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Micro-cantilever based biosensor for electrical actuation and detecting molecular interactions

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Micro-cantilever based biosensor for electrical actuation and detecting molecular interactions

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ABSTRACT

Biosensors that are based on Atomic force microscope (AFM) cantilevers have recently been attracting interest and attention because of its portability and high sensitivity, the gold coated cantilever surface enable researchers to functionalize the cantilever by immobilizing biomolecules like enzyme, DNA, RNA. Reactions between the immobilized molecules and its target molecules on the cantilever can lead to change in measurable signal (e.g. deflection, resonant frequency). By measuring the deflections or resonant frequency change, researchers are able to detect the presence and quantity of target molecules. This property of AFM cantilever allows itself to have potential application in DNA/Protein detection.

We designed an optical system to monitor the deflection of cantilever subjected to electrochemical stimuli in solution. The cantilever was used as the working electrode of a three-electrode cell in order to add an electrical potential onto the functionalized cantilevers for an external stimuli. In my three experiments, Cantilevers with different functionalized surfaces were tested under positive and negative electrical potentials. Several conclusions were obtained based on the measured cantilever deflection. First, immobilized thrombin aptamer can result in a compressive surface stress (compare to gold cantilever coated with alkanethiol) on gold coated cantilever when negative voltage was applied, and a tensile surface stress when positive voltage was applied. Secondly, different molecules immobilized on gold coated cantilever can lead to different deflections under square wave of -100mV and +100mV voltage. The conformational change of thrombin aptamer caused extra compressive surface stress under -100mV and extra tensile surface stress under +100mV. Also the thrombin molecules that bind with aptamers may inhibit the conformational change of aptamer under electrical potential. Because the adenine base pair on poly A does not form a structure like the G-quadruplex form of thrombin aptamer

molecules, the poly A immobilized cantilever does not have deflection change as large as thrombin aptamer cantilever under electrical potential. Thirdly, at concentrations lower than 0.1nM, the deflection of aptamer functionalized cantilever under electrical field decays rapidly as the concentration increases. This phenomenon may have potential application in detecting extremely low thrombin concentration.

CHAPTER I INTRODUCTION

1.1 Biosensors

Biosensors are important devices for quantifying the molecules that we are interested to measure, they have been widely used in many fields, especially in disease diagnosis and drug delivery. A typical biosensor consists of six components, the target molecule, the recognition element, the transducer, the detector, the display and the end user. In the detection process, 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 is responsible to capture this new signal form and transport it to the display for the end user to analyze the signal.

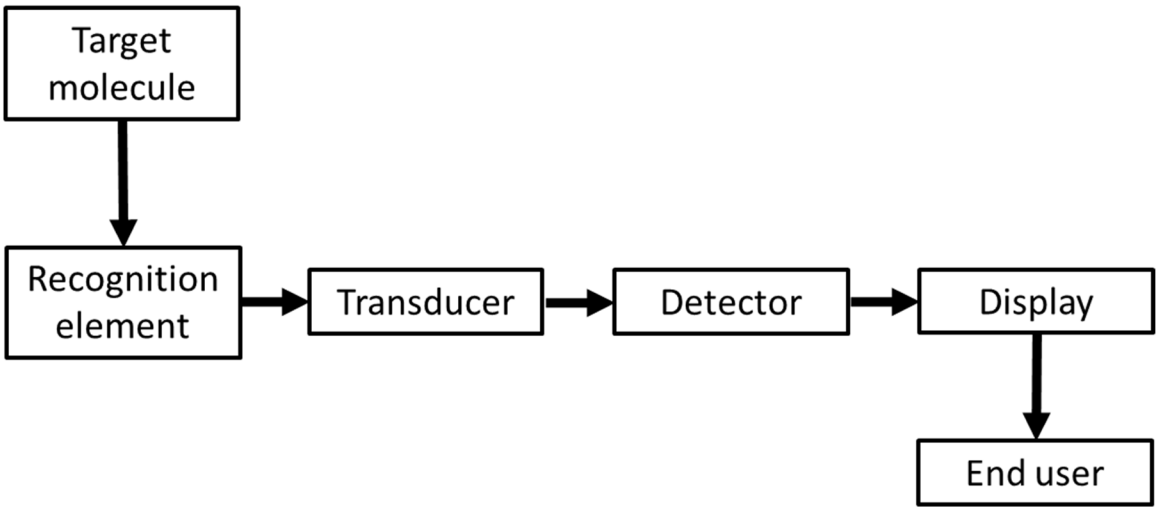

Figure 1 Six components of a biosensor

Most existing biosensors can be classified into three types based on the transduction and detection methods they use: optical biosensors, electrical biosensors and mechanical

biosensors(Arlett, Myers et al. 2011). A brief introduction about different biosensors is provided in this chapter.

1.1.1 Optical biosensors

Optical biosensors can be divided into label-free real-time detection (e.g. microring resonator) and labelled detection (e.g. enzyme-linked immunosorbent assays).

A microring 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 nano-molar and a short response time (2 mins).(Washburn, Luchansky et al. 2009)

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. (Arlett, Myers et al. 2011)

1.1.2 Electrical biosensors

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. (Arlett, Myers et al. 2011)

1.1.3 Mechanical biosensors

One main group of biosensor 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 microchannels, 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 (von Muhlen, Brault et al. 2010).

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 (Kim, Kim et al. 2009), but labeled QCM can achieve femtomolar level detection limit (Kurosawa, Nakamura et al. 2004).

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 (Thundat, Warmack et al. 1994), it was also utilized to detect the formation of alkanethiol self-assembled monolayers (SAM) (Berger, Delamarche et al. 1997) and biomolecules, like single-stranded DNAs (Fritz, Baller et al. 2000). Microcantilever based biosensors have great 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

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 (Stoney 1909):

$$\sigma = \frac{Eh^2}{6R(1-v)}$$

Where σ 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, v is Poisson's ratio of cantilever. The curvature radius change, R is dependent on L (cantilever length) and δ (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, $\frac{Eh^2}{3L^2(1-v)}$ 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:

$$\varphi = {K_d[B]}\big/{K_d[B]+1} \Rightarrow \varphi \approx K_d[B] for K_d[B] << 1$$

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 $[B]$. So 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 Influence of electrostatic field on molecules

The transduction between biological signal and mechanical signal of a biological system is the essence of a mechanical biosensor, which has attracted interest in the past decades. The objective of this research is to investigate this transduction. In our experiment, we use 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 nano deflection on the cantilever, thus the cantilever can be actuated by the changes in electrostatic field. Also, by doing experiment in solution of different thrombin concentration, we expect to see the influence of thrombin concentrations on the deflection under electrical potential. In all the cases, the cantilever deflection was measured with a customized phase-shift laser interferometer.

In this 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 its complementary thrombin. This phenomenon has been observed in previous research using in-situ electrochemical atomic force microscopy (ECAFM) based DFS experiment (Ma and Shrotriya 2015).

Previous research has found that other electrochemical 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 of Na+ and the pH change can results in the change of Thrombin aptamer's G-quadruplex motif (Hianik, Ostatna et al. 2007). The binding process of DNA can also be affected by temperature change, and the affinity reaches the highest value at certain temperature zone (Nguyen, Pei et al. 2011), hence, in this experiment, the laboratory temperature was monitored and the temperature was 22±1 Celsius degree. Compared to the above methods, electrostatic actuation has its own advantages in wide range of biomedical applications (Ma and Shrotriya 2015), it makes it possible to induce rapid and controllable conformational change on charged bio-molecules with no destructive influence on the bio-molecules. 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 (Rant, Arinaga et al. 2004).

Other research has showed that not only biomolecules can be influenced by electrostatic field but also the alkanethiol molecules with charged head-groups can change their conformation under application of electrical field (Lahann, Mitragotri et al. 2003). In this research, the deflection of micro-cantilever covered with alkanethiol under electrostatic field is measured to evaluate the influence of electrostatic field on thrombin aptamer functionalized micro-cantilever.

1.4 The coagulation catalytic enzyme, thrombin

The human coagulation catalytic enzyme, the thrombin, is an enzyme that is responsible for the conversion of fibrinogen to fibrin to complete the clotting process of blood. Thrombin is associated with healthy functions, higher or lower concentration of thrombin than the normal level can cause thrombosis or hemorrhage, respectively. The fibrinogen binding site of thrombin catalyzes the conversion of fibrinogen to fibrin to complete clotting process, while the heparin binding site of thrombin can bind to heparin to inhibit coagulation process(Bode, Mayr et al. 1989).

1.5 Thrombin aptamer

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. Thrombin aptamer has a hairpin structure because its eight guanine bases attract each other, this structure is called G-quadruplex form (fig.2).

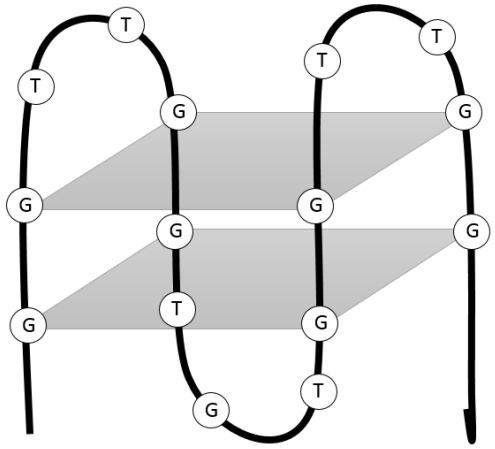

Figure 2 G-quadruplex form of thrombin aptamer

Bode, Mayr et al. found that the heparin binding site of thrombin can bind with thrombin aptamer, and a high affinity was also observed in their research (Bode, Mayr et al. 1989) (Tsiang, Gibbs et al. 1995), which makes thrombin and its DNA aptamer an ideal material for study in interaction between binding molecules. The binding process is the essential part of my experiments, two of my experiments (2.4.2 and 2.4.3) involves binding process between aptamer and thrombin. To understand the binding process, the aptamer and thrombin binding may be modeled as a reversible reaction equation as written as below:

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB$$

Where A is aptamer and B is the thrombin, AB is the bound aptamer/thrombin complex. The rate constants kon and koff determines the reaction rate of forward and backward reactions.

$$V_{forward} = k_{on} * [A] * [B]$$

$$V_{backward} = k_{off} * [AB]$$

Where [A] [B] and [AB] are the concentration of aptamer, thrombin and complex.

When the reaction reaches equilibrium, the rate of forward and backward reactions become equal:

$$V_{forward} = V_{backward}$$

$$k_{on} * [A] * [B] = k_{off} * [AB]$$

This expression can be rearranged:

$$K_d = \frac{k_{off}}{k_{on}} = \frac{[A] * [B]}{[AB]}$$

Kd is the equilibrium dissociation constant with unit of molarity, and it is also the thrombin concentration at which 50% of aptamers are bound to thrombin. According to previous research, thrombin aptamer has a strong affinity with thrombin with a dissociation constant of 1 to 6 nM (Wu, Tsiang et al. 1992, Tsiang, Gibbs et al. 1995).

Another quantity, half-time, is used to describe how long it takes for half of aptamers to bound to thrombin, which equals to:

$$t_{1/2} = \frac{Ln2}{k_{off}}$$

It can be seen that when Kd and kon are known, koff can be estimated in order to calculate half-time. Because for most binding reaction, kon falls in the range of 10^6 to10^8, and the Kd is 1 to 6 nM (Wu, Tsiang et al. 1992, Tsiang, Gibbs et al. 1995), the half-time is estimated to have a range between seconds to ten minutes. Based on this half-time, a reaction time of one hour (longer than

five times of half-time) was used to ensure that the binding reaction between the thrombin/aptamer is close to equilibrium.

1.6 Thrombin aptamer based biosensor

Both aptamer and antibody are commonly used as the recognition element in biosensors, but compared to antibody, aptamer is easier and more economical to produce, hence widely used in biosensing.

