These sandwich structures will be moved to a magnetic gold nanoisland film electrode (M-AuE). The M-AuE works as a and working electrode. The electrochemical reaction on the working electrode can trigger the electroluminescence (ECL) effect on ENs, allowing the virus to be detected. Zhen Wu et al. applied this electroluminescent nanosphere method for Ebola virus detection with detection limit of 5.2pg/mL Ebola virus [25].

Nanoporous Membrane method anchored upconversion nanoparticles (UCNPs) on a nanoporous alumina (NAAO) membrane to form a sensitive surface. Amino-modified oligo
probes are immobilized on UCNPs, while the Ebola virus oligo conjugates onto gold nanoparticles (AuNPs), which leads to the hybridization between the oligo probes and virus oligo. Under the 980nm diode laser excitation, the upconversion emissions of UCNPs are absorbed by AuNPs, leading to an LRET process between UCNPs and AuNPs. The high surface area to volume ratio of NAAO allows large amount of UCNPs to be anchored on the membrane surface for high optical intensity. This Ebola biosensor was developed by Ming-Kiu Tsang et al. to achieve a detection limit of 300 fM Ebola oligonucleotide [26].

Three of above methods reported detection limits of GP1,2 amount, [17, 18, 20]. However, all of them involves using antibody as the recognition element, which is more expensive and time consuming to produce compared to aptamer-based biosensor. In this research, we developed an economical and portable aptamer based biosensing system to allow Ebola detection at the point-of-care.

1.6 Acute Kidney Injury (AKI) and Neutrophil gelatinase–associated lipocalin (NGAL)

Acute kidney injury (AKI) is a serious condition, which is commonly found after cardiac surgery and renal damage due the exposition to toxic agent and drugs, thus gained an increasing attention in the last years [27]. While the lack of reliable biomarker results in a delay in diagnosis and limits the proper treatment [28].

Traditional AKI diagnosis is based on the serum creatinine measurement. However, creatinine cannot be an ideal AKI biomarker because it increases only after half of the kidney function is lost, which results in a delay in detection[29]. Hence, a more reliable biomarker is needed for early diagnosis of AKI[27, 30-32].
The neutrophil gelatinase-associated lipocalin (NGAL), a molecule of 178 amino acids, belongs to lipocalin family. It is responsible for binding and transporting small hydrophobic molecules[32]. Moreover, NGAL is an important component for immunity to bacterial infection[33]. The level of NGAL can rise 1000-fold in human in the presence of renal injury, which makes it an ideal early biomarker of AKI [34].

1.7 Current NGAL based biosensors

Currently, only a few measurement methods that can accurately evaluate NGAL has been reported. The majority of NGAL measurements has not provided sufficient data to prove their reliability and efficiency. The most common measurement methods are human urine or serum tests based on the ELISA [35] and immunoblotting assay [36]. Even though ELISA methods usually provide an extremely low detection limit and a large dynamic range, it’s too time-consuming because the kidney can lose function within 7 days under AKI [37]. The typical ELISA methods take about 24 hours [35, 38] and the most rapid ELISA takes 3-4 hours [39, 40]. Chemiluminescent microparticle immunoassay (CMIA) has also been used in the detection of NGAL, the current CMIA used anti-NGAL antibody as the recognition element and provides a dynamic range close to ELISA (2 to 1500 μ g/L) within 30min[41]. However, this rapid detection method requires anti-body, which is relatively expensive to make and difficult to store. Due to the limitation of the existing NGAL measurement method, a low-cost NGAL measurement method is needed to deliver a rapid point-of-care (POC) detection of acute kidney injury.
CHAPTER II EBOLA BIOSENSOR BASED ON GLYCOPROTEIN APTAMER AND MINI INTERFEROMETER

2.1 Introduction

Immobilization of aptamer on a surface can lead to change in configuration, electrical charge and steric hindrance between neighboring aptamer, thus produce a surface stress change. When the immobilized aptamer binds with its ligand, the above effects will be affected, resulting a new surface stress change.

To develop an economical label-free biosensor that is capable of rapid and portable detection of Ebola virus disease, a functionalized gold Atomic Force Microscope (AFM) cantilever was used to transduce the interaction between aptamer and Ebola glycoprotein molecules into surface stress change. And a miniature laser interferometer was used to measure the cantilever deflection that is proportional to the surface stress change.

2.2 Methods

2.2.1 Experiment materials

The DNA aptamer (oligo 6011) was developed and synthesized by Dr. Soma Banerjee from Professor Marit Nilsen-Hamilton’s lab in the department of Biochemistry, Biophysics and Molecular Biology of Iowa State University. The reference DNA (oligo 3806) with the similar length was purchased from Integrated DNA Technologies (https://www.idtdna.com). The Zaire Ebola Virus (Zebov) envelop glycoprotein 1,2 (GP1,2) and the secretory glycoprotein were purchased from BPS bioscience (http://bpsbioscience.com). The alkanethiol (6-mercaptop-1-hexanol) and other chemicals were purchased from Sigma Aldrich (www.sigma.com). The
cantilevers used in this experiment were purchased from Nano World (www.nanoworld.com), with width of 100μm, length of 500μm and thickness of 1μm (Figure 1), these Atomic Force Microscope (AFM) cantilevers are made of monolithic silicon and coated with 5nm titanium and 30nm gold on the top, which makes the top side of cantilever conductive.

![Figure 1 Images of microcantilevers (Nanoworld 2011)](image)

Before experiments, all cantilevers were cleaned by being rinsed in ethanol and then DDH2O for 10 minutes in each, and then dry in air.

2.2.2 The Mini Phase-shift interferometric deflection measurement system

Interferometers are widely-used optical devices that use the interference of laser beams to measure a nano-scale deflection, it has a high sensitivity and stability. Due to a variety of measurement requirements, a customized interferometer is needed. In this research, a Mini interferometer (fig. 2) was designed based on the previous version of phase-shift interferometer (in my Master’s research) to measure the deflection on the micro-cantilever. The addition of the quarter wave plate between calcite and cantilevers reduced the size of entire system by removing one of the two beamsplitters, as a result, making the system more compact.
Figure 3 shows the flow chat of a phase-shifting interferometer. In the interferometer, a laser generator sends a 1mW laser beam at 632nm wavelength to a polarizer, which polarizes the laser beam at 45°, as written as:
\[ I = A(i + j)e^{i\omega t} \]

Where \( i \) and \( j \) are the unit vector in horizontal and vertical direction, \( i\omega t \) is the phase of the laser beam, \( A \) is the amplitude of laser beam. Then the polarized laser passes through a beam splitter and a calcite. A calcite will tilt the vertical component of the incident laser, as a result, this will separate the incident laser beam into two laser beams, one is the vertical component of the original laser, and the other one is the horizontal components:

\[
I_1 = \frac{\sqrt{2}}{2} Ae^{i\omega t} \quad I_2 = \frac{\sqrt{2}}{2} Ae^{i\omega t}
\]

Both laser beams will be focused by a lens and hit on the tip of two cantilevers (one is sensing, and the other one is reference) in the PVC chamber, because the front and back of PVC chamber is made of anti-reflective glass, the laser beams can come in and the reflected laser beams can exit. When one cantilever has deflection (d) relative to the other one, one of the reflected laser beam will travel an extra distance of 2d than the other laser, which results in a phase difference \( \phi \), the reflected lasers can be written as:

\[
I_1 = \frac{\sqrt{2}}{2} Ae^{i\omega t + \phi} \quad I_2 = \frac{\sqrt{2}}{2} Ae^{i\omega t}
\]

