SURFACE-STRESS-BASED MICROCANTILEVER

APTASENSOR

by

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BE (Hons)

Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

Deakin University
June 2012
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ABSTRACT

Biosensors based on microcantilevers convert biological recognition events into measurable mechanical displacements and benefit from small size, low sample volume, label-free detection, ease of integration, high-throughput analysis, and low development cost. The specificity of a biosensor is determined by the immobilized bioreceptors. Antibodies are commonly used as bioreceptors for biomolecule detection however, are unstable, very difficult, and costly to produce. Aptamers are synthetic nucleic acids that exhibit superior specificity to antibodies due to their small size and physicochemical stability. Furthermore, aptamers are less susceptible to denaturation, have less batch variation, and long storage life. These advantages have prompted the development of aptamer-based biosensors known as aptasensors.

This thesis presents the design and development of a microcantilever-based aptasensor employing SU-8 polymer as the fabrication material. The work is carried out in five stages: (i) design of the aptasensor, (ii) mathematical modelling and simulation of the aptasensor, (iii) fabrication of the aptasensor using surface micromachining, (iv) experimentation on aptasensor surface modification and immobilisation, and (v) characterisation and evaluation of the aptasensor using thrombin molecules.

The thesis presents the first reported work on aptasensors with aptamer immobilized on a bare SU-8 surface. The main findings and contributions include:
(i) employing aptamers as the biorecognition elements for surface-stress based microcantilever biosensor, (ii) optimizing aptasensor parameters through modelling and simulation studies, (iii) implementing SU-8 polymer as the fabrication material for aptasensor, (iv) developing a surface functionalisation technique for aptamer immobilisation on bare SU-8 surface, (v) investigating and identifying an ideal thin cantilever release method to achieve the highest yield, (vi) characterizing the fabricated SU-8 microcantilever and comparing it to its silicon counterpart, and (vii) demonstrating proof-of-concept for the developed aptasensor via biological trials using thrombin molecules.

Binding specificity of the developed biosensor is improved using aptamers. Design parameters of the aptasensor are optimised using finite element method to improve sensitivity through modelling and simulation studies. Fabrication parameters are optimised to reduce residual stress in the microcantilever and the methods are presented. Experiments on different microcantilever release methods are conducted and include: (i) a dry release approach using a fluorocarbon polymer layer, and (ii) a wet release approach using (a) Omnicoat and (b) PMMA as a sacrificial layer. The results demonstrated that the wet release approach using PMMA produced the highest yield of 90% for the release of 2µm thick structures.

Functionalisation of the aptasensor surface is achieved using gas plasma treatment instead of conventional wet chemical methods. Surface modification using the plasma technique is more advantageous as it is a dry single step method, simple to implement, and has good reproducibility.
Characterisation of the fabricated SU-8 polymer aptasensor is presented. The spring constant of the microcantilever is measured at 1.389 mN/m, which closely corresponds to the calculated theoretical value of 1.604 mN/m. In contrast, the calculated theoretical spring constant of silicon based aptasensors is 67.6 mN/m. This demonstrates that the lower Young’s modulus of SU-8 polymer results in a spring constant that is 48 times lower compared to that of silicon microcantilevers of similar dimensions. Label-free detection of thrombin molecules using the aptasensor is successfully demonstrated. Thrombin molecules with a concentration of 1 µM produced a measured deflection of 1900 nm. The deflection is an order of a magnitude higher than reported works using silicon nitride microcantilevers, resulting in a sensitivity of 130.683 µm/ N/m.
ACKNOWLEDGEMENT

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Being a student with engineering degree, it was very challenging for me to adapt myself to conduct biological experiments. Thanks to Professor Wei Duan for his support and advice in this aspect and this helped me progress smoothly.