The current aptamer based thrombin sensing methods include electrochemical method, optical method (e.g. fluorescence), nano-structure method (gold nano-particle, nano magnetic beads). Most current sensing methods apply a combination of above methods and can achieve a lower detection limits.

Generally, electrochemical methods use an working electrode (usually gold) that was functionalized by immobilizing aptamer on the electrode surface, after submerging the three electrodes (working, reference and counter) in a cell that contains buffer, a voltammetry was conducted and the plot of voltage Vs. current was recorded. By analyzing and comparing the results with and without injection of thrombin, the concentration of thrombin can be estimated.

Optical methods generally use thrombin DNA aptamer labeled with a fluorophore on one end and a quencher on the other end. After adding thrombin to the buffered solution of aptamer, the binding process between the aptamer and thrombin can cause the conformational change on aptamer, the conformational change leads to the change of relative distance between the fluorophore and quencher, resulting an increase or decrease in the fluorescent signal. By detecting the fluorescent signal change, one can quantify the thrombin in the solution (Li, Fang et al. 2002).

While nano-structure methods can use a wide range of nano-devices, such as micro cantilever, gold nano particles, magnetic beads and nano porous structure. Thiolated DNA aptamer will be immobilized on the surface of one of above nano-structure that has high sensitivity to molecular reactions. When the immobilized aptamers bind with protein on the nano-structure, the nanostructure will have detectable signal change such as natural frequency change (micro-cantilever), electrical impedance change (nano porous structure).

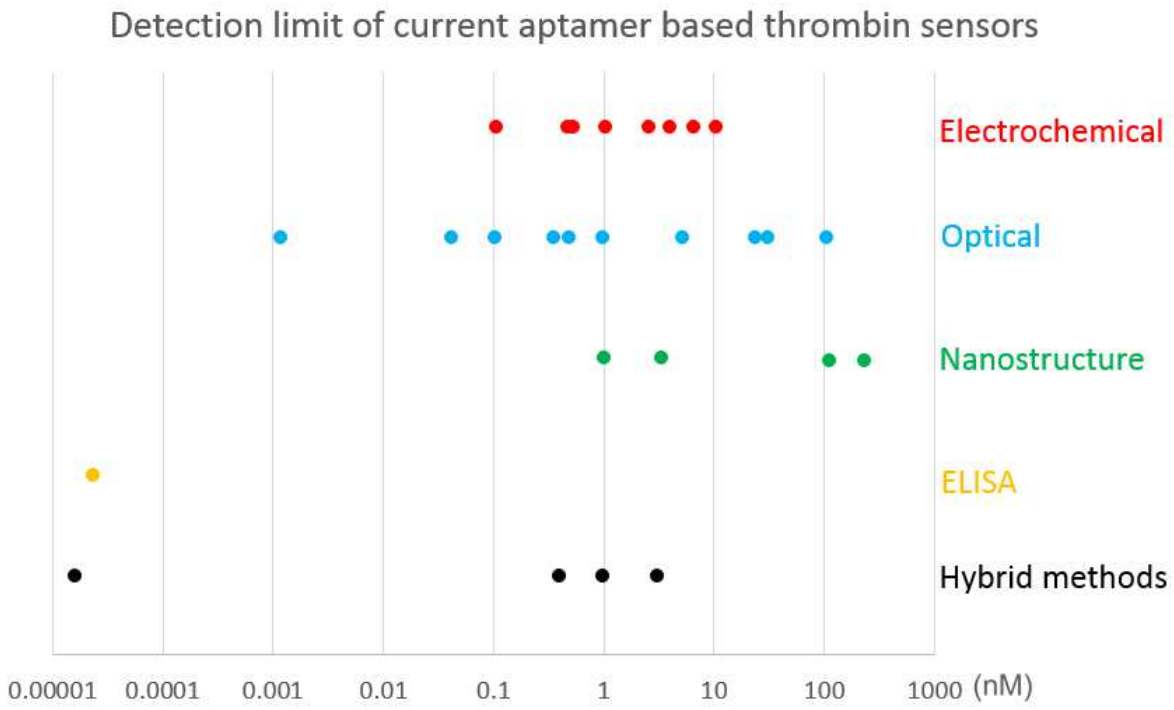

Figure 3 Detection limit of reported aptamer based thrombin sensors (in nanomolar)

Most aptamer based biosensor combines above methods to achieve higher sensitivity (lower detection limit). A literature review was done to collect the detection limits of current existing aptamer based thrombin sensor. (Potyrailo, Conrad et al. 1998, Li, Fang et al. 2002, Ho and Leclerc 2004, Heyduk and Heyduk 2005, Hianik, Ostatna et al. 2005, Radi, Sanchez et al. 2005, Xiao, Lubin et al. 2005, Cai, Lee et al. 2006, Lin, Katilius et al. 2006, Mir, Vreeke et al.

2006, Centi, Tombelli et al. 2007, Tombelli, Minunni et al. 2007, Vannela and Adriaens 2007, Centi, Messina et al. 2008, Fischer, Tarasow et al. 2008, Kim, Kerman et al. 2008, Pandana, Aschenbach et al. 2008, Huang and Zhu 2009, Pu, Huang et al. 2009, Dai and Kool 2011, Goji and Matsui 2011, Zhang, Zhao et al. 2011, Zhang, Zhao et al. 2011, Zhang, Li et al. 2011, Zhang, Li et al. 2011, Evtugyn, Kostyleva et al. 2012, Sarpong and Datta 2012, Goda and Miyahara 2013, Tang, Zhao et al. 2013, Muller and Konig 2014, Park, Cho et al. 2014, Park, Kwon et al. 2014, Wu, Ren et al. 2014, Yan, Wang et al. 2015, Yan, Wang et al. 2015).

In fig.3, it can be seen that most sensors have a detection limit higher than nano molar, and only two biosensors achieve femto molar level, one of them is hybrid method that requires radioactive chemical, and the other one uses a branch of enzyme-linked immunosorbent assay (ELISA). The hybrid method uses combination of different methods, and achieves lower detection limit, this phenomenon motivates us to investigate if it's possible to develop a new method that use a combination of electrochemical and micro-cantilever to detect thrombin and other target molecules without using different kinds of biomolecules required by ELISA.

CHAPTER II ELECTROSTATIC ACTUATION OF FUNCTIONALIZED CANTILEVERS

2.1 Introduction

Before developing a hybrid method that use a combination of electrochemical and micro-cantilever to detect target molecules, the effect of electrical field on functionalized microcantilever needs to be investigated.

The transduction between mechanical signal, electrical signal and biochemical signal is recently attracting attention because it is an important part of biotic-abiotic interface, which has broad potential application in biochemical and biomechanical filed, such as controlling biological system and biomolecules activities (Wong, Almquist et al. 2010). Electrical actuation, a vital method of biotic-abiotic signal transduction, can apply conformational transition upon biomolecules without affecting the chemical stability of the biomolecules. One part of my thesis is to produce conformational transition of thiolated thrombin aptamers on a micro cantilever by applying electrical field and using interferometry system to measure the differential deflection produced by this conformational transition, the sensing cantilever was functionalized by immobilizing its gold surface with thiolated thrombin aptamers. Because the DNA phosphate backbone is negatively charged, when a positive electrical potential is applied on the sensing and reference cantilevers, the thiolated thrombin aptamers on the sensing cantilever will be flatly attracted to the gold surface of sensing cantilever, and when a negative electrical potential is applied, the thiolated thrombin aptamers will be pulled up vertically or even pulled apart from the cantilever, this conformational transition may cause a surface stress change that results in the

cantilever deflection. This conformational transition may also influence the ability of thrombin aptamers to bind with thrombin protein that has affinity with thrombin aptamers.

Electrical actuation on cantilevers has the potential ability to control the adsorption and desorption process of thrombin protein by changing the conformation of thrombin aptamers on the gold surface of cantilever. It also makes it possible to transduce electrical signal into biological signal and mechanical signal.

2.2 Methods

2.2.1 Experiment materials

The DNA aptamer (HSGCCTTAACTGTAGTACTGGTGAAATTGCTGCCATTG GTTGG TGTGGTTGG) was purchased from Integrated DNA Technologies (www.idtdna.com). The human thrombin, the alkanethiol (6-mercapto-1-hexanol), and other chemicals were purchased from Sigma Aldrich (www.sigma.com). The cantilevers used in this experiments were purchased from Nano World (www.nanoworld.com), with width of 100μm, length of 500μm and thickness of 1μm (Figure 2), 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.

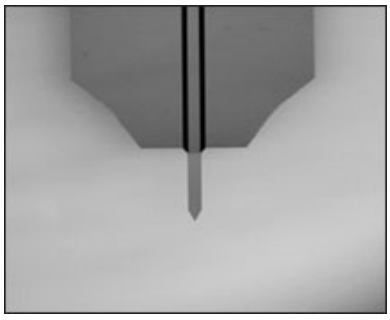

Figure 4 Images of microcantilevers (Nanoworld 2011)

Before experiments, all cantilevers were cleaned by being rinsed in acetone, methanol, isopropanol, sterilized DI water for 10 minutes in each, and then dry in air.

2.2.2 The three-electrode system

The electrostatic field was applied on the functional cantilevers with a Gamry potentiostat Reference 600. The cantilevers were attached on two ceramic holders and were wired to the working electrode and working sensing electrode, a silver wire coated with silver chloride was wired to the reference electrode, and a platinum wire was wired to the counter electrode in order to stabilize the applied voltage. When the cantilevers, the platinum wire and the silver wire were submerged in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 1 mM MgCl2•6H2O, pH 7.4) contained by a polyvinyl chloride (PVC) chamber, an electrochemical system was obtained (fig.5). A cyclic electrical potential was applied on the working electrode for several cycles, in the meantime, the deflection change of cantilever was measured by a customized phase-shift interferometer.

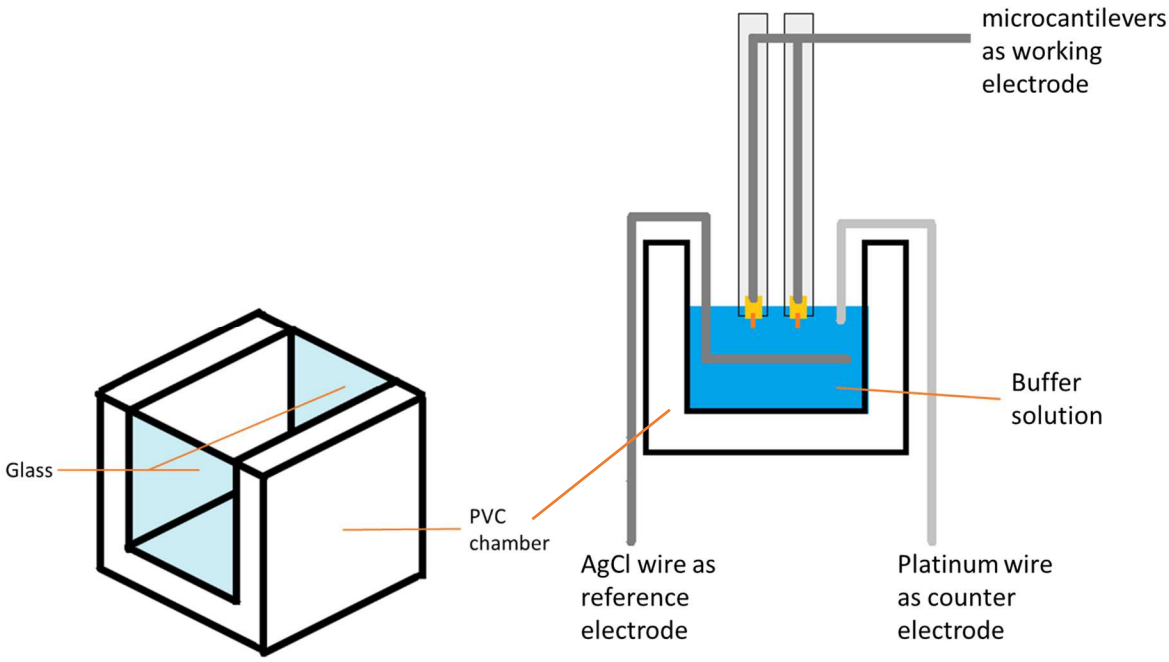

Figure 5 PVC chamber (left) and Electrochemical system (right)

2.2.3 The 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 my research, a customized interferometer (figure 6) was setup to measure the deflection (about several hundred nm) on the micro-cantilever.