The QWP between the calcite and the cantilever is aimed to rotate polarizations (by making a HWP as beams will go through it twice) in such a way that the reflected beams will not overlap on the input beam. The calcite then sends the two reflected laser beams to two mirrors, the mirrors reflect laser to a polarizing beam splitter, which separate the laser beam into two lasers, \( I_1 \) and \( I_2 \), \( I_2 \) will pass through a quarter wave plate, which can slow the -45° component of \( I_2 \), by 90° to form a new \( I_2' \):
\[ I_{2\text{ new}} = I_{2+45 \degree} + I_{2-45 \degree} = \frac{1}{2} A(i - j)e^{i\omega t} + \frac{1}{2} A(i + j)e^{i\omega t + \frac{\pi}{2}} \]

I1 also has two components in +45° and -45° direction:
\[ I_1 = \frac{\sqrt{2}}{2} A e^{i\omega t + \varphi} = \frac{1}{2} A(-i + j)e^{i\omega t + \varphi} + \frac{1}{2} A(i + j)e^{i\omega t + \varphi} \]

After the quarter wave plate, I1 and new I2 are combined in the beam splitter, which sends out two identical laser beams. After that, polarizer 1 and polarizer 2 only keep the -45° and +45° components of the combination of I1 and new I2 respectively. As result, the laser beams received by the two photodiodes can be written as:
\[ I_{\text{photo}1} = \frac{1}{2} A(i + j)e^{i\omega t + \frac{\pi}{2}} + \frac{1}{2} A(i + j)e^{i\omega t + \varphi} \]
\[ I_{\text{photo}2} = \frac{1}{2} A(-i + j)e^{i\omega t + \varphi} + \frac{1}{2} A(i - j)e^{i\omega t} \]

The power of laser beams is \( P=I^2 \), so the powers of laser beams captured by the two photodiodes can be written as:
\[ P_1 = A(1 + \cos(\frac{\pi}{2} - \varphi)) \quad P_2 = A(1 + \cos(\pi - \varphi)) \]

P1 and P2 are two sinusoidal waves with 90° phase difference. Two photodetectors were used to capture power P1 and P2, and generates currents that are proportional to the captured light power, which will be recorded by a data acquisition system for future analysis. As the deflections increases linearly over time (upper part in fig. 4), the two signals P1 and P2 captured by two photodetectors (lower part in fig. 4) will change sinusoidally with a constant phase.
difference of \( \pi/2 \). Theoretically, one signal from one photodetector would be sufficient to calculate the phase change \( \phi \), the purpose of using two signals is to easily determine the direction of deflection.

Figure 4 If deflection increases linearly over time (upper), the signals captured by photodetectors (lower) will change sinusoidally with a constant \( \pi/4 \) phase difference.
Since the phase difference is caused by deflection, by counting the number of periods P1 and P2 traveled (φ, the phase change) using, the deflection can be calculated by using the following equation:

\[ \Delta = \frac{\phi \lambda}{4\pi} \]

Where \( \lambda \) is the wavelength of laser (632nm), and \( \phi \) is the phase change due to deflection change.

2.3 The Experiment Results

2.3.1 Experiment 1: Ebola envelope glycoprotein (GP1,2) and DNA aptamer on micro-cantilever

The dry thiolated Ebola DNA aptamer was dissolved in deionized and distilled water to obtain 100 μM aptamer in water, a small amount of aptamer in water was moved into several micro tubes. Before functionalization, heat one micro tube to 95°C for 5 min, and while it is hot, rapidly add the mixture of 10x PBS buffer and ddH2O to obtain 0.5 μM aptamer in 1x PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 1 mM MgCl2•6H2O, pH 7.4) to ensure a rapid cooling. The purpose of this rapid cooling is to ensure that the aptamer will fold into its desired conformation when ions were present.

Before experiment, the cleaned sensing cantilever was incubated in a solution containing 0.5 μM thiolated Ebola DNA aptamer in PBS for 5 hours [42] in order to immobilize thiolated aptamer on the sensing cantilever surface, this step was conducted on a petri dish cleaned with ethanol and ddH2O. A raised humidity is required to prevent PBS drying that cause ion concentration change. During this time period, the thiol group on aptamers will be immobilized.
on the gold surface through strong covalent bonds. While the cleaned reference cantilever was incubated in a solution containing 0.5 μM thiolated reference DNA in PBS for 5 hours, the reference DNA has similar length as the aptamer but has no affinity to Ebola virus Glycoproteins.

Both sensing and reference cantilevers were then submerged in solution containing 3mM alkanethiol (6-mercapto-1-hexanol) in ddH2O for 1 hour, the empty area not covered by DNA will be covered by the alkanethiol. The purpose of this step is to minimize the electron exchange and non-specific binding or protein. After that, both cantilevers were rinsed in PBS buffer to remove molecules that were not immobilized on the surface.

Before measurement, both sensing and reference cantilevers were clamped on a polyethylene (PE) plastic holder and submerged in the PBS buffer. A detailed real picture and schematic are shown above.

Figure 5 real picture (upper) and schematic (lower) of reaction chamber and cantilevers
When measurement starts, the incident laser was reflected by a mirror and then focused by a convex lens onto the tip of both sensing and reference cantilevers, which were submerged by the PBS buffer in the reaction chamber (fig. 5). After the photodetector signal stabilized, several injections that contain small amount of Ebola virus glycoprotein in PBS buffer will be carefully added into the reaction chamber to raise the concentration of Ebola GP1,2 in the chamber step by step. The time between each injection is about 30 minutes to allow most aptamer on the cantilever bind with GP1,2, during these time periods, the sensing cantilever will deflect due to the binding process, and the mini interferometer will monitor the deflection of sensing cantilever relative to the reference cantilever. The photodetector output data will be analyzed in Excel. A representative result data was shown in fig. 6.

![Surface stress (mN/m) vs. time (s)](image)

**Figure 6** A representative result data of measured stress over time
The concentrations and the corresponding stress changes will be recorded. After a series of experiments, the concentrations and stresses were plotted in the below chart (fig. 7). In this chart, the negative surface stress refers to a tensile surface stress on the gold coated side.
From fig. 7, we can see that as the concentration of GP1,2 increases, a tensile surface stress was produced on the sensing cantilever. This stress saturates at about 250mN/m at a concentration of about 200nM. A fitting based on Langmuir equation was used to estimate the coefficient of dissociation, Kd. According to the fitting, the Kd of GP1,2 is 118.6nM.

2.3.2 Experiment 2: Ebola secretory glycoprotein (sGP) and its DNA aptamer

The experiment 2 was very similar to experiment 1, the only difference is the envelope glycoprotein (GP1,2) was replaced with secretory glycoprotein (sGP), while the aptamer, the cantilever functionalization, and the laser measurement method remain the same. Compared to the molecular weight of GP1,2, which is 74.5 kDa, the sGP has smaller molecular weight, 41.2kDa.
From below chart (fig. 8), we can see that as the concentration of sGP increases, the binding between Ebola virus DNA aptamer and sGP produces a compressive surface stress on the sensing cantilever. The surface stress saturates at only about 20mN/m, according to the fitting, the Kd of sGP is only 2.14nM, indicating that high affinity between the DNA aptamer and sGP.