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<th>Abbreviation</th>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>$BF_4^-$</td>
<td>tetrafluoroborate</td>
<td></td>
</tr>
<tr>
<td>$ClO_4^-$</td>
<td>perchlorate</td>
<td></td>
</tr>
<tr>
<td>$PF_6^-$</td>
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</tr>
<tr>
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<td>AgCl</td>
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</tr>
<tr>
<td>AuNP</td>
<td>gold nanoparticle</td>
<td></td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscope</td>
<td></td>
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<tr>
<td>C$_4$F$_8$</td>
<td>fluorocarbon</td>
<td></td>
</tr>
<tr>
<td>CCD</td>
<td>charged-coupled device</td>
<td></td>
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<tr>
<td>CMOS</td>
<td>complementary metal–oxide–semiconductor</td>
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<tr>
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<td>carbon nanotube</td>
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</tr>
<tr>
<td>DNA</td>
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<tr>
<td>DRIE</td>
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<tr>
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<td>ISFET</td>
<td>ion-sensitive field effect transistors</td>
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<tr>
<td>ITO</td>
<td>indium tin oxide</td>
<td></td>
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<tr>
<td>Li</td>
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<td>LOC</td>
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<td>MEMS</td>
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<td>NaClO₄</td>
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<tr>
<td>NaDBS</td>
<td>dodecylbenzenesulfonate sodium salt</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>polyimide</td>
<td></td>
</tr>
<tr>
<td>PID</td>
<td>proportional, integral and derivative</td>
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</tr>
<tr>
<td>PMMA</td>
<td>poly(methylmethacrylate)</td>
<td></td>
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<tr>
<td>PSD</td>
<td>photosensitive detector</td>
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<td></td>
</tr>
<tr>
<td>SOI</td>
<td>silicon-on-insulator</td>
<td></td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
<td></td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>Ti</td>
<td>titanium</td>
<td></td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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</tr>
</tbody>
</table>
LIST OF PUBLICATIONS

Journals:


Book chapters:


Conference papers:

CHAPTER 1

INTRODUCTION

1.1 Background

Applications of micro-sensing in chemical, biological, environmental, and food monitoring have been gaining increasing interests from interdisciplinary researchers. Integration, miniaturisation, and parallelisation are the key characteristics of a successful micro-sensing system to address the increasing demand in low cost, high throughput, and high sensitivity micro-sensing systems. These characteristics can be achieved by combining a MEMS platform and microfluidics technology on a single system referred to as a lab-on-a-chip (see Figure 1-1 (a)). MEMS technology enables the integration of sensors and electronic read-out circuitry on a single chip. On the other hand, the microfluidics technology allows the addition of fluidics components on-board the chip for sample and fluid handling [1].

An ideal lab-on-a-chip system is capable of performing standard laboratory functions such as crude sample handling, sample and reagent mixing and reacting, separation and subsequent detection of analyte of interest [2, 3]. To achieve the aforementioned functions, the lab-on-a-chip system requires functional components (as shown in Figure 1-1(b)) that has been classified by the
author as injector, preparator, transporter, mixer, reactor, separator, detector, controller, and power supply [4].

The detector identifies and quantifies analyte via a host of different methods. It consists of a transducer that acquires physical signals from analyte and transforms them into electrical signals for analysis. The detector is the most crucial component in a LOC system as it determines the functionality and performance of the entire system. The LOC system would not be complete without a detector. Thus, the work in this thesis focuses on the detector component for biosensing applications. For real-world applications, it is necessary
for the sensing device to meet the stringent requirements such as high selectivity, the ability to detect and identify molecular species from a single molecule up to high concentrations in crude sample in real-time.

A biosensor is a device that is capable of providing qualitative and/or quantitative information of biomolecular interactions via coupling of biological recognition element to a transducer. A biosensor consists of two main components: a sensing layer and a transducer. The sensing layer is usually made up of bioreceptors. To achieve high selectivity and specificity, the sensing layer is typically immobilised with a layer of receptor molecules that recognises and binds the molecules of interest. Biomolecules such as antibodies, proteins, peptides, enzymes and aptamers can be used as the receptor molecules. Most of the biosensors found in the literature employ antibodies as the sensing layer. However, antibodies are laborious to prepare, costly and unstable. Conversely, aptamers are a promising class of agents for biomolecules detection compared to antibodies. Aptamers are short synthetic nucleic acids that are isolated from very large combinatorial libraries of oligonucleotides via an iterative in-vitro process of selection and amplification. This selection procedure is known as systematic evolution of ligands by exponential enrichment (SELEX). Due to their small size (5-25 kDa), they exhibit superior specificity and physicochemical stability. They are also less susceptible to denaturation, low cost, have less batch variation, have long storage life, and easy to modify facilitating covalent bonding to material surfaces. These advantages have spurred exciting developments in aptamer-based sensors that are also referred to as aptasensors. It is notable that the aptasensor
binding affinity and specificity is influenced by the surface immobilisation technique. Binding affinity describes the strength with which a ligand binds to a receptor. High affinity binding denotes that the ligand has a greater tendency to bind to the receptor relative to its dissociation from the receptor. Specificity is important to prevent false-positive results due to the non-specific binding of impurities in the assay. High specificity denotes that receptor molecules only bind to the target ligand.