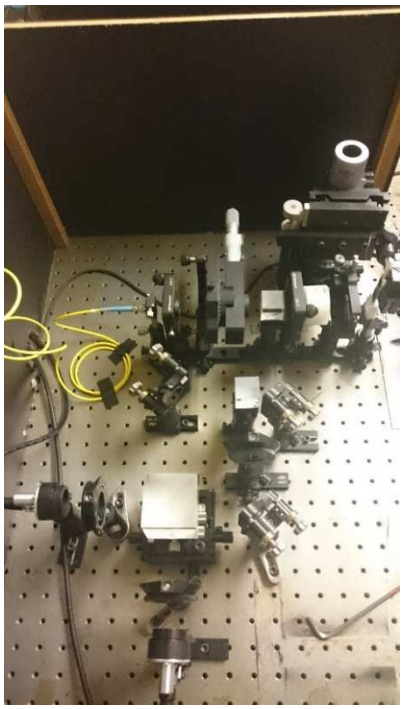

Figure 6 Phase-shift interferometer

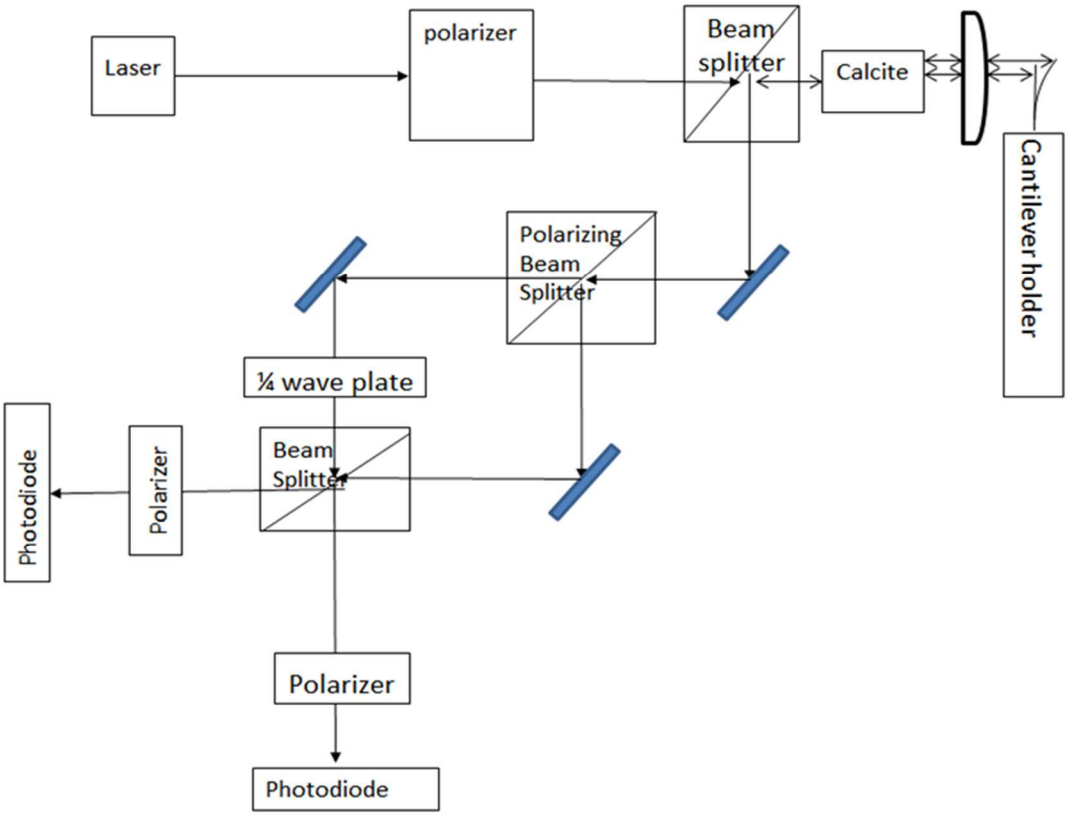

Figure 7 Flow-chart of interferometer

Fig. 7 shows the flow chat of a phase-shifting interferometer. In the interferometer, a laser generator sends a 2mW laser beam at 632nm wavelength to a polarizer, which polarizes the laser beam at 45 degree, as written as:

$$I = A(i+j)\mathrm{e}^{iwt}$$

Where i and j are the unit vector in horizontal and vertical direction, *iwt* 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 separate the laser beam into two laser beams, one is the vertical component of the original laser, and the other one is the horizontal components:

$$I_1 = \frac{\sqrt{2}}{2} A\mathrm{j}\mathrm{e}^{\mathrm{i}wt}$$, and

$$I_2 = \frac{\sqrt{2}}{2} A\mathrm{i}\mathrm{e}^{\mathrm{i}wt}$$

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 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 φ, the reflected lasers can be written as:

$$I_1 = \frac{\sqrt{2}}{2} Aj\mathrm{e}^{iwt+\varphi}$$, and

$$I_2 = \frac{\sqrt{2}}{2} A\mathrm{i}\mathrm{e}^{\mathrm{i}wt}$$,

the calcite then combines the two reflected laser beams and the beam splitter reflect the laser beam to a mirror, the mirror reflects it to a polarizing beam splitter, which separate the laser beam into two laser, and the laser

$$I_1 = \frac{\sqrt{2}}{2} A j \mathrm{e}^{iwt+\varphi}$$, and

$$I_2 = \frac{\sqrt{2}}{2} A \mathrm{ie}^{\mathrm{i}wt}$$,

I2 will pass through a quarter wave plate, which can slow the -45 degree component of I2 by 90 degree to form a new I2:

$$I_{2\,\mathrm{new}} = I_{2+45\,degree} + I_{2\,-45\,\mathrm{degree}}$$
$$= \frac{1}{2} A(i-j)\mathrm{e}^{iwt} + \frac{1}{2} A(i+j)\mathrm{e}^{iwt+\frac{\pi}{2}}$$

I1 also has two components in +45 and -45 degree direction:

$$I_1 = \frac{\sqrt{2}}{2} A j \mathrm{e}^{iwt+\varphi} = \frac{1}{2} A(-i+j)\mathrm{e}^{iwt+\varphi} + \frac{1}{2} A(i+j)\mathrm{e}^{iwt+\varphi}$$

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 degree components of the combination of I1 and new I2 respectively. As result, the laser beams received by the two photodiodes can be written as:

$$I_{photo1} = \frac{1}{2} A(i+j)\mathrm{e}^{iwt+\frac{\pi}{2}} + \frac{1}{2} A(i+j)\mathrm{e}^{iwt+\varphi}$$

$$I_{photo2} = \frac{1}{2} A(-i+j)\mathrm{e}^{iwt+\varphi} + \frac{1}{2} A(i-j)\mathrm{e}^{iwt}$$

The power of laser beams is P=I^2, so the powers of laser beams captured by the two photodiodes can be written as:

$$P_1 = A\left(1 + \cos\left(\frac{\pi}{2} - \varphi\right)\right)$$

$$P_2 = A(1 + \cos(\pi - \varphi))$$

P1 and P2 are two sinusoidal waves with 90 degree 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. 8), the two signals P1 and P2 captured by two photodetectors (lower part in fig. 8) will change sinusoidally with a constant phase difference of $\pi/4$. Theoretically, one signal from one photodetector would be sufficient to calculate the phase change φ, the purpose of using two signals is to easily determine the direction of deflection.

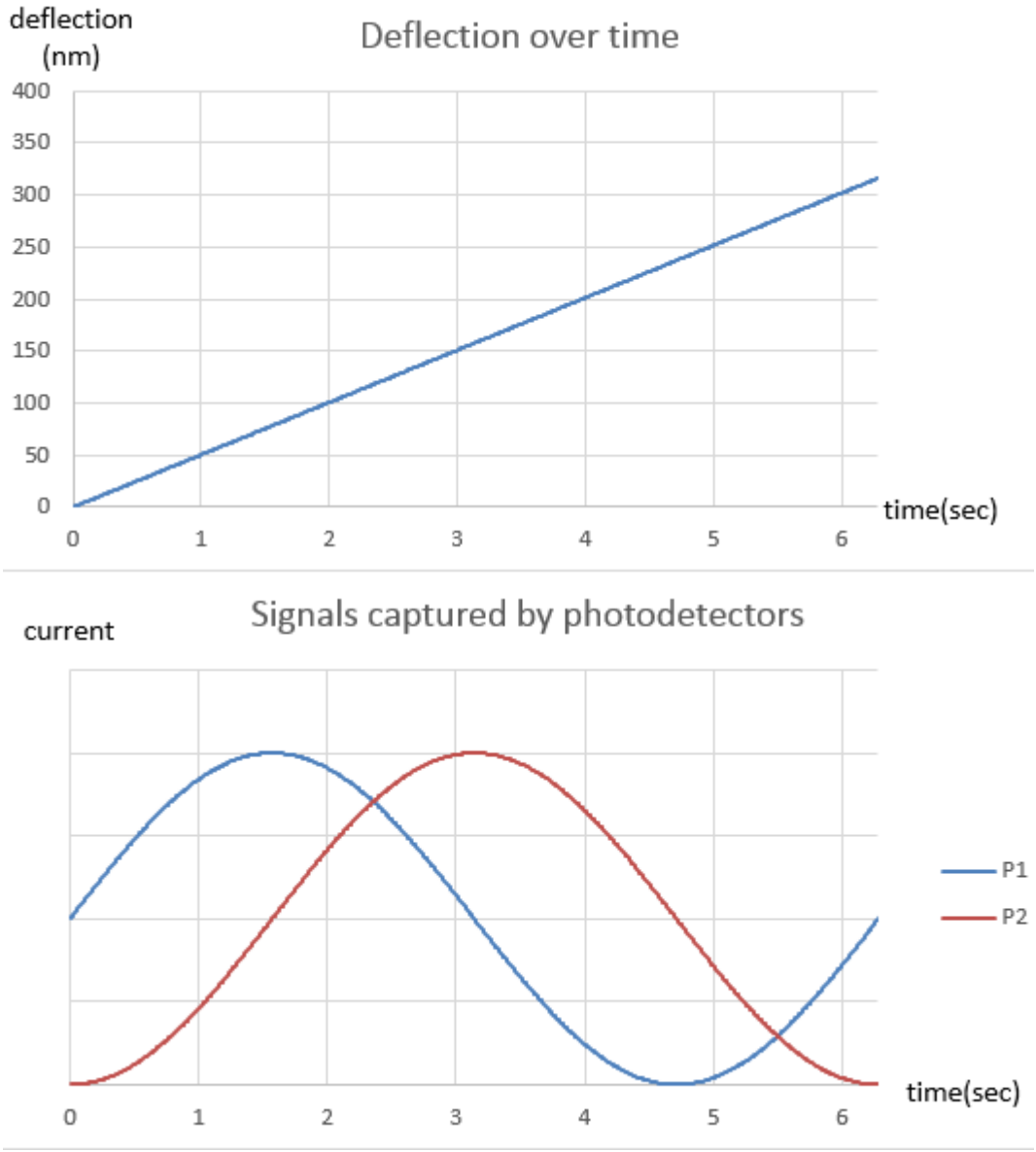

Figure 8 If deflection increases linearly over time (upper), the signals captured by photodetectors (lower) will change sinusoidally with a constantπ/4 phase difference.