![Surface stress vs. sGP concentration](image)

**Figure 8 Stress change at corresponding sGP concentrations**

2.4 Conclusion

1) Experiment 1

From the GP1,2 chart, we can see that as more GP1,2 is presented in the reaction chamber, more Ebola aptamer will bind with the DNA aptamer because of its affinity to aptamer.
The binding between aptamer changed the original equilibrium of electrical charge, the hydration effect and the steric hindrance, thus a tensile surface stress was produced on the sensing cantilever. A fitting based on Langmuir equation was used to estimate the coefficient of dissociation, $K_d$. The physical meaning of $K_d$ is the concentration at which 50% of aptamer binds with its target molecules, thus producing 50% of saturated surface stress.

According to the fitting, the $K_d$ of GP1,2 is 118.6\text{nM}. This result is close to the $K_d$ (66\text{nM}) measured with filter binding assay when the aptamer was synthesized by my colleague, Dr. Soma Banerjee.

2) Experiment 2

From the sGP chart, we can see that as the concentration of sGP increases, the binding between Ebola virus DNA aptamer and sGP produces a compressive surface stress on the sensing cantilever, which is opposite to that of GP1,2, this might be due to the different charge carried by sGP at pH of 7.4. Due to difference amino acid combination, GP1,2 and sGP have different isoelectric points (pI). The isoelectric point (pI), is the pH at which a particular molecule carries no net electrical charge. At a pH below their pI, proteins carry a net positive charge; above their pI they carry a net negative charge. The pI of sGP is 9.2 while the pI for GP1,2 is 6.3 [43], showing that sGP is positively charged and GP1,2 is negatively charged. The surface stress saturates at only about 20mN/m, which is probably because of the less steric effect produced by the smaller size of sGP. According to the fitting, the $K_d$ of sGP is only 2.14\text{nM}, indicating that high affinity between the DNA aptamer and sGP. Compare to the $K_d$ (100\text{nM}) measured with filter binding assay, this cantilever biosensing method shows significantly high sensitivity.
CHAPTER II

MATHEMATICAL MODELING OF THE BINDING INDUCED DEFLECTION

3.1 Introduction

In the previous chapter, we found that the opposite deflection direction of GP1,2 and sGP cannot be explained using proteins charge or steric effect. The surface was negatively charged due to the negative charge carried by the DNA aptamer, and these aptamers can repel each other to create a compressive surface stress before binding. Hence, upon binding, the negatively charged GP1,2 theoretically should increase the negative charge of surface, and result in even a larger compressive surface stress (bending down), while the positively charged sGP should neutralize the negatively charged aptamer surface, causing relaxation on the compressed surface (bending up). The GP1,2 is also larger than the sGP, so upon binding, the steric effect from the GP1,2 should create larger compressive stress compared to sGP. However, these theoretical speculations are not consistent to our experimental observation.

3.2 Methods

To investigate the cause of opposite direction of deflection, a mathematical modeling was established. In the model, the aptamers were assumed to be distributed on the cantilever surface in a hexagonal pattern, which was proved by Strey et. al. using x-ray diffraction [44]. After immobilization of aptamer, the immobilization density is significantly dependent on the salt concentration and the length of DNA sequence [45]. The relation between the immobilization density, the DNA length and the salt concentration were found by Stachowiak et. al using fluorescence measurements [45]. The Ebola aptamer is 100-mer ssDNA, hence, an
immobilization density of 0.045/nm² was derived based on the ratio of immobilization densities of 10-mer and 30-mer at 200mM salt concentration. Based on the hexagonal pattern and the immobilization density, the average initial separation between each aptamer was calculated to be 5.07nm. The DNA molecule length is approximately 34nm, which is much larger than the DNA separation, so we expect that they are standing parallel on the surface of the cantilever.

This model is based on finding the equilibrium deflection by the minimizing the total energy cantilever system, which include cantilever bending energy and inter-DNA interaction energy.

\[ E_{total} = E_{bending} + \sum_{all\ DNA\ pairs} E_i \]

The micro-cantilever has multiple layers including SiNₓ base layer, the gold layer and the aptamer layer on the gold surface. Because the thickness of SiNₓ is much larger than other layers, micro-cantilever was modeled as a single layer beam with bending energy as:

\[ E_{bending} = \frac{Et^3}{12R^2(1-v^2)} \]

where \( R = \frac{L^2}{2\Delta} \), E is the Young’s modulus for the cantilever, L is the cantilever length, v is the Poisson Ratio, and \( \Delta \) is the cantilever deflection.

Each type of energy should be modeled in three different situations: the interaction between bound DNAs, the interaction between unbound DNAs, and the interaction between one bound and one unbound DNAs.

\[ \sum E_i = \sum E_{i,\text{bound}} + \sum E_{i,\text{unbound}} + \sum E_{i,\text{bound,unbound}} \]
Strey et al. found the function of the interaction energy based on the analysis of nematically ordered charged polymers [44, 46]. The total interaction energy includes the direct interactions $F_0$, and the entropy fluctuation $F$. The direct interactions $F_0$ consists of the hydration energy and the electrostatic energy.

\[
E_i = F_0 + F
\]

\[
F_0 = a \sqrt{\frac{\pi \lambda_{el}}{2d}} e^{-\frac{d}{\lambda_{el}}} + b \sqrt{\frac{\pi \lambda_h}{2d}} e^{-\frac{d}{\lambda_h}}
\]

\[
F = c T \frac{k_b}{\sqrt{k_c}} \left[ \delta^2 F_0 \frac{\delta F_0}{\delta d^2} - \frac{1}{d} \frac{\delta F_0}{\delta d} \right]
\]

The electrostatic energy was caused by the negative charges carried by the DNA molecules and the hydration energy are due to a hydration bonding between the DNA molecules in water based environment. It can be seen that the electrostatic energy is an exponential function dependent on the coefficient $a$ and the decaying length $\lambda_{el}$ [47], while the hydration force has the same structure but dependent on coefficient $b$ and the decaying length $\lambda_h$ [48].

In the function of entropy fluctuation $F$, $k_b$ is the Boltzmann constant, $T$ is temperature, $d$ is the average DNA separation, $k_c = k_b T l_p$, is the intrinsic bending stiffness of the DNA molecules, and $l_p$ is the persistence length. The coefficient $a, b, c$ and decaying length $\lambda_{el}, \lambda_h$ were measured experimentally by Strey et al [44, 46] as following: $a=1.1e-7$ J/m, $b=4.1e-10$ J/m, $c=0.8$, $\lambda_h=0.288$nm, $\lambda_{el}=0.974$nm.
The total interaction energy should be calculated for each pair of DNA molecules on the cantilever. To calculate the total interaction energy for each pair of DNA molecules, we need to consider the sum of first four levels of neighbor DNA for each DNA center, because beyond the fourth level neighbor, the DNA separation becomes so large that interaction energy is negligible.