Successful interaction event between the receptors and target molecules at the sensing layer is then converted to measurable electronic signals via the transducer component. The transducer can further be subdivided into two components: a reporter and a detector. The reporter acts as signalling interface between the sensing layer and the detector. However, some biosensors may not employ the reporter in their transducer components.

Bioreceptors, particularly aptamers, undergo conformational change upon target binding. By incorporating signalling or reporter molecules into the aptamers, the target binding event can be detected. This labelling approach is widely implemented in optical-based sensing whereby fluorescent labels are attached to the target for molecular sensing. Electrochemical sensing is yet another label-based technique which employs fluorescent and catalytic labels to amplify the electron transfer signal. Although labelling can enhance the detection signal, it could affect the binding properties and thus the yield of ligand-target reaction. Furthermore, labelling is cumbersome as it requires time and sample handling. A label-free approach is more attractive for point-of-care diagnostics.
applications as it eliminates the labelling step and provides real-time sensing. Examples of label-free sensing are FET sensors, impedance-based sensors and micromechanical sensors. Among the label-free sensing techniques, microcantilever sensing is deemed the most promising device for point-of-care applications as it is sensitive, can be easily integrated into lab-on-a-chip platforms, offers high throughput analysis via microarray sensing and is capable of batch production.

1.2 Motivations

Microcantilever-based sensing has become the key focus of chemical and biological sensing systems. This is largely due to its inherent benefits such as small size, low cost, low sample volume, label-free detection and ease of integration with microfluidics devices. Furthermore, high-throughput analysis is easily achievable via fabrication of microcantilever arrays for parallel processing. With these advantages, the microcantilever sensors are deemed the ideal sensing platform [5, 6].

However, the microcantilever sensing device is still far-fetched from commercialization. A key reason is that its performance is limited by the sensitivity of the deflection detection mechanism. Since the microcantilever-based sensors have flexible structures, they are easily affected by the surrounding environment and this makes accurate measurements of its deflection considerably challenging. Also, the limit of detection of the existing microcantilever biosensors is still inadequate for real-world applications. Limit of detection refers to the
smallest concentration of target molecules that can be detected by the sensor. Different applications have different limit of detection requirements. For medical diagnostics, typical concentrations for sensing of antibody-antigen interactions are in ng/ml, metabolites in the blood are in the mg/ml range and viruses in blood are in the several hundred per ml range [7]. Since these target compounds exist in a heterogeneous sample format, whereby other non-target compounds are of higher concentration the achievable limit of detection is limited by the binding affinity between the target and receptor molecules. It is noteworthy to mention that the performance of sensors is often limited by the non-specific binding of molecules rather than the inherent sensing device. Therefore, careful considerations are essential when designing the sensor surface immobilisation protocols. Surface immobilisation affects the binding affinity and specificity of the sensing device.

1.3 Aims and objectives

In this thesis, the aim is to design and develop a microcantilever-based aptasensor employing SU-8 polymer as the fabrication material. SU-8 is a negative photoresist epoxy-acrylate polymer which is highly transparent in the UV region and is biocompatible.

The objectives of the research work are listed as follows:

i. **Achieve label-free sensing**

ii. **Improve sensor sensitivity and limit of detection**

   a) Reduce false negative signals caused by non-specific binding and environmental effects.
b) Improve binding affinity between receptor and target molecules.

iii. **Develop a suitable surface functionalisation method to generate an immobilisation layer with high biomolecules density**

   a) Achieve good linkage between sensor surface and immobilised biomolecules so that it is stable in the biological medium.

   b) Control orientation of biomolecules so that they are easily accessible by target molecules.

   c) Obtain reproducibility.

   d) Enable immobilised biomolecules maintain their biological activity and retain their native 3D structure.