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 λ is the wavelength of laser (632nm), and φ is the phase change due to deflection change.

2.3 The Experiment Results

In order to investigate the effect of electrical potential on thrombin and its aptamer, two experiments (2.3.1 and 2.3.2) have been done with the interferometer and electrochemical systems in order to investigate the effect of electrical potential (2.3.1) and functionalization method (2.3.2) on the microcantilever deflection.

2.3.1 Experiment 1: The surface stress change induced by conformational change of DNA under different electrical potentials

Before experiment, the cleaned sensing cantilever was incubated in a solution containing 0.5 μM 5' thiolated thrombin aptamer in sterilized DI water for 3 hours (Ma and Shrotriya 2015) in order to immobilize thiolated aptamer on the sensing cantilever surface,. During this time period, the thiol group on aptamers will be immobilized on the gold surface through strong covalent bonds. Both sensing and reference cantilevers were then submerged in solution containing 3mM alkanethiol (6-mercapto-1-hexanol) in sterilized DI water for 1 hour, the empty area not covered by aptamer will be covered by the alkanethiol. After that, both cantilevers were heated in sterilized DI water to the melting temperature (80 celsius degree) and slowly cool down, the purpose of this step is to separate the hybridized neighboring DNA molecule.

In this experiment, both cantilever was attached on a ceramic holder and submerged in the PBS buffer to form a three-electrode system. The open circuit potential (OCP) was first measured, and then a cyclic square wave of electrical potential above this OCP was applied on both reference cantilever and sensing cantilever. The OCP was measured before every time we applied different cyclic square wave of electrical potential (-100mV, -75mV, -50mV, -25mV, +25mV, +50mV, +75mV, +100mV) above the OCP.

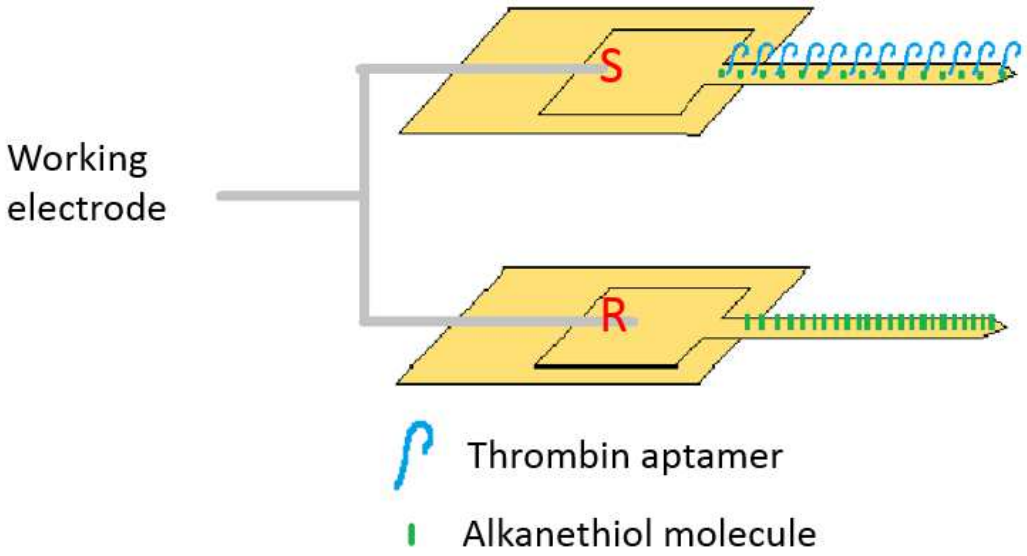

Figure 9 The sensing cantilever (upper) was coated with thrombin aptamer and alkanethiol, the reference cantilever (lower) was coated with alkanethiol only, both of them are connected to the working electrode through silver wire.

When the electrical potential was applied, the laser interferometer was monitoring the deflection of the sensing cantilever relative to the reference cantilever. The figure 10 shows the deflections

and electrical potential change.

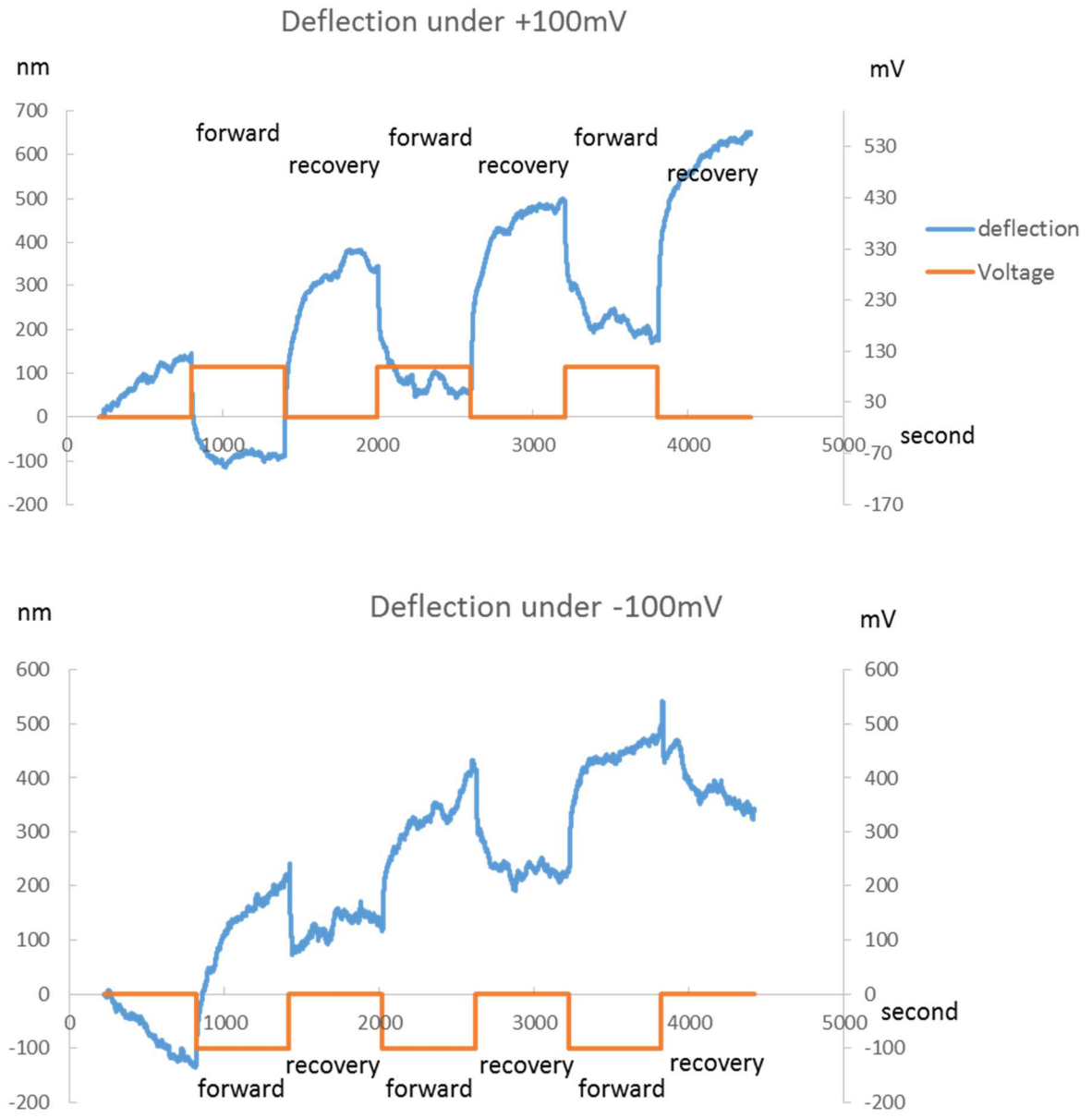

Figure 10 A representative observation of actuation of +100mV (upper) and -100mV (lower), electrical potential was applied on sensing and reference cantilevers (positive deflection means compressive surface stress)

A three-cycle square electrical potential with period of 1200 seconds was applied on both reference and sensing cantilevers. It was observed that when +100mV was applied, the sensing cantilever was bended up (tensile surface stress on gold surface) relative to reference cantilever,

and the sensing cantilever recovered when the potential came back to 0mV (upper part of figure 10). The sensing cantilever showed the opposite deflection and recovery in the -100mV cycles (lower part of figure 10).

The deflections of three cycles was plotted for following voltages: -100mV, -75mV, -50mV, -25mV, +25mV, +50mV, +75mV, +100mV (figure 11). Figure 11 shows that positive voltage can result in negative deflection (cantilever bending up, tensile surface stress) and negative voltage can result in positive deflection (cantilever bending down, compressive surface stress), this deflection difference between sensing and reference cantilevers was caused by the conformational change of thrombin aptamer on the cantilever surface. When positive voltage was applied on the surface of cantilevers, the negatively charged thrombin aptamers on the sensing cantilever was attracted to the surface of cantilever and forced to lie down (Rant, Arinaga et al. 2004) the strong electrical field produced by the double layer close to the cantilever surface may affect the G-quadruplex form, as a result the repulsive force between each neighboring aptamer is lost, this might be the reason that we observed tensile surface stress when +100mV voltage was applied and compressive stress when -100mV was applied.

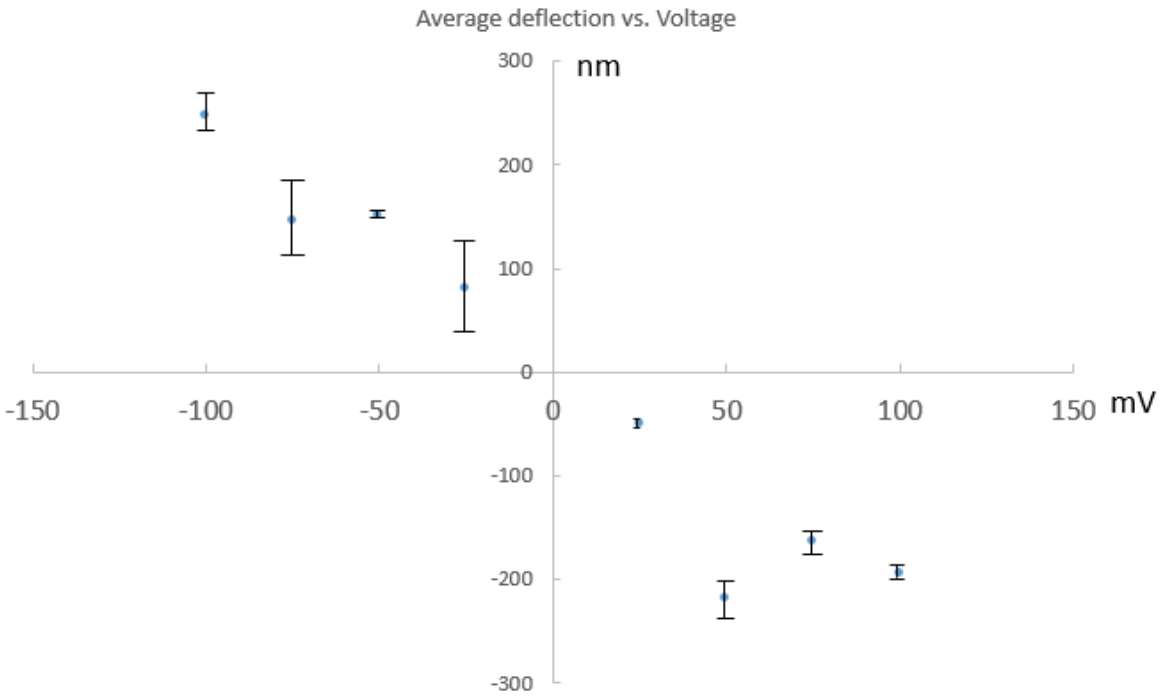

Figure 11 Deflections under different voltages (starting from -100mV to +100mV, with error bar of ±standard deviation)

One Canadian research group also performed an electrical actuation on gold coated micro-cantilevers that were immobilized with thiolated DNA molecules (Nagai, Carbajal et al. 2013), and the direction of deflection they observed is opposite to above experiment, that is, compressive surface stress under positive voltage and tensile surface stress under negative voltage. One possible reason is that, in experiment 1, the voltage was applied above open circuit potential (OCP), while the Canadian research group applied voltage directly on zero relative to reference electrode, because their OCP was not reported, the voltage could be very far from OCP and different factors such as oxidization/reduction can be dominating the deflection direction. Another possible reason can be that a different buffer (10 mM Tris-HCl, 50 mM NaCl, pH 7.4) was used in their experiment. To investigate the influence of different buffers, a voltammetric scanning experiment was conducted. In this experiment, a cleaned new bare cantilever was

connected to the working electrode of a potentiostat through a silver wire, then the cantilever was submerged in buffer, the reference electrode (Ag/AgCl wire) and counter electrode (platinum wire) were also submerged in buffer, the voltage applied on working electrode changed from -1000mV to +1000mV at 10mV/s, the current going through the cantilever was recorded, this voltammetric scanning was repeated three times until a repeatable plot was observed. Both PBS buffer and the Tris-HCl buffer was tested using above procedure and the third time of voltammetric scanning was plotted in Fig. 12.