As the cantilever bends down, the average separation between each DNA molecules can increase compared to the original separation due to the surface stretching, so the cantilever bending energy can also be expressed as a function of DNA separation.

\[ E_{bending} = \frac{Et}{3(1-v^2)d_0^2} (d - d_0)^2 \]

where \( d \) is the simultaneous DNA separation and \( d_0 \) is the initial DNA separation (5.07nm). Now all energy included in the total energy can be expressed as functions of DNA separation. As the DNA separation increases, the cantilever deflection also increases, resulting in larger bending energy in the cantilever; while the electrostatic energy and hydration energy will decay exponentially as the DNA separation increase. Due to above reason, for each decaying lengths of
electrostatic energy or hydration energy, we can find one simultaneous DNA separation that minimize the total energy, then we can use the corresponding simultaneous DNA separation to calculate the cantilever deflection. When the calculated cantilever deflection is consistent to what we observed in the GP1,2 or sGP experiments, the corresponding decaying lengths of electrostatic energy and hydration energy is our estimation of the decaying lengths of electrostatic energy and hydration energy upon binding of GP1,2 or sGP.

Finding the decaying lengths of electrostatic energy and hydration energy upon the binding of GP1,2 and sGP can help us to understand the mechanism of deflection cantilever.

3.3 Modeling Result

By iterating the value of decaying length of electrostatic energy, we found the decaying length of electrostatic energy upon binding of GP1,2 or sGP that gives close estimation of experimental results. We estimate that upon GP1,2 binding, the decaying length of electrostatic changes from the initial 0.974nm to 0.690nm; while upon sGP binding, the decaying length of electrostatic
changes from 0.974nm to 0.985nm. The decaying length of hydration energy was not considered as the variable because we found it negligible compared to electrostatic energy due to its small
initial decaying length $\lambda_h = 0.288 \text{nm}$. The modeled deflection upon GP1,2 and sGP binding at 0% to 100% binding fraction is plotted with the experimental results.

### 3.4 Conclusion

From the GP1,2 experiments, we can see that as more GP1,2 is presented in the reaction chamber, more Ebola aptamer will bind with the DNA aptamer because of the its affinity to aptamer. The binding between aptamer changed the original equilibrium of electrical charge, the hydration effect and the steric hindrance, thus a tensile surface stress was produced on the sensing cantilever. A fitting based on Langmuir equation was used to estimate the coefficient of dissociation, Kd. The physical meaning of Kd is the concentration at which 50% of aptamer binds with its target molecules, thus producing 50% of saturated surface stress. According to the fitting, the Kd of GP1,2 is 118.6nM. This result is close to the Kd (66nM) measured with filter binding assay when the aptamer was synthesized by my colleague, Dr. Soma Banerjee.

From the sGP chart, we can see that as the concentration of sGP increases, the binding between Ebola virus DNA aptamer and sGP produces a compressive surface stress on the sensing cantilever, which is opposite to that of GP1,2, this might be due to the different charge carried by sGP at pH of 7.4. Due to difference amino acid combination, GP1,2 and sGP have different isoelectric points (pI). The isoelectric point (pI), is the pH at which a particular molecule carries no net electrical charge. At a pH below their pI, proteins carry a net positive charge; above their pI they carry a net negative charge. The pI of sGP is 9.2 while the pI for GP1,2 is 6.3 [43], showing that sGP is positively charged and GP1,2 is negatively charged. The deflection saturates at only about 80nm. According to the fitting, the Kd of sGP is only 2.14nM, indicating that high affinity
between the DNA aptamer and sGP. Compare to the Kd (100nM) measured with filter binding assay, this cantilever biosensing method shows significantly high sensitivity.
CHAPTER IV THROMBIN BIOSENSING BASED ON MICRO INTERFEROMETER

4.1 Introduction

In the case of Ebola outbreak in West Africa, a rapid and portable biosensing system that can be used in the point-of-interest can significantly shorten the time for diagnostic, thus lower the chance of disease spreading. A compact micro interferometer of shoe-box size was built to allow this biosensing system to be carried to West Africa.

A measurement was conducted using alpha thrombin and its DNA aptamer to verify the function of this compact micro interferometer system.

4.1.1 Thrombin and its aptamer

The human coagulation catalytic enzyme, alpha thrombin, is an enzyme that is responsible for the conversion of fibrinogen to fibrin to complete the clotting process of blood. Alpha thrombin is associated with healthy functions, higher or lower concentration of alpha thrombin than the normal level can cause thrombosis or hemorrhage, respectively. The fibrinogen binding site of alpha thrombin catalyzes the conversion of fibrinogen to fibrin to complete clotting process, while the heparin binding site of alpha thrombin can bind to heparin to inhibit coagulation process[49].

Alpha thrombin has good affinity to its aptamer (a single strand DNA molecule with sequence of GGTTGG TGTGGTTGG), and this property enables scientists to use its binding process to develop medical application. Compare to antibody and its ligand, protein and its aptamer have advantages in micro sensing and controlling experiments because aptamer has high
affinity to its protein at low concentration even though the sequence of aptamer is short. Alpha thrombin aptamer has a hairpin structure because its eight guanine bases attract each other, this structure is called G-quadruplex form (fig. 11).

Bode, Mayr et al. found that the heparin binding site of alpha thrombin can bind with alpha thrombin aptamer, and a high affinity was also observed in their research [49] [50], which makes alpha thrombin and its DNA aptamer an ideal material for study in interaction between binding molecules.

4.2 Methods

4.2.1 Experiment materials

The thrombin aptamer (HSGCCTTAACCTGTAGTACTGCTGGTGAATAATTGCTGCCATTGGTTGG) TGTGGTTGG was purchased from Integrated DNA Technologies (www.idtdna.com).
The human alpha thrombin, the alkanethiol (6-mercaptop-1-hexanol), and other chemicals were purchased from Sigma Aldrich (www.sigma.com). The cantilevers used in this experiment were the same cantilevers in Chapter 2 from Nano World (www.nanoworld.com). Before experiments, all cantilevers were cleaned by being rinsed in ethanol and then DI water for 10 minutes in each, and then dry in air.

4.2.2 The Micro Phase-shift interferometric deflection measurement system

In the previous chapter, the size of our biosensing system was relative small (2 by 3 feet) but still cannot be used as a handheld tool. The interferometer part takes a big part of the system size. In order to further shrink the size of the system, we requested Kylia, a French optics company, to integrate all optical components together by fusing them onto one piece of glass (fig. 12). To isolate the system from ambient disturbance and dust, the integrated system was protected by a compact aluminum package. To allow accurate laser input and output, fiber optic input and output were provided.

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Figure 12 Optical components (left) and Kylia micro interferometer compared to a quarter coin (right)
Unlike the Mini interferometer, the Kylia micro interferometer has a size of hand but lose the ability of laser alignment because all optical components are fixed on one piece of glass. To enable accurate adjustment of reflected laser beam, we also design a multi-axis adjustable cantilever holder to adjust the translation and rotation of micro-cantilever in multiple axis (fig. 13).

![Image](image13.png)

**Figure 13** The Micro Phase-shift interferometric deflection measurement system with a multi-axis adjustable cantilever holder to allow the laser to hit the cantilever tip and reflect along the incident path

In this micro interferometer, cantilevers are placed vertically instead of horizontally in the reaction chamber, the front and back of reaction chamber is made of anti-reflection glass to increase the signal intensity. The working principle of micro interferometer is basically the same as the mini interferometer in last chapter. As the sensing cantilever deflection increases linearly over time, the two signals from the two photodetectors will change sinusoidally with a constant
phase difference of $\pi/2$. Same as the mini interferometer, the purpose of using two signals is to easily determine the direction of deflection.