1.4 Thesis contributions

The main findings and contributions from this thesis are listed as follows:

i. **Employed aptamers as the biorecognition elements for surface-stress based microcantilever biosensor**

Aptamers improve the biomolecule binding affinity and specificity which leads to aptasensor sensitivity enhancement.

ii. **Optimised aptasensor parameters through modelling and simulation studies**

To improve the sensitivity of the aptasensor, modelling and simulation of the proposed design geometry was carried out using the finite element method with Coventorware, a multiphysics software.
iii. **Implemented SU-8 polymer as the fabrication material for the aptasensor**

The benefits of applying polymer materials to fabricate the aptasensor include the ease of fabrication, low cost, and low Young’s modulus. SU-8 has a Young’s modulus as low as 4 GPa which makes it more flexible compared to silicon which resulted in an improvement in the aptasensor response.

iv. **Investigated and identified an ideal thin microcantilever release method to achieve the highest yield**

Methods of fabricating the prototype aptasensor using SU-8 were presented. The fabrication parameters were optimised to reduce residual stress in the microcantilever. Experiments on different release methods were conducted to release 2 µm thick microcantilevers. They include the dry release approach using a fluorocarbon polymer layer and the wet release approach using Omnicoat and PMMA as a sacrificial layer. Results demonstrated that the wet release approach using PMMA produced the highest yield percentage of 90%.

v. **Developed a surface functionalisation technique for aptamer immobilisation on SU-8 surface**

Surface functionalisation of the aptasensor surface was achieved using gas plasma treatment instead of conventional wet chemical methods. Surface modification using the plasma technique is more advantageous as it is a dry single step method, and simple to implement. It has good reproducibility and enhances immobilisation density of aptamers that leads to sensitivity enhancement of the aptasensor.
vi. **Characterised the fabricated SU-8 microcantilever and compared it to its silicon counterpart**

The method of characterising the fabricated SU-8 polymer aptasensor was presented. The spring constant of the microcantilever was measured at 1.389 mN/m, which closely corresponded to the calculated theoretical value of 1.604 mN/m. In contrast, the calculated theoretical spring constant of silicon based aptasensors was 67.6 mN/m. This demonstrates that the lower Young’s modulus of SU-8 polymer resulted in a spring constant that is 48 times lower compared to that of silicon microcantilevers of similar dimensions.

vii. **Demonstrated proof-of-concept for the developed aptasensor via biological trials using thrombin molecules**

Label-free thrombin molecules detection using the developed aptasensor was successfully demonstrated. A displacement of 1900 nm was detected for a 1 µM thrombin molecule concentration. This is an order of a magnitude higher than the reported aptasensors based on silicon nitride microcantilevers.

### 1.5 Thesis layout

**CHAPTER 2** presents the literature review on the existing biosensing works developed thus far. The chapter starts with the introduction of the basic architecture of a sensing device. Then, various types of receptor molecules used and different sensor transduction mechanisms are presented and compared. As the microcantilever-based transducer is the main focus of this thesis, a dedicated
section that describes different types of microcantilever-based sensor operation modes and the state-of-the-art read-out approaches are presented.

CHAPTER 3 describes the theory of mechanical bending of microcantilevers. The theory of surface stress is described followed by a review on the related works describing the underlying operating principle of the adsorption-induced surface stress. A mathematical model elucidating the relationship between the surface stress and the mechanical deformation of microcantilevers concludes the chapter.

CHAPTER 4 presents sensor design considerations in terms of the microcantilever dimensions, material and shapes. Modelling of the device and simulations results are presented at the end of the chapter.

CHAPTER 5 outlines the methods employed to fabricate the aptasensor prototype. The background of the existing fabrication techniques is reviewed followed by a listing of the required tools, chemicals and equipments. Device fabrication process flow is listed with the aid of diagrams. This chapter also presents the problems encountered during the fabrication process and gives the proposed troubleshooting solutions.

CHAPTER 6 focuses on the aptasensor surface immobilisation techniques. Existing surface functionalisation techniques on different types of surfaces are discussed. Then a detailed step-by-step surface functionalisation and immobilisation protocol is presented.

CHAPTER 7 reports the aptasensor characterization, testing and evaluation. The characterization methods are presented and the obtained results are discussed.
This chapter continues with a demonstration of the biological trials using thrombin molecules. The experimental procedures, the calibration methods and the obtained measurement results are also reported in this chapter.

CHAPTER 8 concludes the thesis by giving a discussion on the developed aptasensor and providing suggestions on the future developments of the aptasensor.
CHAPTER 2

LITERATURE REVIEW

2.1 Background

An extensive literature search has been carried out based on the aims and objectives of this research before deciding on designing a microcantilever-based aptasensor. The current state-of-the-arts biological sensing technologies are presented in this chapter. Firstly, Section 2.2 gives a brief background on the sensor architecture before proceeding to the elucidation of different transduction methods implemented in the existing works. A comprehensive review is presented on microcantilever-based sensors in Section 2.3 which is related to the main focus of the work presented in this thesis.