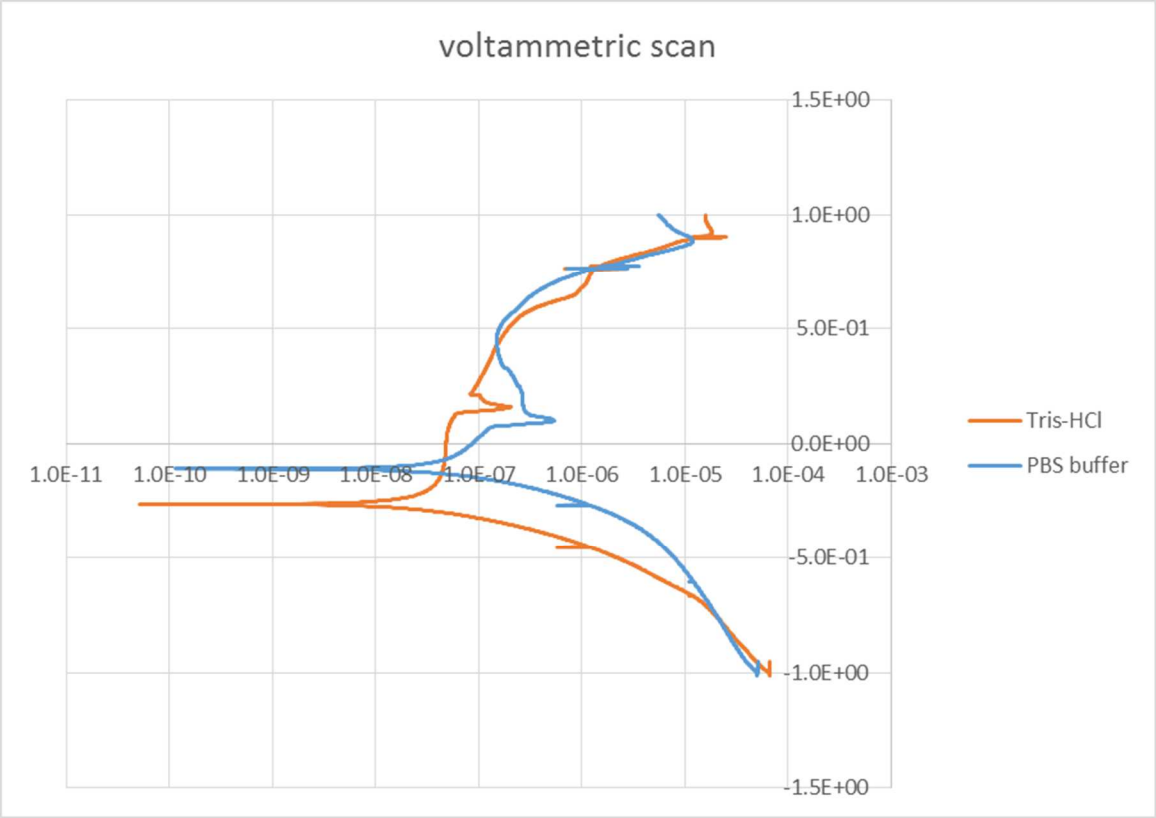

Figure 12 Voltammetric scan from -1000mV to +1000mV at 10mV/s on new bare gold cantilever

From fig. 12, it can be seen that the cantilever behaves differently in PBS buffer and Tris-HCl buffer when voltage was applied. In the PBS buffer, gold cantilever has high OCP (the large sharp peak toward left) than tris-HCl buffer, and after the voltage exceeded OCP the current

increases more rapidly in PBS buffer. Different current direction under same voltage can lead to different ion adsorption on cantilever, as a result, change the deflection direction.

2.3.2 Experiment 2: The surface stress change induced by electrical potentials for different cantilever pairs

The experiment 2 was conducted in order to further test the influence of electrostatic field on different cantilever surface and the binding process between thrombin and thrombin aptamer. In this experiment, a bare gold sensing cantilever was connected to the working electrode, while the reference cantilever was not, so only the sensing cantilever was actuated. The Square wave of -100mV and +100mV in experiment 1 were applied above the open circuit potential (OCP) on the sensing cantilever to actuate it. Similar to experiment 1, an interferometer was used to monitor the relative deflection between the sensing and reference cantilever when the electrical potential was applied. The sensing cantilever was deflected forward when the electrical potential goes to -100mV or +100mV, and recovered when the electrical potential come back to 0mV.

Afterwards, Sensing cantilever coated with different kinds of molecules were all actuated, while the reference was the always the alkanethiol cantilever (figure 13).

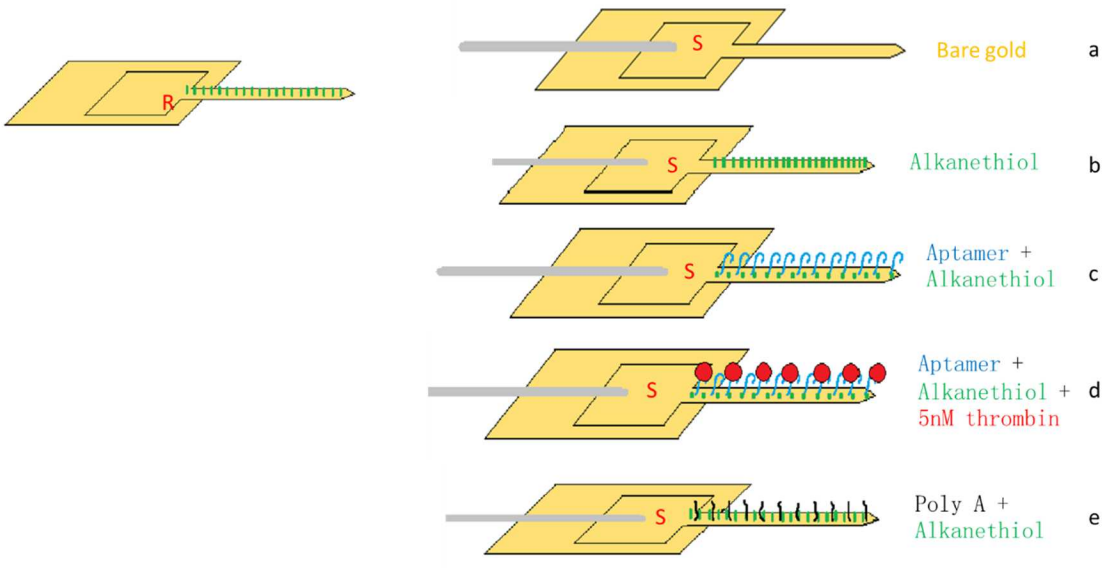

Figure 13 In experiment 2, reference cantilever (left) was coated with alkanethiol, and various sensing cantilevers (right) include cleaned bare gold cantilever (a), cantilever coated with alkanethiol (b), cantilever coated with thrombin aptamer and alkanethiol (c), cantilever coated with thrombin aptamer and alkanethiol in 5mM thrombin solution (d), and cantilever coated with poly-A and alkanethiol (e).

The alkanethiol cantilever used as the sensing cantilever (b. in figure 13) was the same as reference cantilever.

The thrombin aptamer cantilever (c. in figure 13) was the same as the sensing cantilever in experiment 1, that is, incubating a cleaned bare gold cantilever in a solution containing 0.5 µM 5' thiolated thrombin aptamer in sterilized DI water for 3 hours(Ma and Shrotriya 2015) and then submerged in solution containing 3mM alkanethiol (6-mercapto-1-hexanol) in sterilized DI water for 1 hour. In order to break the hybridization between neighboring aptamers on sensing cantilever, the cantilever was heated to the melting temperature of the DNA (80°C) in DI water, and slowly cooled down to room temperature.

The cantilever coated with thrombin aptamer and alkanethiol in 5mM thrombin solution (d. in figure 13) is the same as the thrombin aptamer cantilever (c. in figure 13), but a concentrated solution of thrombin in PBS buffer was injected into the PBS buffer in the PVC chamber to obtain a concentration of 5nM. And measurement was conducted 1 hour after

injection, which is a sufficient time for the reaction to reach equilibrium, the derivation of this time was discuss in the introduction part (1.3). Thrombin aptamer has a strong affinity with thrombin with a dissociation constant of 1 to 6 nM (Wu, Tsiang et al. 1992, Tsiang, Gibbs et al. 1995), so in a concentration of 5nM, part of thrombin aptamers on the cantilever surface should be bound with thrombin molecules.

The cantilever coated with poly A and alkanethiol (e. in figure 13) was made by incubating a cleaned bare gold cantilever in a solution containing 0.5 μM 5' thiolated 5' single stranded poly A (pHS-A30) in sterilized DI water for 3 hours(Ma and Shrotriya 2015) and then submerged in solution containing 3mM alkanethiol (6-mercapto-1-hexanol) in sterilized DI water for 1 hour.

Because in the first cycle of actuation, ions and other unbounded molecules on the surface of cantilever will be removed, so only the deflection in the second cycle for all different sensing cantilevers were chosen and plotted together below.