4.3 The Experiment Results

The dry thiolated thrombin DNA aptamer was dissolved in deionized and distilled water to obtain 100 μM aptamer in water, a small amount of aptamer in water was moved into several micro tubes. Before functionalization, heat one micro tube to 95°C for 5 min, and while it is hot, rapidly add the mixture of 10x PBS buffer and ddH2O to obtain 1 μM aptamer in 1x PBS buffer without MgCl2 (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) to ensure a rapid cooling. (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 1 mM MgCl2•6H2O, pH 7.4)

Before experiment, the cleaned sensing cantilever was incubated in a solution containing 1 μM thiolated thrombin DNA aptamer in PBS for 5 hours [42] in order to immobilize thiolated aptamer on the sensing cantilever surface, this step was conducted on a petri dish cleaned with ethanol and ddH2O. A raised humidity is required to prevent PBS drying that cause ion concentration change. During this time, the thiol group on aptamers will be immobilized on the gold surface through strong covalent bonds. While the cleaned reference cantilever was incubated in a solution containing 1 μM thiolated reference DNA in ddH2O for 5 hours, the reference DNA has similar length as the aptamer but has no affinity to alpha thrombin.

Both sensing and reference cantilevers were then submerged in solution containing 3mM alkanethiol (6-mercapto-1-hexanol) in ddH2O for 1 hour, the empty area not covered by DNA will be covered by the alkanethiol. The purpose of this step is to minimize the electron exchange
and non-specific binding of protein. After that, both cantilevers were rinsed in PBS buffer to remove molecules that were not immobilized on the surface.

Before measurement, both sensing and reference cantilevers were clamped on a polyethylene (PE) plastic holder and submerged in the PBS buffer.

When measurement starts, the laser generator sends 1mW 632nm laser to the input fiber optics of Kylia micro interferometer. After passing through the components of the Kylia micro interferometer, a laser of 2cm focal length hits the tip of both sensing and reference cantilevers, which were submerged by the PBS buffer in the reaction chamber. The direction of reflected lasers will be adjusted with the multi-axis adjustment to ensure that the reflected laser come back in the original path and finally reach the photodetectors. The photodetectors transduce the light energy into current amplitude and deliver it to the data acquisition system (DAQ) to be shown and saved in computer.

After the photodetector signal stabilized, several injections that contains small amount of alpha thrombin in PBS buffer will be carefully added into the reaction chamber to raise the concentration of alpha thrombin in the chamber step by step. The time between each injection is about 10 minutes to allow most thrombin aptamer on the cantilever bind with alpha thrombin, during these time, the sensing cantilever will deflect due to the binding process, and the Kylia
micro interferometer will monitor the deflection of sensing cantilever relative to the reference cantilever. The photodetector output data will be analyzed in Excel.

The concentrations and the corresponding stress changes will be recorded. After a series of experiments, the concentrations and stresses were plotted in fig. 14. In this plot, a positive surface stress refers to a tensile surface stress on the gold coated side.

We can see from the chart that as the concentration of alpha thrombin increases, a tensile surface stress was produced on the sensing cantilever. This stress saturates at about 35mN/m at a concentration of about 2nM.

To investigate the influence of interfering molecules like serum, a substitute of human serum, bovine serum was used to test the stress change when the bovine serum is present. We did
one alpha thrombin binding measurement when diluted bovine serum was present (1% of original bovine concentration) as the background, and very little surface stress change was observed (fig. 15).

1% and 10% Human serum albumin (HSA) was also used as the background to see the influence of this interfering molecule on thrombin binding (fig. 15).

As the control experiments, Gamma thrombin, a degradation pattern of alpha thrombin which has a low affinity with thrombin aptamer, was used to replace the alpha thrombin injections. The noise level of the experiment setup was also tested by using stress change caused by PBS buffer before each experiment (shaded area in fig. 15).

![Stress change vs. Thrombin concentration](image)

**Figure 15** Stress change at corresponding alpha thrombin concentrations (with and without HSA) and gamma thrombin concentration
4.4 Conclusion

As the concentration of alpha thrombin increases, a tensile surface stress that saturates at about 35mN/m at a concentration of about 2nM was observed for the alpha thrombin. A fitting based on Langmuir equation was used to estimate the coefficient of dissociation, Kd. According to the fitting, the Kd of alpha thrombin is 0.321nM. This value is relatively smaller compared to the 1 to 6 nM Kd value reported by Wu [50, 51]. This difference can be caused by difference between the immobilized aptamer and the free aptamer in liquid in Wu’s assay.

The bovine serum (BS) result shows that the bovine serum is a strong interfering solution because it inhibited the stress change caused by the binding between thrombin and aptamer. This might be because of the non-specific binding on aptamer or adsorption on cantilever surface of components of bovine serum.

Human serum albumin (HSA) has a Physiological concentration of 500 μM in human body [52]. Due to the difficulty to remove HSA from blood sample, it is necessary to check the interfering from HSA on the biosensing results. A 5 μM (1%) and 50 μM (10%) Human serum albumin (HSA) was also used as the background to see the influence of this interfering molecule on thrombin binding. Very small influence on the binding stress change was found when 1% HSA and 10% HSA were present. Because the 50 μM concentration of HSA is 5000-fold higher than the concentration of human alpha thrombin, the influence from HSA on the biosensing results is negligible.

Like the BS experiment, the control experiments with Gamma thrombin shows very little stress change due to the low affinity with thrombin aptamer, this also confirms that the stress change was induced by the specific binding between alpha thrombin and aptamer, indicating the high specificity of this biosensing method.
CHAPTER V NGAL BIOSENSING BASED ON MICRO INTERFEROMETER

5.1 Introduction

The verification experiment with thrombin showed a promising potential of the micro interferometer based biosensing system. Because acute kidney injury (AKI) requires an accurate and rapid biosensing method, we will use this micro biosensing system to estimate the concentration of the biomarker of AKI, the neutrophil gelatinase-associated lipocalin (NGAL).

5.1.1 The Aptamer of Neutrophil gelatinase–associated lipocalin (NGAL)

NGAL aptamer has a good affinity to its ligand, the NGAL. It is a single strand RNA molecule (Oligo 3832) developed by Professor Marit’s group using systematic evolution of ligands by exponential enrichment (SELEX), in which the aptamer was selected by repeating the SELEX cycle including binding, partition, elution, amplification, and conditioning. Its high affinity enables us to use the binding process between NGAL and its aptamer to develop the transducer of biosensor application.

5.2 Experiment materials

The NGAL aptamer was developed by Professor Marit’s group. The recombinant glycosylated NGAL and recombinant non-glycosylated NGAL are purchased from outside of our lab, the alkanethiol (6-mercapto-1-hexanol), and other chemicals were purchased from Sigma Aldrich (www.sigma.com). The cantilevers used in this experiment were the same cantilevers in Chapter 2. Before experiments, all cantilevers were cleaned by being rinsed in ethanol and then DI water for 10 minutes in each, and then dry in air.
The NGAL aptamer is RNA sequence that has not been thiolated, because it is usually difficult and expensive to thiolate long sequence, especially on RNA sequence. In order to immobilize this non-thiolated RNA aptamer to the surface of cantilever, a thiolated linker DNA with complementary sequence was first immobilized on the cantilever, then upon the introduction of NGAL aptamer, the complementary part of DNA linker will hybridize with the complementary part of the NGAL aptamer on the cantilever surface, resulting in that the aptamer grafted on the cantilever surface.

Due to the different mechanism of immobilization, the functionalization procedure is slightly different to the thrombin experiment. The detailed immobilization procedure works as below.