2.2 Biosensors

Miniaturization of biosensors enables rapid analysis, low cost and real-time detection. A biosensor is a device that is capable of providing qualitative and/or quantitative information of molecular interactions via coupling of recognition element to a transducer (see Figure 2-1). A biosensor comprises of two main components: a bioreceptor layer and a transducer. The bioreceptor layer consists of biomolecules that bind specifically to the analyte of interest. The transducer translates the binding event into detectable and measurable signals such as an electrical signal, an optical emission or a mechanical signal, and outputs the data
to the user interface. The transducer can be further subdivided into two sub-components: a reporter and a detector. The reporter acts as a signalling interface between the bioreceptor layer and the detector. However, some biosensors may not employ the reporter in their transducer components. The type of output signals generated by the transducer is dependent on the transduction mechanism. The transduction mechanism can be categorised into various groups according to their detection physics, and is further elaborated in Section 2.2.2.

Figure 2-1: Schematic representation of biosensor architecture.

### 2.2.1 Bioreceptor layer

The bioreceptor layer can be attached or immobilised onto a wide range of surfaces including metals [8-10], carbon nanotubes [11-13], gold nanoparticles [14-16], and polymers [17-19]. The bioreceptor layer can be made up of antibodies [20-22], proteins [23], peptides [5, 24], enzymes [25], nucleic acids [26-28] or aptamers [8, 29-31]. Antibodies have been the popular choice of capture molecules in biosensing applications for the past three decades [32]. Aptamers are short synthetic nucleic acids that are isolated from very large
combinatorial libraries of oligonucleotides via an iterative in vitro process of selection and amplification. This selection procedure is known as systematic evolution of ligands by exponential enrichment (SELEX) [33]. Aptamers [32, 34] are very small in size (5–25 kDa), mimic antibodies properties while exhibiting superior specificity and physicochemical stability. Compelling features of aptamers include low susceptibility to denaturation, low cost and less batch variation, ease of modification to facilitate covalent bonding to materials surfaces as well as long storage life. The properties comparison between aptamers and antibodies are summarised in Table 2-1. These advantages have spurred exciting developments in aptamer-based sensors which is also known as aptasensors [35-37]. Based on these advantages, biosensors that employ aptamers as the bioreceptor layer produce a sensing device that is of higher sensitivity and specificity compared to bioreceptor layer made up of antibodies. Therefore, aptamers are chosen as the bioreceptor layer in this work.
Table 2-1: Comparison of aptamers and antibodies (Adapted from [34]).

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Aptamers</th>
<th>Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding affinity</td>
<td>Binding affinity in low nanomolar to picomolar range</td>
<td>Binding affinity in low nanomolar to picomolar range</td>
</tr>
<tr>
<td>Selection process</td>
<td>Entire selection is a chemical process carried out in vitro and can therefore target any protein</td>
<td>Selection requires a biological system, therefore difficult to raise antibodies to toxins (not tolerated by animal) or non-immunogenic targets</td>
</tr>
<tr>
<td>Conditions for observations</td>
<td>Can select for ligands under a variety of conditions for in vitro diagnostics</td>
<td>Limited to physiologic conditions for optimising antibodies for diagnostics</td>
</tr>
<tr>
<td>Screening methods</td>
<td>Iterative rounds against known target limits screening processes</td>
<td>Screening monoclonal antibodies time consuming and expensive</td>
</tr>
<tr>
<td>Synthesis</td>
<td>Uniform activity regardless of batch synthesis</td>
<td>Activity of antibodies vary from batch to batch</td>
</tr>
<tr>
<td>PK parameters</td>
<td>PK parameters can be changed on demand</td>
<td>Difficult to modify PK parameters</td>
</tr>
<tr>
<td>Investigator</td>
<td>Investigator determines target site of protein</td>
<td>Immune system determines target site of protein</td>
</tr>
<tr>
<td>Molecular modifications</td>
<td>Wide variety of chemical modifications to molecule for diverse functions</td>
<td>Limited modifications of molecule</td>
</tr>
<tr>
<td>Temperature stability</td>
<td>Return to original conformation after temperature insult</td>
<td>Temperature sensitive and undergo irreversible denaturation</td>
</tr>
<tr>
<td>Shelf-life</td>
<td>Unlimited shelf-life</td>
<td>Limited shelf-life</td>
</tr>
<tr>
<td>Immunogenicity</td>
<td>No evidence of immunogenicity</td>
<td>Significant immunogenicity</td>
</tr>
<tr>
<td>Cross-reactive compounds</td>
<td>Cross-reactive compounds can be isolated utilising toggle strategy to facilitate pre-clinical studies</td>
<td>No method for isolating cross-reactive compound</td>
</tr>
<tr>
<td>Specific antidote</td>
<td>Aptamer-specific antidote can be developed to reverse the inhibitory activity of the drug</td>
<td>No rational method to reverse molecules</td>
</tr>
</tbody>
</table>