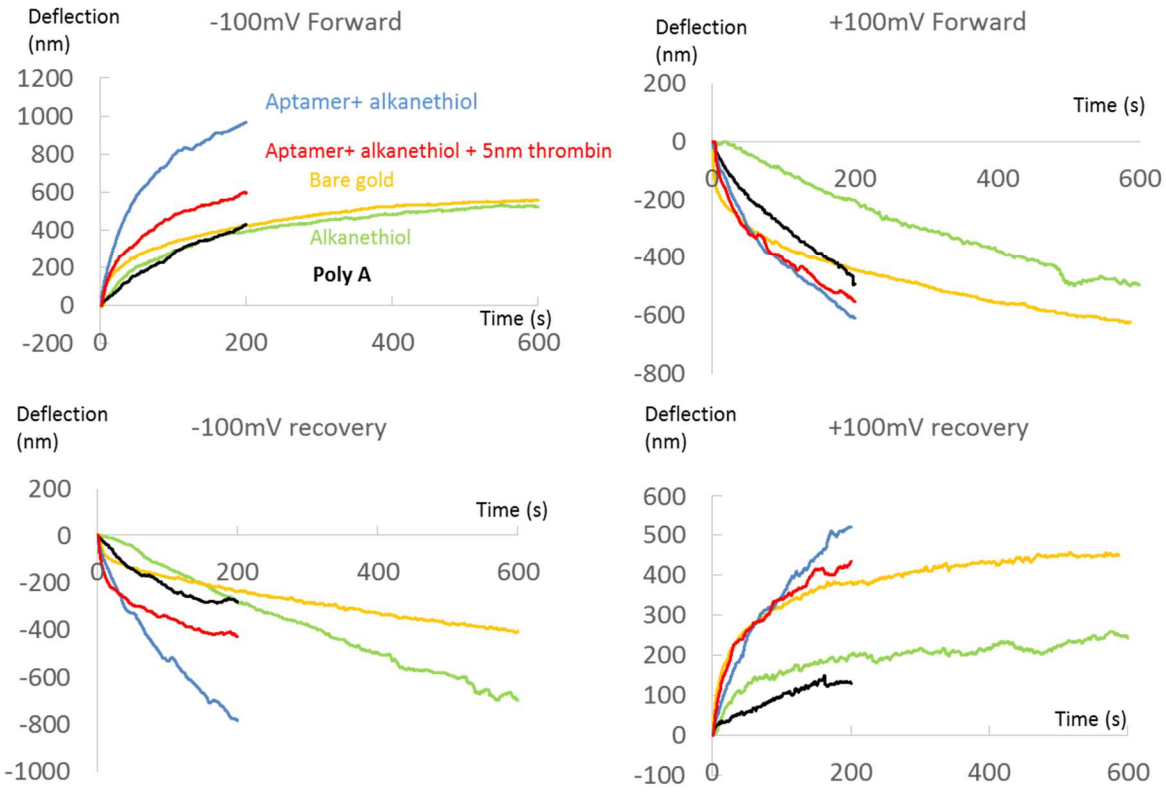

Figure 14 Deflection over time for five different sensing cantilevers, positive deflection means bending down

From figure 14, we can see that the deflection is much larger than deflections in previous experiment, because in this experiment, only sensing cantilever was actuated. The aptamer cantilever (blue) and aptamer cantilever in 5nM thrombin (red) deflected more than other cantilevers, especially when -100mV was applied, this may be because the conformational change of thrombin aptamer caused extra compressive surface stress under -100mV and extra tensile surface stress under +100mV, and this is consistent with the conclusion of experiment 1. Also the thrombin molecules that bind with aptamers may inhibit the conformational change of aptamer under electrical potential, that is probably why the thrombin aptamer cantilever in 5nM thrombin (red) showed less deflection than the thrombin aptamer cantilever (blue).

The poly A showed smaller deflection than the aptamer cantilevers (red and blue). Because the adenine base pair on poly A does not form a structure like the G-quadruplex form of

thrombin aptamer molecules, the DNA molecules on the cantilever do not have conformational change under electrical potential, which results in less surface stress change to deflect the cantilever.

2.4 Conclusion

1) Experiment 1

   Immobilized thrombin aptamer can result compressive surface stress (compare to gold cantilever coated with alkanethiol) on gold coated cantilever when negative voltage was applied, and tensile surface stress when positive voltage was applied. For positive voltage, the magnitude of surface stress change increases rapidly at low voltage and saturated at higher voltage

   Experiment 2

   Different molecules immobilized on gold coated cantilever can lead to different deflections under square wave of -100mV and +100mV voltage. The conformational change of thrombin aptamer caused extra compressive surface stress under -100mV and extra tensile surface stress under +100mV. Also the thrombin molecules that bind with aptamers may inhibit the conformational change of aptamer under electrical potential. Because the adenine base pair on poly A does not form a structure like the G-quadruplex form of thrombin aptamer molecules, the DNA molecules on the cantilever do not have conformational change under electrical potential.

CHAPTER III THROMBIN APTAMER BASED BIOSENSOR

3.1 Introduction

The difference between the aptamer cantilever (blue in fig.14) and aptamer cantilever in 5nM thrombin (red in fig.14) inspired us to investigate the influence of thrombin concentration on the cantilever deflection under voltage, also to develop an aptamer based biosensor for thrombin.

3.2 Experimental Methods

In order to investigate the relation between the thrombin concentration and surface stress under electrical, in this experiment, concentrated solution of thrombin in PBS buffer was injected into the PVC chamber to obtain a certain concentration of thrombin one hour before every time we applied the square wave of electrical potential.

With the same protocol as experiment 1, The sensing cantilever and reference cantilever were the same as the sensing and reference cantilevers in experiment 1, that is, incubating a cleaned bare gold cantilever in a solution containing 0.5 μM 5' thiolated thrombin aptamer in sterilized DI water for 3 hours(Ma and Shrotriya 2015) and then submerged in solution containing 3mM alkanethiol (6-mercapto-1-hexanol) in sterilized DI water for 1 hour, which is a sufficient time for the reaction to reach equilibrium, the derivation of this time was discuss in the introduction part (1.3).

In order to break the hybridization between neighboring aptamers on sensing cantilever, the cantilever was heated to the melting temperature of the DNA (80°C) in DI water, and slowly cooled down to room temperature.

After functionalization, only the sensing cantilever covered with aptamer and alkanethiol was connected to the working electrode and submerged in a PVC chamber containing PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 1 mM MgCl2•6H2O, pH 7.4).

Concentrated solution of thrombin was injected into the chamber 1 hour before each time we applied electrical potential and measured the deflection. Several sensing and reference cantilever pairs were used for this experiment, and each cantilever pair was used for four times of actuation, each actuation includes a cyclic square wave of -100mV and then +100mV.

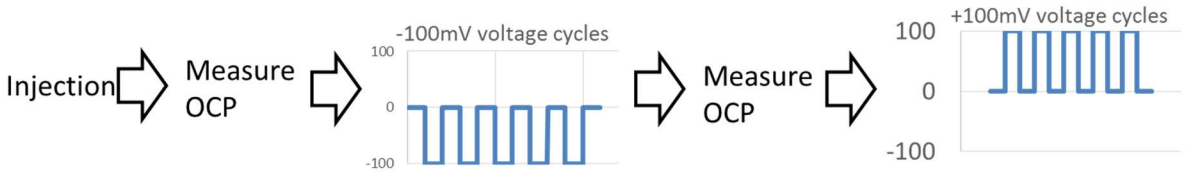

Figure 15 Flowchart of one of the four actuations

In the first actuation, only PBS buffer was injected in order to measure the deflection at zero concentration of thrombin, and for the next three actuations, a small amount of thrombin in PBS buffer was injected 1 hour before each actuations to obtain four different incremental concentrations starting from 0nM.

3.3 Results

The average deflection of only the last four cycles was calculated. The first cycle was excluded because the deflection in the first cycle was always larger than other cycles, this is

probably because the ion or molecule adsorbed on the cantilever before the experiment was repelled in the first cycle. A representative observation is in the figure 16.

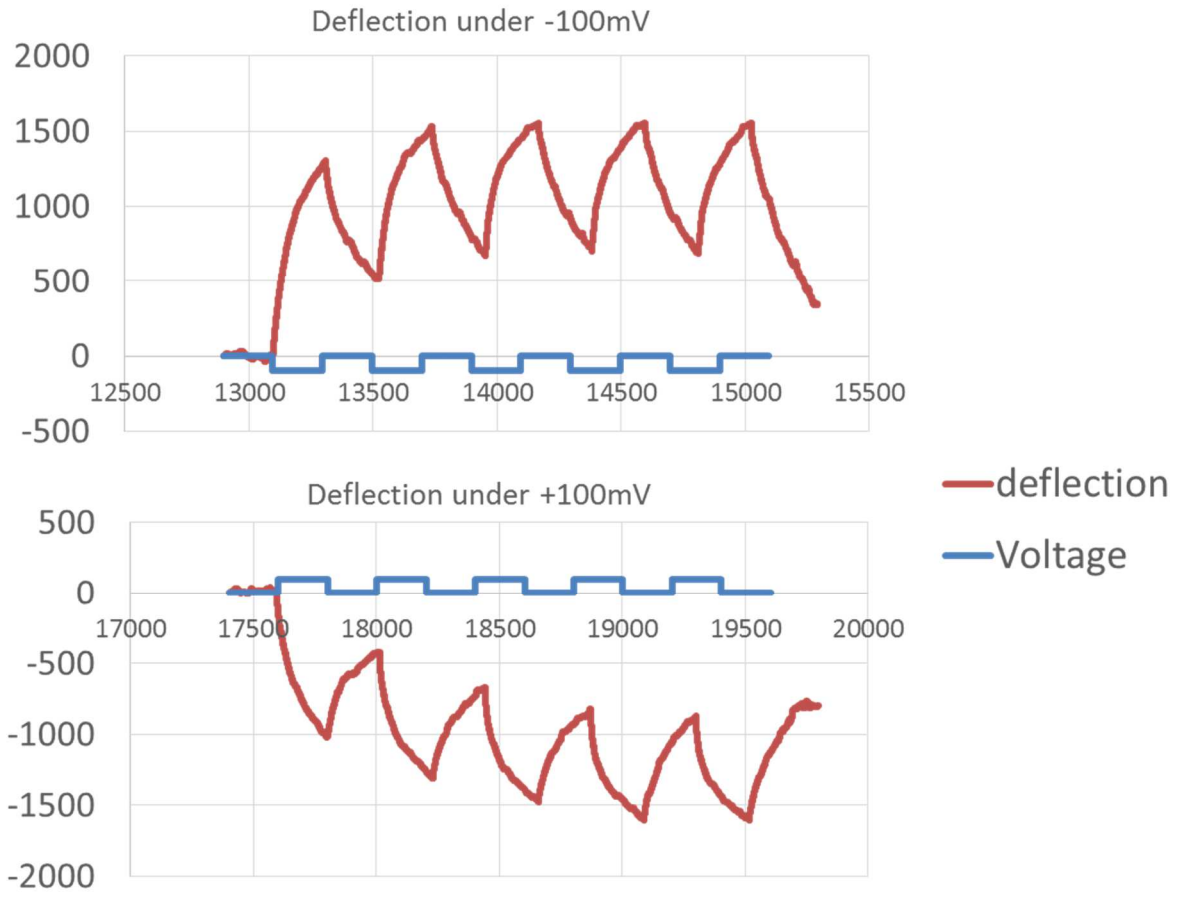

Figure 16 A representative observation of deflection and applied voltage

During experiments, it was found that not only the concentration of thrombin, but also the OCP of cantilever can affect the deflection under cyclic electrical potential. In order to rule out the effect of different OCP, the deflection of each actuation was plotted with the OCP measured before the actuation (fig.17). The deflections were obtained by averaging the deflections of each cycle in an actuation, however, the first cycle of each actuation was not used because it has much larger deflection than other cycles, that's probably because the ion adsorption was cleared away in the first cycle of actuation.