For the sensing cantilever, 1uM Linker was heated on a heating plate to 95 degree for 5 min and then cooled down to room temperature. Then 50 uL 1uM linker in 1X PBS was pipetted out from the microtube onto a petri dish to form a droplet. Cleaned cantilever was inserted into the droplet. After 5 hours, cantilever was taken out and rinsed in ddH2O. 50uL 3 mM Alkanethiol (6-Mercapto-1-Hexanol) in DDwater was pipetted out onto a petri dish to form a droplet. cantilever from previous step is inserted into the droplet. After the 1 hour, cantilevers are taken out and rinsed in ddH2O, the empty area not covered by DNA will be covered by the alkanethiol. The purpose of this step is to minimize the electron exchange and non-specific binding of protein. After that, both cantilevers were rinsed in PBS buffer to remove molecules that were not immobilized on the surface. 1uM aptamer in 1X SELEX (137mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 2mM KH2PO4, pH 7.4) was heated to 95 degree for 5 min, and then cooled down to room temperature. 50 uL 1uM aptamer in 1X SELEX buffer was pipetted out from the microtube
onto a petri dish to form a droplet. The cantilever from previous step was inserted into the droplet. After the 1 hours, cantilever was taken out without washing.

For the reference cantilever, the functionalization procedure is similar but without the hybridization step of aptamer.

Before measurement, both sensing and reference cantilevers were clamped on the ceramic holder and submerged in the SELEX buffer.

When measurement starts, the laser generator sends 1mW 632nm laser to the input fiber optics of Kylia micro interferometer. After passing through the components of the Kylia micro interferometer, a laser of 2cm focal length hits the tip of both sensing and reference cantilevers, which were submerged by the SELEX buffer in the reaction chamber. The direction of reflected lasers will be adjusted with the multi-axis adjustment to ensure that the reflected laser come back in the original path and finally reach the photodetectors. The photodetectors transduce the light energy into current amplitude and deliver it to the data acquisition system (DAQ) to be shown and saved in computer.

After the photodetector signal stabilized, several injections that contains small amount of NGAL in SELEX buffer will be carefully added into the reaction chamber to raise the concentration of NGAL in the chamber step by step. The time between each injection is about 5 minutes to allow most NGAL aptamer on the cantilever bind with NGAL, during these time, the sensing cantilever will deflect due to the binding process, and the Kylia micro interferometer will monitor the deflection of sensing cantilever relative to the reference cantilever. The photodetector output data will be analyzed in Excel.
5.3 The Experiment Results

Figure 16 Stress vs non-glycosylated NGAL (upper) and glycosylated (lower) concentration
The concentrations of recombinant non-glycosylated NGAL and the corresponding stress changes was recorded. After a series of experiments, the concentrations and stresses were plotted in fig. 16. In this plot, a negative surface stress refers to a compressive surface stress on the gold coated side of cantilever.

We can see from the plot that as the concentration of recombinant non-glycosylated NGAL increases, a compressive surface stress was produced on the sensing cantilever. This stress saturates at about 70mN/m at a concentration of about 1uM. By fitting the plot using Langmuir equation, a coefficient of disassociation Kd of 288nM was found.

To investigate the influence of non-specific molecules, a control experiment was used to test the stress change when human serum albumin was injected into the reaction chamber instead of NGAL, and very little surface stress change was observed (fig. 16).

The influence of interfering molecules was also tested using bovine serum background (10% of its physiological concentration), and no significant different was observed.

The noise level of the experiment setup was also tested by using stress change measured when SELEX buffer before each experiment (shaded area in plot). The detection limit (LOD), that is, the minimum concentration that cause stress change more than the noise level, was found to be 0.1uM.

The recombinant non-glycosylated NGAL was prepared in laboratory environment, however, the natural NGAL secreted by human body is glycosylated, if the glycosylate group is unfortunately attached on the binding site on the natural NGAL, the RNA aptamer may not bind with the natural NGAL because the RNA aptamer was prepared in the SELEX cycles that uses non-glycosylated NGAL. To ensure that the glycosylate group does not affect the binding between glycosylated NGAL and RNA aptamer, several experiments using glycosylated NGAL
were conducted, and the concentration of glycosylated NGAL and corresponding stress were plotted. The plot has very similar saturating concentration and stress, a coefficient of disassociation Kd of 277nM was found by fitting the plot with Langmuir equation, which is very close to the Kd in the non-glycosylated NGAL experiments, so we can confirm that the glycosylate group does not affect the binding between NGAL and its aptamer.

5.4 Conclusion

As the concentration of non-glycosylated NGAL or glycosylated NGAL increases, a tensile surface stress that saturates at about 70mN/m at a concentration of about 1uM was observed for the non-glycosylated NGAL and glycosylated NGAL. A fitting based on Langmuir equation was used to estimate the coefficient of dissociation, Kd. According to the fitting, the Kd is 0.288uM for non-glycosylated NGAL and 0.277uM for glycosylated NGAL. This value is relatively smaller compared to the Kd measured by Prof. Marit’s group using filter binding assay. This difference can be caused by the transduction mechanism or/and the difference between the immobilized aptamer and the free aptamer in liquid.

The bovine serum (BS) experiment shows very similar results of experiment without bovine serum. Hence, the components in bovine serum will not interfering the biosensing.

Like the BS experiment, the control experiments with Human serum albumin (HSA) shows very little stress change due to the low affinity with Human serum albumin (HSA), this also confirms that the stress change was induced by the specific binding between alpha thrombin and aptamer instead of non-specific binding and manual injection, indicating the high specificity of this biosensing method.
CHAPTER VI CANTILEVER BASED ELECTORCHEMICAL BIOSENSOR

6.1 Introduction

Except above work, we are also exploring a novel electrochemical biosensing method based on the Kylia micro interferometer.

In my Master’s research, we applied changes in electrostatic field to change the conformation of thrombin aptamer (DNA molecules) immobilized on a micro-cantilever, and use the conformational change to induce nanometer deflection on the cantilever, thus the cantilever can be actuated by the changes in electrostatic field. Also, by measuring the deflection amplitude in the solution at different alpha thrombin concentrations, we were able to see the influence of alpha thrombin concentrations on the deflection under electrical potential.

In that study, we observed that electrostatic field can produce controllable surface stress change on gold coated silicon micro-cantilever. This surface stress change causes the deflection and may be induced by several factors including the reconstruction of gold surface under electrical potential, the ion adsorption/desorption on gold surface, and the conformational transition of the aptamer molecules (DNA molecules) immobilized on the cantilever. The conformational changes can affect the specific binding between aptamer and alpha thrombin. This phenomenon has been observed in previous research using in-situ electrochemical atomic force microscopy (ECAFM) based DFS experiment [42].

Previous research has found that other stimuli such as changes in Na+ ions concentration or pH can influence the binding of thrombin and thrombin aptamer. Changes in Na+ ion concentration may change the shielding effect of Na+ and the pH change can results in the change of Thrombin aptamer’s G-quadruplex motif [53]. The binding process of DNA can also
be affected by temperature change, and the affinity reaches the highest value at certain temperature zone [54]. Compared to the above methods, electrostatic actuation has its own advantages in wide range of biomedical applications [42], it makes it possible to induce rapid and controllable conformational change on charged bio-molecules with no destructive influence on the bio-molecules and transducer. In the buffer environment, the double-layer with thickness of nanometers produces a local region of large electrical fields only near the surface of cantilevers, and thus, the DNA molecules immobilized on the surface of cantilever are subjected to the electrical field. Previous research has demonstrated that short double-strand DNA molecules can be switched between “lying” status and “standing” status by applying positive and negative voltage because DNA molecules are negatively charged [55].