### 2.2.2 Transducers

Transducers can be categorised into two main approaches: label-based and label-free. The transducers that have reporter molecules incorporated into the transduction system are categorised as the label-based approach. Meanwhile, the transducers that operate without reporter molecules are categorised as the label-free approach. Figure 2-2 gives an overview of the classification of biosensors based on their transduction methods. As aptamers are used in the bioreceptor layer...
in this work, a table (see Table 2-2) summarising the existing aptamer-based biosensors is given to compare the performance of different sensing technologies.

![Flowchart illustrating an overview of biosensor categories.](image)

**Label-based approach**

Label-based approach exploits explicit properties of the incorporated reporter or signalling molecules. Reporter molecules are secondary molecules that are attached to the target molecules, converting the binding event to measurable signals detected by the detector. Reporter molecules with specific properties such as fluorescence, chemiluminescence, or radioactive are usually used to detect and
quantify the target molecules. The label-based approach is the most commonly applied approach in the optical-based transduction mechanism. Examples of optical transduction mechanism that employ the label-based approach are presented in the following.

**Fluorescent**

In the fluorescent detection method, the receptor is labelled with a fluorophore on one end and a quencher molecule at the opposite end. This approach exploits the conformational change of receptor molecule that occurs upon target binding. By incorporating signalling or reporter molecules into the receptor molecules, the target binding event can be detected. This method was demonstrated in [38] to detect L-argininamide using fluorescence resonance energy transfer method. A fluorophore and a quencher molecule are attached to the DNA aptamer 5'-end and 3'-end respectively. DNA aptamer formed a random structure without the presence of L-argininamide, leading to the detection of fluorescence signal. When L-argininamide was added, the fluorescence intensity decreased with increasing concentration of L-argininamide. This was due to the quenching of the fluorescence when target binds to the DNA aptamer domain caused by the stabilization of the stem-loop structure. This strategy has its drawback as not all receptor-target interactions produce adequate, if any, conformational change that results in a detectable signal from a molecular beacon.

Heyduk and Heyduk [26] introduced an improved molecular beacon design that can be implemented on a variety of target proteins. The authors implemented molecular beacon with bivalent thrombin aptamers to recognise two distinct
epitopes (G15D aptamer binds to heparin binding exosite while 60-18 aptamer binds to fibrinogen-binding exosite) in thrombin with higher affinity. The aptamers each contained short fluorophore-labeled complementary signalling oligonucleotides attached via a non-DNA linker. The two signalling oligonucleotides were brought in close proximity when the two aptamers associate with the protein resulting in a change in fluorescence signal.

Another variation of the fluorescent detection is reported by Li and Ho [39]. This system consists of a molecular recognition element (an unmodified aptamer specific to target) and a signal transduction element (a competitor oligonucleotide). The signaling oligonucleotide can be completely or partially complementary to the aptamer. In the absence of target, competitor oligonucleotide binds to aptamer. A change in optical readout is observed, when target binds to aptamer. This is due to displacement of competitor oligonucleotide which is the signal transduction element from aptamer. Both signal-on or signal-off scheme (see Figure 2-3) can be applied in this detection strategy.

Recently, quantum dots have been used as alternatives to organic fluorescent dyes due to their superior robustness, photostability and the ability to tune via size variations [40]. Liu et al. [41] introduced multiplexed sensing of adenosine and cocaine in a single solution using QD-encoded aptamer sensor (see Figure 2-4). The system is encoded with QDs of different emission wavelengths so that the identity of the analytes can be distinguished. QDs with emission at 585 nm are used for cocaine detection while QDs with emission at 525 nm are used for adenosine detection. AuNPs are also incorporated into the system to serve as
quenchers. Increased emission from QDs is observed when target analytes are added and disassembled the aggregates.

Figure 2-3: Fluorescent detection strategy based on top: signal-on; bottom: signal-off (Reprinted (adapted) with permission from [39]. Copyright 2008, American Chemical Society).