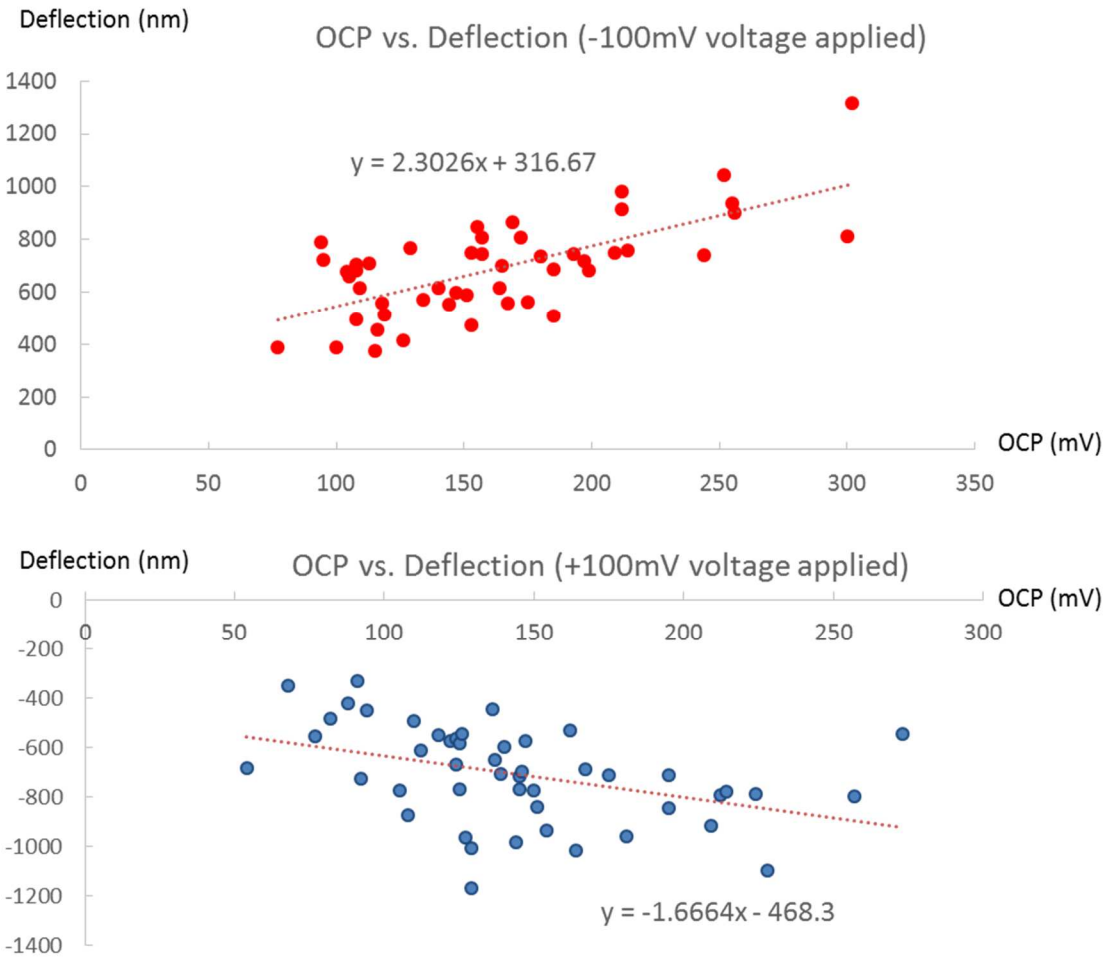

Figure 17 Average deflection of each actuation Vs. the OCP measured before the actuation of cyclic square wave of -100mV (upper) +100mV (lower)

From fig. 17, it can be seen that the negative voltage has larger effect on OCP than positive voltage, this is probably because the surface condition of cantilever was changed when negative voltage was applied. In order to rule out the effect of OCP, a linear fitting (y=m*x + b) was used to find the relation between OCP and deflection, the measured deflection was corrected by subtracting the deflection with the product of slope and OCP:

$$D_{corrected} = D - m * OCP$$

Concentration of thrombin (nM)	Average Deflection (nm) of -100mV	Average Deflection (nm) of +100mV
0	901.75	-788.75
0.1	758.25	-712.25
0.5	571.75	-572.5
5	551.5	-546.5

Figure 18 Deflections at four thrombin concentrations

After correction, the deflections at different thrombin concentrations was obtained, a representative observation was shown in fig. 18.

Afterwards, the corrected deflections were normalized by subtracting the corrected deflection and then dividing the deflection at zero concentration of thrombin in the same actuation:

$$D_{corrected,normalized} = \frac{D_{corrected} - D_0}{D_0}$$

After normalization, negative normalized deflection refers to smaller deflection than deflection at zero concentration, and positive normalized deflection refers to larger deflection than deflection at zero concentration. The normalized deflections in cyclic square wave of +100mV was plotted versus concentrations.

In order to prove that the change of deflection was caused by the binding of aptamer and alpha thrombin, control experiments were conducted. In 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 injection that we used before. The noise level of the experiment setup was also tested by using PBS buffer injection to replace the alpha thrombin injection.

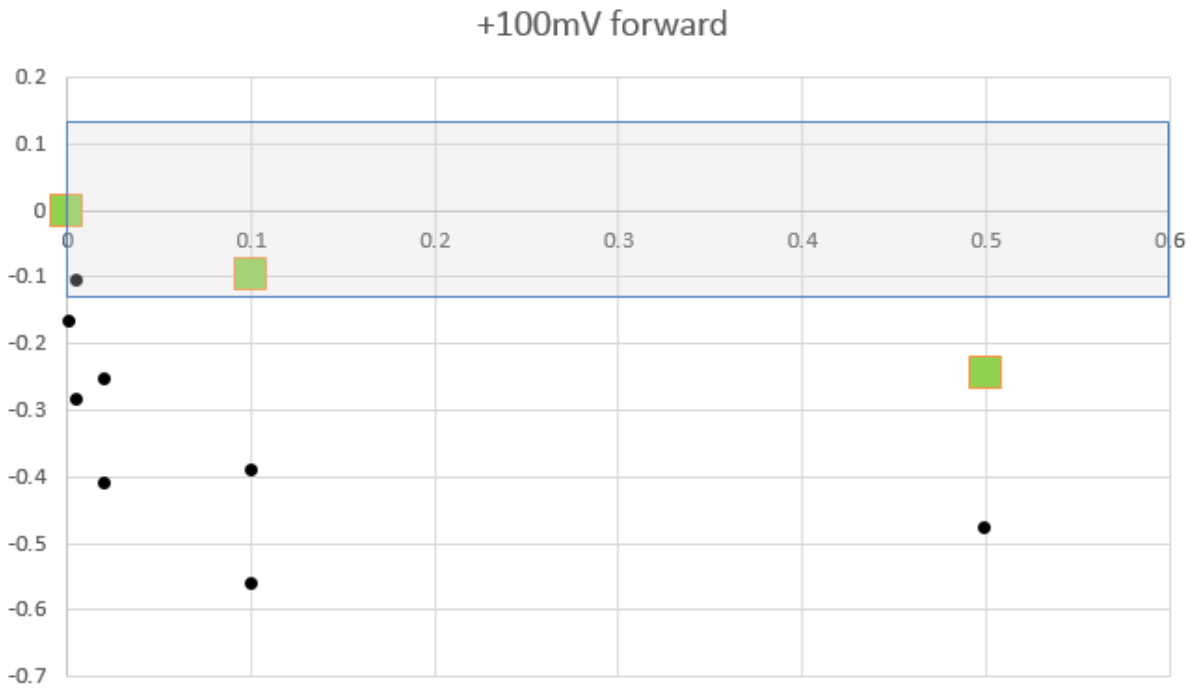

Figure 19 The corrected and normalized deflection under cyclic square wave of +100mV in different concentrations of thrombin. Dots are alpha thrombin data, squares are gamma thrombin data, and shaded area refers to noise level.

The -100mV was not used because it was observed that the OCP was seriously changed when negative voltage was applied. In the figure It can be seen that as the concentration of thrombin increases, the sensing cantilever lose ability to deflect under electrical potential (dots in fig.19), the deflection starts to saturate at concentration of 0.1nM, which is one tenth of the dissociation constant (KD) of thrombin. At concentrations lower than 0.1nM, the deflection decays rapidly as the concentration increases, and this phenomenon may have potential application in detecting extremely low thrombin concentration. Compared to alpha thrombin, the gamma thrombin (squares in fig.19) shows smaller slower decaying deflections. The shaded area is the noise level, which is ±standard deviation of corrected and normalized deflections after each injection of similar amount of PBS buffer.

3.4 Conclusion

Following conclusion can be drawn based on the above experiment on the electrical actuation of functionalized AFM cantilever.

As the concentration of thrombin around aptamer immobilized cantilever increases, the cantilever lose ability to deflect under electrical potential, and this trend starts to saturate at concentration of 0.1nM, which is one tenth of the dissociation constant (KD) of thrombin. At concentrations lower than 0.1nM, the deflection of aptamer functionalized cantilever under electrical field decays rapidly as the concentration increases. This phenomenon may have potential application in detecting extremely low thrombin concentration. Compared to alpha thrombin, the gamma thrombin shows smaller and slower decaying deflections, which proves that the deflection decaying was related to the affinity between aptamer and alpha thrombin.

CHAPTER IV CONCLUSION

Several conclusions can be obtained based on the above experiments.

First, immobilized thrombin aptamer can result in a compressive surface stress (compare to gold cantilever coated with alkanethiol) on gold coated cantilever upon the application of negative voltage, and a tensile surface stress upon the application of positive voltage.

Secondly, different molecules immobilized on gold coated cantilever can lead to different deflections under square wave of -100mV and +100mV voltage. The conformational change of thrombin aptamer caused extra compressive surface stress under -100 mV and extra tensile surface stress under +100mV. Also the thrombin molecules that bind with aptamers may inhibit the conformational change of aptamer under electrical potential. The adenine base pair on poly A does not form a structure like the G-quadruplex form of thrombin aptamer molecules, so the poly A immobilized cantilever does not have deflection change as large as thrombin aptamer cantilever under electrical potential.

Thirdly, at concentrations lower than 0.1nM, the deflection of aptamer functionalized cantilever under electrical field decays rapidly as the concentration increases. This phenomenon may have potential application in detecting extremely low thrombin concentration.

It can be seen that the noise level of the experiment in chapter 3 is relatively large compared to the normalized deflection, this is because of the limitations of current experiment setup.

First, the cantilever drifts in PBS buffer, which takes uncontrollable time (minutes to hours) to stabilize, during this time, the molecules immobilized on cantilevers surface can be affected by the environment, e.g. contamination in air.

Secondly, the PVC chamber was not sealed, because the cantilevers have to be mounted on a ceramic holder that was controlled by a micro-controller in order to helps aligning the cantilevers to the focus of laser beam. Because the PVC chamber is open, the PBS buffer in the chamber can evaporate during the experiment, each experiment has different time duration (determined by time the cantilevers take to stabilize), the PBS concentration in the PVC chamber can slightly vary. As we know, the binding between thrombin and aptamer is significantly dependent on buffer concentration, the difference in buffer concentration may affect the binding process, as a result, affect the deflections.

Thirdly, the current interferometer using a large optical path between cantilever and photodetector to obtain high sensitivity, so change of ambient light intensity in the lab environment can lead to noise in the final signal.

Fourthly, the cantilevers were wired with silver wires through contacting, which may be a weak connection when voltage was applied.

A fiber optic interferometer with a new buffer container and a new cantilever fixation will be designed to eliminate the above disadvantages.

REFERENCES

Arlett, J., E. Myers and M. Roukes (2011). "Comparative advantages of mechanical biosensors." Nature nanotechnology **6**(4): 203-215.

Arlett, J. L., E. B. Myers and M. L. Roukes (2011). "Comparative advantages of mechanical biosensors." Nature Nanotechnology **6**(4): 203-215.

Berger, R., E. Delamarche, H. P. Lang, C. Gerber, J. K. Gimzewski, E. Meyer and H. J. Guntherodt (1997). "Surface stress in the self-assembly of alkanethiols on gold." Science **276**(5321): 2021-2024.

Bode, W., I. Mayr, U. Baumann, R. Huber, S. R. Stone and J. Hofsteenge (1989). "The Refined 1.9 a Crystal-Structure of Human Alpha-Thrombin - Interaction with D-Phe-Pro-Arg Chloromethylketone and Significance of the Tyr-Pro-Pro-Trp Insertion Segment." Embo Journal **8**(11): 3467-3475.