In my Master’s research, we applied a constant square-wave of voltage on the cantilever immobilized with thrombin aptamer. We observed that the maximum surface stress (or deflection) at equilibrium decays as the concentration of alpha-thrombin increases. We also found that the time it takes for the cantilever to reach a certain portion of the maximum stress, that is, the first order system time constant, is also affected by the thrombin concentration. We believe that single strand DNA used in that experiments behave like freely jointed chains in unbound state and, binding with protein has significant increase in the DNA stiffness. This increase on specific binding results in a quicker conformation change and thus faster deflection under applied voltage. While in the presence of gamma-thrombin molecules, the stiffness of single stranded aptamer was unchanged, Hence the time constant for cantilever deformation were unchanged for low concentrations and show a slight increase for higher concentrations. We speculate that the specific binding caused the stiffness change to result in a faster deflection, in conclusion, a biochemical signal change caused a mechanical signal change. This conclusion
inspired us that maybe the biomolecular signal change also caused an electrical signal change (e.g. electrical impedance) that is measurable with our existing instruments. The second biosensing method that uses different transduction mechanism (biochemical to electrical), but the same device can be a powerful proof for the previous biosensing method (biochemical to mechanical). By using both methods in one experiment, we can significantly reduce the false detection rate of the system.

6.1.1 Electrochemical Impedance Spectroscopy (EIS)

Electrochemical impedance spectroscopy (EIS) is a powerful measurement method to investigate the components of an electrochemical system. It has several advantages compared to other electrochemical methods. It contains much larger information content than DC techniques or single frequency methods and reveal the information like diffusion, capacitive behavior and electron transferring within an assembled system. This advantage allows EIS to be applied in the investigation of metal corrosion and electrode surface condition.

EIS applies a small sinusoidal voltage change at one frequency to the electrochemical system and measures the current passing through the system,

\[ E = E_0 \sin(\omega t) = E_0 e^{j(\omega t - \phi)} \]

\[ I = I_0 \sin(\omega t - \phi) = I_0 e^{j(\omega t - \phi)} \]

After that, the impedance of the system at that frequency can be calculated.
Then EIS repeats this step for a wide range of frequencies. By fitting the impedance at a wide range of frequencies to an equivalent circuit, the composite and the value of each component (resistance, capacitance or inductance) inside this system can be estimated.

6.2 Experimental Method

Because of the low price and familiarity of thrombin and thrombin aptamer, the same functionalization on the sensing cantilever in the thrombin experiments was used in this electrochemical method. That is, the cantilever was immobilized with thrombin aptamer (5 hours) first and then treated with 6-mercapto-1-hexanal (1 hour).

![Figure 17 Three-electrode system in PE reaction chamber](image)
To apply voltage on the cantilever surface, a three-electrode electrochemical cell will be added to the PE reaction chamber (fig. 17). With the sensing cantilever connected to the working electrode, an Ag/AgCl reference electrode and a platinum counter electrode were combined to protect the AgCl coating and stabilized the position of electrode. All three electrodes will be submerged in the PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 1 mM K₃Fe(CN)₆, pH 7.4) in the reaction cell when voltage was applied, ferri/ferrocyanide redox couple works as an electrochemical mediator. A Gamry Reference600 potentiostat was used to apply desired potential on the working electrode.

Before the experiment, the cantilever was submerged in the PBS buffer containing ferricynide for 6 hours.

When the experiment starts, first, PBS buffer was injected into the reaction chamber, 15 min after the injection, the EIS started with measuring the open circuit potential (OCP) of the cantilever vs. reference electrode, and then applied small (5mVrms) sinusoidal voltage waves at a specific frequency on the OCP to the cantilever surface, the current passing through the cantilever was recorded to calculate the impedance at that frequency. A wide range of frequency from 0.05Hz to 1MHz was repeated.

Secondly, PBS buffer that contains alpha thrombin was injected into the reaction chamber to raise the concentration of alpha thrombin, and EIS was applied 15 min after the injection, then the EIS response over the frequency range was recorded for that alpha thrombin concentration. This step was repeated for several thrombin concentrations.
6.3 Results and Discussion

The EIS results at different concentration is the Nyquist plot and the Bode plot, with real impedance vs. imaginary impedance and absolute impedance vs. frequency, respectively. A representative EIS result was given in fig. 18. We can see that the impedance varies as the frequency that we applied changes due to the effect of the capacitance in the system.

Figure 18 Bode plot (upper) and Nyquist plot with equivalent circuit (lower)
To investigate the components inside the system, different theoretical circuit was used to fit the Nyquist plot and Bode plot, such as CPE, Randals, CPE with diffusion, which turns out that CPE with diffusion gives the smallest goodness of fitting, that is, the minimum square error, also each components inside the CPE with diffusion can be explained and related to the physical conditions in the electrochemical system, the CPE with diffusion model was selected to fit the EIS results. The CPE with diffusion model is shown in the figure 18.

The CPE with diffusion model includes several components including the electrolyte resistance $R_u$, the double layer capacitance $Y_0$, the surface resistance $R_p$ and the Warburg diffusion element $W_d$. Each component can be related to the physical interfaces in the system. The double layer capacitance here is modeled as a constant phase element, because a perfect capacitor has impedance of $\frac{1}{j\omega C}$, where $j$ is the $\sqrt{-1}$, $C$ is capacitance and $\omega$ is the frequency, but an imperfect capacitor (due to surface roughness) behaves like $\frac{1}{(j\omega)^n Y}$, where $Y$ is the impedance value of the capacitor and $n$ is an empirical constant, when $n=1$, the constant phase element act as a perfect capacitor. The surface resistance is the resistance that the electrons must overcome to pass through the double layer capacitor and the electrode interface. The electrons also have to overcome the resistance of the electrolyte pass through the system. The Warburg diffusion element is related to the resistance due to the concentration gradient, that is, the diffusion process.

After fitting with the equivalent circuits, the value of each component can be calculated. What interests us is the surface resistance $R_p$ at different thrombin concentrations, because as more and more thrombin binding to the aptamer on the surface, the positively charged thrombin molecules forms a charged layer that may inhibit the electrons from passing through the electrode interface, thus, act like a resistor.
After several experiments, the fitted \( R_p \) at different thrombin concentration is recorded, the \( R_p \) is then normalized by subtracting and divided by the \( R_p \) in the absence of thrombin. The normalized \( R_p \) is plotted with the corresponding thrombin concentrations.

![Normalized impedance vs. concentration](image)

**Figure 19** Normalized surface resistance at different thrombin concentrations

We can see that the surface resistance increases as the thrombin concentration increases, which indicates that built-up of bound thrombin on the cantilever surface makes the cantilever surface more isolating. One control experiment using gamma thrombin was done to see the effect of non-specific binding, in the control experiment, a small resistance increase was observed, this is probably due to the absorption of the gamma thrombin on the cantilever surface. The noise range
of the experiments was calculated based on the injection of PBS buffer, and the minimum concentration that cause the impedance change larger than the noise level is 0.05nM, that is, the detection limit of the biosensing system.