Figure 2-4: QD-encoded aptamer linked nanostructures for multiplex detection. (Reprinted (adapted) with permission from [41]. Copyright 2007, American Chemical Society).
Wang et al. [42] developed a biosensor operating based on surface enhanced Raman scattering with a sandwich type system. When target thrombin is immobilised on the electrode, one of its binding sites interacts with the thrombin aptamers immobilised on a gold electrode. Meanwhile, the gold nanoparticles labelled with thrombin aptamer and Raman reporters bind to the other binding site of thrombin target. Upon thrombin binding with aptamer, electromagnetic hot spots are generated on the AuNPs surface and Raman signals are amplified via deposition of silver nanoparticles on AuNPs surface. The work in ref. [43] gave a comprehensive review comparing the conventional and recent labelling molecules for fluorescent detection.

**Chemiluminescent**

Unlike the fluorescent method that requires a light source to excite the fluorophore, the chemiluminescent method does not involve an excitation light source. Instead, it uses chemically reactive species that are able to emit light upon oxidation. This transduction approach was applied by Li and Zhang [44] for the detection of D-amino acid in human serum. The detection limit was 0.45 µmol/l. D-amino acid oxidase immobilised onto an amine-modified silica gel was used as a catalyst for the oxidation of D-amino acid using oxygen, producing hydrogen peroxide. D-amino acid was then detected by the chemiluminescence reaction between hydrogen peroxide with luminol and ferricyanide. The chemiluminescence was converted into electrical signals using an R456 photomultiplier tube. The same transduction principle was implemented for lactid
acid sensing using natural animal tissue porcine kidney as the bioreceptor layer [45]. The lactid acid detection limit was 0.2 µmol/l.

Another variation of the chemiluminescent method is the electrogenerated chemiluminescence or also known as ECL. ECL involves the generation of species at electrode surfaces and then undergo electron-transfer reactions to form exited states that emit light [46]. This transduction method was employed by Zhang et al. [47] for the detection of target ssDNA, and the obtained detection limit was 90 pmol/l. Hairpin DNA was tagged with ruthenium complex and immobilised on the surface of a gold electrode via probe-thiol-Au linkage. In the absence of target, a strong ECL signal was measured at the electrode as the ECL probe was in folded configuration bringing its termini in close proximity to the electrode. The ECL intensity reduced as the target ssDNA increased due to the conformational change of the ECL probe from a stem-loop into a linear double-helix configuration. One major disadvantage of the chemiluminiscent method is the limited lifetime of the biosensor. This is because the reactive species employed for light generation can only be regenerated for a limited period.

**Radioactive**

In this radioactive method, radioactive labels are used to detect target molecules [48]. This method has several drawbacks such as high handling and disposal cost, short half-life, and slow sample processing [49]. Therefore, this method has been replaced by non-radioactive labels such as enzyme-based or fluorescent method.
Label-free approach

Labelling or tagging could alter the properties of the host molecule. This could impact the binding affinities and associated kinetics of protein molecules [50] which is undesirable especially under low-concentration detection. Unlike label-based approach whereby reporter molecules are tagged to the receptors molecules, no labels or reporter molecules is involved in the label-free biosensing approach. This leads to cost and time saving. The label-free approach can be classified into different groups such as optical, electrochemical, impedance, field-effect transistor, mass-sensitive, and micromechanical based on the transduction physics. The existing works for each of the groups are described in the following.

Optical

The surface plasmon resonance [51-54] is a label-free optical transduction technique. This method measures the variations in the reflective angle upon adsorption of molecules on the metal surface. Information on the affinity and kinematics of biomolecular interaction is obtainable using this technique. For example, the interfacial binding characteristics between thrombin molecule and 15-mer thrombin DNA aptamers had been studied using the SPR technique [55]. Kinetic measurement mode was implemented, whereby the angle or angle shifts are monitored over time during molecular adsorption on the gold-coated glass substrate. The shift angle corresponds to the amount of adsorbed molecules on the surface. Wang et al [56] implemented a curvette-based SPR to characterise the interaction between IgE and aptamer. Streptavidin and anti-IgE antibody were introduced during sensing to amplify the SPR signals. Detection of *B. anthracis*
spores was demonstrated using a commercial SPR sensor, Biacore® 3000 using a subtractive inhibition assay by Wang et al. [57]. In the developed method, cells and antibodies were pre-incubated, followed by the removal of cell-bound antibodies. Biacore® biosensor was then used to quantify the remaining free antibodies related to the initial cell concentration. This subtractive method claimed to improve SPR sensitivity when detecting large cells. This is because large cells do not cover the sensor surface uniformly and prevent effective penetration depth of evanescent field. An eight-channel SPR sensor based on spectral modulation and wavelength division multiplexing is developed by Ostatná et al [53]. These sensing channels were formed by combining the wavelength division multiplexing of pairs of sensing channels with four parallel light beams.