Cai, H., T. M. H. Lee and I. M. Hsing (2006). "Label-free protein recognition using an aptamer-based impedance measurement assay." Sensors and Actuators B-Chemical **114**(1): 433-437.

Centi, S., G. Messina, S. Tombelli, I. Palchetti and M. Mascini (2008). "Different approaches for the detection of thrombin by an electrochemical aptamer-based assay coupled to magnetic beads." Biosensors & Bioelectronics **23**(11): 1602-1609.

Centi, S., S. Tombelli, M. Minunni and M. Mascini (2007). "Aptamer-based detection of plasma proteins by an electrochemical assay coupled to magnetic beads." Analytical Chemistry **79**(4): 1466-1473.

Dai, N. and E. T. Kool (2011). "Fluorescent DNA-based enzyme sensors." Chemical Society Reviews **40**(12): 5756-5770.

Evtugyn, G. A., V. B. Kostyleva, A. V. Porfireva, M. A. Savelieva, V. G. Evtugyn, R. R. Sitdikov, I. I. Stoikov, I. S. Antipin and T. Hianik (2012). "Label-free aptasensor for thrombin determination based on the nanostructured phenazine mediator." Talanta **102**: 156-163.

Fischer, N. O., T. M. Tarasow and J. B. H. Tok (2008). "Protein detection via direct enzymatic amplification of short DNA aptamers." Analytical Biochemistry **373**(1): 121-128.

Fritz, J., M. K. Baller, H. P. Lang, H. Rothuizen, P. Vettiger, E. Meyer, H. J. Guntherodt, C. Gerber and J. K. Gimzewski (2000). "Translating biomolecular recognition into nanomechanics." Science **288**(5464): 316-318.

Goda, T. and Y. Miyahara (2013). "Label-free and reagent-less protein biosensing using aptamer-modified extended-gate field-effect transistors." Biosensors & Bioelectronics **45**: 89-94.

Goji, S. and J. Matsui (2011). "Direct detection of thrombin binding to 8-bromodeoxyguanosine-modified aptamer: Effects of modification on affinity and kinetics." Journal of nucleic acids **2011**.

Heyduk, E. and T. Heyduk (2005). "Nucleic acid-based fluorescence sensors for detecting proteins." Analytical Chemistry **77**(4): 1147-1156.

Hianik, T., V. Ostatna, M. Sonlajtnerova and I. Grman (2007). "Influence of ionic strength, pH and aptamer configuration for binding affinity to thrombin." Bioelectrochemistry **70**(1): 127-133.

Hianik, T., V. Ostatna, Z. Zajacova, E. Stoikova and G. Evtugyn (2005). "Detection of aptamer-protein interactions using QCM and electrochemical indicator methods." Bioorganic & Medicinal Chemistry Letters **15**(2): 291-295.

Ho, H. A. and M. Leclerc (2004). "Optical sensors based on hybrid aptamer/conjugated polymer complexes." Journal of the American Chemical Society **126**(5): 1384-1387.

Huang, H. P. and J. J. Zhu (2009). "DNA aptamer-based QDs electrochemiluminescence biosensor for the detection of thrombin." Biosensors & Bioelectronics **25**(4): 927-930.
Kim, D.-K., K. Kerman, H. M. Hiep, M. Saito, S. Yamamura, Y. Takamura, Y.-S. Kwon and E. Tamiya (2008). "Label-free optical detection of aptamer–protein interactions using gold-capped oxide nanostructures." Analytical biochemistry **379**(1): 1-7.
Kim, N., D. K. Kim and Y. J. Cho (2009). "Development of indirect-competitive quartz crystal microbalance immunosensor for C-reactive protein." Sensors and Actuators B-Chemical **143**(1): 444-448.
Kurosawa, S., M. Nakamura, J. W. Park, H. Aizawa, K. Yamada and M. Hirata (2004). "Evaluation of a high-affinity QCM immunosensor using antibody fragmentation and 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer." Biosensors & Bioelectronics **20**(6): 1134-1139.
Lahann, J., S. Mitragotri, T. N. Tran, H. Kaido, J. Sundaram, I. S. Choi, S. Hoffer, G. A. Somorjai and R. Langer (2003). "A reversibly switching surface." Science **299**(5605): 371-374.
Li, J. W. J., X. H. Fang and W. H. Tan (2002). "Molecular aptamer beacons for real-time protein recognition." Biochemical and Biophysical Research Communications **292**(1): 31-40.
Lin, C. X., E. Katilius, Y. Liu, J. P. Zhang and H. Yan (2006). "Self-assembled signaling aptamer DNA arrays for protein detection." Angewandte Chemie-International Edition **45**(32): 5296-5301.
Ma, X. and P. Shrotriya (2015). "Molecular Dynamics Simulation of Conformational Transition and Frictional Performance Modulation of Densely Packed Self-Assembled Mono layers Based on Electrostatic Stimulation." Langmuir **31**(24): 6729-6741.
Mir, M., M. Vreeke and L. Katakis (2006). "Different strategies to develop an electrochemical thrombin aptasensor." Electrochemistry Communications **8**(3): 505-511.
Muller, A. and B. Konig (2014). "Vesicular aptasensor for the detection of thrombin." Chemical Communications **50**(84): 12665-12668.
Nagai, Y., J. D. Carbajal, J. H. White, R. Sladek, P. Grutter and R. B. Lennox (2013). "An Electrochemically Controlled Microcantilever Biosensor." Langmuir **29**(32): 9951-9957.
Nguyen, T., R. J. Pei, D. W. Landry, M. N. Stojanovic and Q. Lin (2011). "Label-free microfluidic characterization of temperature-dependent biomolecular interactions." Biomicrofluidics **5**(3).
Pandana, H., K. H. Aschenbach and R. D. Gomez (2008). "Systematic aptamer-gold nanoparticle colorimetry for protein detection: Thrombin." Ieee Sensors Journal **8**(5-6): 661-666.
Park, J. H., Y. S. Cho, S. Kang, E. J. Lee, G. H. Lee and S. S. Hah (2014). "A colorimetric sandwich-type assay for sensitive thrombin detection based on enzyme-linked aptamer assay." Analytical Biochemistry **462**: 10-12.
Park, K., S. J. Kwon and J. Kwak (2014). "A Label-Free Electrochemical Aptasensor for Thrombin Using a Single-Wall Carbon Nanotube (SWCNT) Casted Glassy Carbon Electrode (GCE)." Electroanalysis **26**(3): 513-520.
Potyrailo, R. A., R. C. Conrad, A. D. Ellington and G. M. Hieftje (1998). "Adapting selected nucleic acid ligands (aptamers) to biosensors." Analytical Chemistry **70**(16): 3419-3425.
Pu, F., Z. Z. Huang, D. Hu, J. S. Ren, S. Wang and X. G. Qu (2009). "Sensitive, selective and label-free protein detection using a smart polymeric transducer and aptamer/ligand system." Chemical Communications(47): 7357-7359.
Radi, A. E., J. L. A. Sanchez, E. Baldrich and C. K. O'Sullivan (2005). "Reusable impedimetric aptasensor." Analytical Chemistry **77**(19): 6320-6323.

Rant, U., K. Arinaga, S. Fujita, N. Yokoyama, G. Abstreiter and M. Tornow (2004). "Dynamic electrical switching of DNA layers on a metal surface." Nano Letters **4**(12): 2441-2445.
Sarpong, K. and B. Datta (2012). "Nucleic-acid-binding chromophores as efficient indicators of aptamer-target interactions." Journal of nucleic acids **2012**.
Stoney, G. G. (1909). "The tension of metallic films deposited by electrolysis." Proceedings of the Royal Society of London Series a-Containing Papers of a Mathematical and Physical Character **82**(553): 172-175.
Tang, X. F., D. Zhao and M. N. Zhang (2013). "Sensitive and reusable electrochemiluminescent aptasensor achieved with diblock oligonucleotides immobilized solely through preferential adenine-Au interaction." Analyst **138**(19): 5706-5712.
Thundat, T., R. J. Warmack, G. Y. Chen and D. P. Allison (1994). "Thermal and Ambient-Induced Deflections of Scanning Force Microscope Cantilevers." Applied Physics Letters **64**(21): 2894-2896.
Tombelli, S., M. Minunni and M. Mascini (2007). "Analytical applications of aptamers." Optical Sensing Technology and Applications **6585**: U245-U253.
Tsiang, M., C. S. Gibbs, L. C. Griffin, K. E. Dunn and L. L. K. Leung (1995). "Selection of a Suppressor Mutation That Restores Affinity of an Oligonucleotide Inhibitor for Thrombin Using in-Vitro Genetics." Journal of Biological Chemistry **270**(33): 19370-19376.
Vannela, R. and P. Adriaens (2007). "DNAzymes in environmental sensing (vol 36, pg 375, 2006)." Critical Reviews in Environmental Science and Technology **37**(4): 379-379.
von Muhlen, M. G., N. D. Brault, S. M. Knudsen, S. Y. Jiang and S. R. Manalis (2010). "Label-Free Biomarker Sensing in Undiluted Serum with Suspended Microchannel Resonators." Analytical Chemistry **82**(5): 1905-1910.
Washburn, A. L., M. S. Luchansky, A. L. Bowman and R. C. Bailey (2009). "Quantitative, label-free detection of five protein biomarkers using multiplexed arrays of silicon photonic microring resonators." Analytical chemistry **82**(1): 69-72.
Wong, I. Y., B. D. Almquist and N. A. Melosh (2010). "Dynamic actuation using nano-bio interfaces." Materials Today **13**(6): 14-22.
Wu, L., J. S. Ren and X. G. Qu (2014). "Target-responsive DNA-capped nanocontainer used for fabricating universal detector and performing logic operations." Nucleic Acids Research **42**(21).
Wu, Q. Y., M. Tsiang and J. E. Sadler (1992). "Localization of the Single-Stranded-DNA Binding-Site in the Thrombin Anion-Binding Exosite." Journal of Biological Chemistry **267**(34): 24408-24412.
Xiao, Y., A. A. Lubin, A. J. Heeger and K. W. Plaxco (2005). "Label-free electronic detection of thrombin in blood serum by using an aptamer-based sensor." Angewandte Chemie-International Edition **44**(34): 5456-5459.
Yan, J., L. Wang, L. Tang, L. Lin, Y. Liu and J. Li (2015). "Enzyme-guided plasmonic biosensor based on dual-functional nanohybrid for sensitive detection of thrombin." Biosensors and Bioelectronics **70**: 404-410.
Yan, J., L. D. Wang, L. H. Tang, L. Lin, Y. Liu and J. H. Li (2015). "Enzyme-guided plasmonic biosensor based on dual-functional nanohybrid for sensitive detection of thrombin." Biosensors & Bioelectronics **70**: 404-410.
Zhang, X., Z. Zhao, H. Mei, Y. Qiao, Q. Liu, W. Luo, T. Xia and X. Fang (2011). "A fluorescence aptasensor based on DNA charge transport for sensitive protein detection in serum." Analyst **136**(22): 4764-4769.

Zhang, X. Y., Z. L. Zhao, H. C. Mei, Y. P. Qiao, Q. L. Liu, W. X. Luo, T. Xia and X. H. Fang (2011). "A fluorescence aptasensor based on DNA charge transport for sensitive protein detection in serum." Analyst **136**(22): 4764-4769.
Zhang, Y., B. Li, C. Yan and L. Fu (2011). "One-pot fluorescence detection of multiple analytes in homogenous solution based on noncovalent assembly of single-walled carbon nanotubes and aptamers." Biosensors and Bioelectronics **26**(8): 3505-3510.
Zhang, Y. F., B. X. Li, C. G. Van and L. H. Fu (2011). "One-pot fluorescence detection of multiple analytes in homogenous solution based on noncovalent assembly of single-walled carbon nanotubes and aptamers." Biosensors & Bioelectronics **26**(8): 3505-3510.