6.4 Conclusion

We managed to apply the electrochemical impedance spectroscopy (EIS) to measure the concentration of thrombin down to 0.05nM in the PBS buffer, which is sufficient to measure the normal physiological thrombin concentration, 1.4uM [56]. The thrombin bound on the cantilever surface behave like resistor to increase the surface resistance. The non-specific binding of gamma thrombin also caused a resistance increase but is relatively smaller than that of alpha thrombin. The plot of resistance and concentration was fitted with Langmuir equation to derive the calibration curve, which gives a coefficient of disassociation (Kd) of 57nM. This Kd is much larger than the Kd reported by previous researches, in which thrombin aptamer has a strong affinity with thrombin with a dissociation constant of 1 to 6 nM [50, 51]. Several factors can be responsible to this difference, such as the buffer condition, the size of the reaction chamber, the transduction mechanism, and the time we allowed the thrombin to bind. After most thrombin aptamer is bound with alpha thrombin, some subsequent thrombin can still absorb to the thrombin-aptamer complex to cause small further resistance increase, which is the transduction reason for larger Kd in this experiment. By checking the surface resistance at different thrombin concentration, this EIS experiment is a validation that proves the existence of thrombin bound on the cantilever surface. Overall, this EIS based biosensing method has a wide dynamic range and detection limit to offer a low-cost and rapid detection, which can be used together with the
electrical actuation based biosensing method (in my master’s program) to significantly decrease the false detection rate.
CHAPTER VII FUTURE WORK

7.1 Introduction

In my master’s research, we applied a constant square-wave of voltage on the cantilever immobilized with thrombin aptamer and monitored the surface stress change under the voltage in a range of thrombin concentration. We observed that the maximum surface stress (or deflection) at equilibrium decays as the concentration of alpha-thrombin increases. We also found that the time it takes for the cantilever to reach a certain portion of the maximum stress, that is, the first order system time constant, is also affected by the thrombin concentration. We believe that single strand DNA used in that experiments behave like freely jointed chains in unbound state and, binding with protein has significant increase in the DNA stiffness. This increase on specific binding results in a quicker conformation change and thus faster deflection under applied voltage. While in the presence of gamma-thrombin molecules, the stiffness of single stranded aptamer was unchanged, Hence the time constant for cantilever deformation were unchanged for low concentrations and show a slight increase for higher concentrations.

The specific binding caused the stiffness change to result in a faster deflection, which bring up our curiosity to see the actuation under a more complicated voltage function, for example. Except the maximum deflection and time constant, more information based on the measurement can be probably found to further characterize the surface condition of cantilever.

In this electrical actuation based biosensing system, there are some improvement we can do to finalize it in order to applied it together with the EIS based biosensor. First, the previous electrical actuation based biosensing method had a low repeatability, which is probably due to the expired thrombin aptamer. Second, the previous electrical actuation based biosensor applied
a square electrical wave to actuate the cantilever and use the deflection amplitude and time constant as the indicator at different thrombin concentration. But compared to square wave, we found that a sine wave is a better choice to actuate the cantilever because it is easier to analyze with mathematical method like Fast Fourier Transform.

In the current experiment, we will apply sinusoidal electrostatic field on a micro-cantilever, a surface stress change will be induced by several factors including the reconstruction of gold surface under electrical potential, the ion adsorption/desorption on gold surface, and the conformational transition of the aptamer molecules (DNA molecules) immobilized on the cantilever. The cantilever will be deflected under this surface stress change, thus can be actuated by the changes in electrostatic field. Also, by doing experiment in solution of different alpha thrombin concentrations, we were able to see the influence of alpha thrombin concentrations on the deflection under electrical potential. The cantilever deflection will be monitored with the Kylia micro interferometer.

7.2 Experimental Setup

To apply sinusoidal voltage on the cantilever surface, a three-electrode electrochemical cell will be added to the previous experimental setup (fig. 20). With the sensing cantilever connected by a gold wire to the working electrode, an Ag/AgCl reference electrode and a platinum counter electrode were attached on a ceramic holder to protect the AgCl coating and stabilized the position of electrode. All three electrodes will be submerged in the reaction cell when voltage was applied. The Kylia micro interferometer will monitor the deflection during this time. A Gamry Reference600 potentiostat was used to apply desired potential on the working electrode.
The same functionalization on the sensing cantilever and reference cantilever in the thrombin experiments was used in this electrochemical method. That is, the cantilever was immobilized with thrombin aptamer (5 hours) first and then treated with 6-mercapto-1-hexanal (1 hour). Both cantilevers were submerged in the PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 1 mM MgCl2•6H2O, pH 7.4), but only the sensing cantilever connected to the working electrode. Before experiment, the open circuit potential (OCP) of the cantilever vs. reference electrode will be measured by the potentiostat, and ten periods of sine waves will be applied above this OCP on the working electrode. Thrombin in PBS buffer will be injected into the reaction cell 15 min before the application of sine wave actuation. The Kylia micro interferometer was monitoring the deflection during this time.
7.3 Results and Discussion

One experiment was conducted using old batch thrombin aptamer. The stress over time at different thrombin concentrations was plotted in the figure 21. It can be seen that the stress changed sinusoidally under the sine wave voltage, however, the signal has some high frequency noise and drifts slowly over time. Thanks to the sine property of this stress change, we can apply Fast Fourier Transform (FFT) to analyze the stress and filter out the noise at other frequency.

![Stress change under the same sinusoidal wave at different thrombin concentrations](image)

Figure 21 Stress change under the same sinusoidal wave at different thrombin concentrations

From the plot of stress vs. time (fig. 21), we can see that the 50mV sine wave was able to actuate the sensing cantilever, and a positive voltage produced a tensile surface stress on the cantilever, while a negative voltage produced a compressive surface stress on the cantilever.

The FFT basically transform the stress in time domain into the stress in frequency domain. Since we know that the applied sine voltage was at 0.025Hz frequency, after the FFT, the magnitude at the 0.025Hz on the frequency domain can be an indicator of noise-free stress amplitude. The noise-free stress amplitude will be normalized by subtracting and then divided by
the stress amplitude at 0nM thrombin concentration, and finally plotted with the thrombin concentrations. Using FFT processed stress, we expect to remove the pneumatic isolation table and noise isolation box to further shrink the size of the system, which should enable a portable detection at point-of-care.

Figure 22 stress in time domain and frequency domain (upper) and normalized FFT stress vs. thrombin concentration (lower)
From the plot of normalized FFT stress vs. concentration, we can see that this actuation biosensing system has an extremely high sensitivity, the normalized stress saturates at -0.1 at 0.1nM alpha thrombin concentration. This is very close to the results observed in my master’s program when square wave was applied.

Unlike the square wave actuation, when applying triangular or sine wave, the relation between stress and voltage can also be investigated since they can be plotted together. We plotted the surface stress vs. the voltage (fig. 23 lower), it can be seen that the surface stress increases with cycle number for the first three cycles, and for the last two cycles, the surface stress starts to stabilize and repeating the same circle. By analyzing the stabilized circles, we can extract information that relates the cantilever surface condition. The circular shape of surface stress indicates a phase difference between the applied voltage and the stress change, which will be probably affected by the stiffness of immobilized aptamer. The stresses at the most positive and negative voltage can also be affected by the surface condition because aptamers are molecules with negative charge due to its phosphate group. More information about the surface
condition affected by the cantilever charge and electron transferring can be found in the stress vs. current curve.

Figure 23 Triangular voltage and stress over time (upper) and applied voltage vs. stress (lower)
REFERENCES