Optical elipsometry is another label-free optical transduction method. Similar to the SPR technique, elipsometry measures the thickness and density of materials on a flat solid substrate. This technique measures the differential changes in phase and magnitude of the reflectivity for p-polarised and s-polarised components of a monochromatic light [58]. Unlike the SPR technique, which requires a thin metal layer coated on a transparent substrate for the SPR angle measurement, the elipsometry technique does not require any special requirements on the solid substrates. Elipsometry is compatible with all glass slides substrate and any other transparent solid substrates.

Absorption spectroscopy is another example of label-free optical transduction employed in sensing. In absorption spectroscopy [59], the intensity
of a beam of light is measured before and after interaction with the analyte. Chandrasekaran and Packirisamy [60] demonstrated absorption spectroscopy for the detection of enzymatic reaction in a lab-on-a-chip device. The authors used a pulsed xenon light source at 470 nm and a broadband light source for wavelengths from 300 to 700 nm to illuminate the sample. A collector fibre output the light from the sample to a spectrometer to study the spectral variations and absorption characteristics due to the enzymatic reaction.

Instead of using wavelength dependent light source, Minas et al. [61] introduced an optical absorption based detector using white light illumination. This was achieved by incorporating an optical filtering system and a detection and read-out system on-board of a lab-on-a-chip system. The optical filtering system is based on highly selective Fabry-Perot optical resonators fabricated using a stack of CMOS process compatible thin-film layers. The optical filters filter the white light to a narrow spectral band centred at the wavelength at which the sample has its absorption maximum. The underlying photodetectors measure the intensity of the selected spectral component. The optical intensity is proportional to the biomolecule concentration.

Ho and Leclerc [62] demonstrated a label-free fluorescent and UV-vis absorption transduction method for human \(\alpha\)-thrombin in femtomole range. A water-soluble, cationic polythiophene derivative acts as a ‘polymeric stain’ was used as the reporting element, converting the binding of aptamer to its target into colorimetric and fluorometric signal (see Figure 2-5). No tagging on the probes or analytes involved in this technique. It is based on different electrostatic
interactions and conformational modifications of the conjugated backbone of a cationic poly (3-alkoxy-4-methylthiophene) derivative upon mixing with anionic single-stranded oligonucleotides and a target protein. The poly (3-alkoxy-4-methylthiophene) displayed colour changes and optical changes when complexed to ssDNA or dsDNA. An aqueous solution of poly (3-alkoxy-4-methylthiophene) is yellow with a maximum absorption at 402 nm wavelength. The colour turned to red when poly (3-alkoxy-4-methylthiophene) in the presence of ssDNA. When α-thrombin molecule bound to the poly (3-alkoxy-4-methylthiophene)-ssDNA complex, the colour changed into orange. The orange colour generated during the formation of 1:1:1 complex between polymer, oligonucleotide ssDNA aptamer, and thrombin yields less fluorescent (measured using spectrofluorometer) compared to the yellow form (only poly (3-alkoxy-4-methylthiophene) in the solution) but more fluorescent than the red-violet form (poly (3-alkoxy-4-methylthiophene), thrombin and non-specific thrombin aptamer), thus a sensitive dual modality detection system.

Colorimetric detection of thrombin based on aptamer-AuNPs conjugates using a dot-blot assay can be used as an alternative mode of reporting of the binding events [15]. AuNPs change colour from colourless to red upon thrombin-aptamer binding and the changes is visible to the naked eye.
Micro ring resonator is also a type of label-free optical transducer. It is made of a closed loop waveguide coupled to one or more input or output waveguides that measure the change in the refractive index. Optical biosensor based on silicon-on-insulator micro-ring cavities was devised by Vos et al. [63] for the detection of different avidin concentrations. SOI material was chosen due to its high refractive index contrast. The SOI was coated with a layer of biotin molecules. Immersion of the device in different avidin concentration caused the shift of resonance wavelength.

**Electrochemical**

A three electrodes setup (working electrode, auxiliary electrode and reference electrode) is used in the electrochemical detection. The working electrode is dipped into the solution containing the analyte. A voltage is applied to facilitate
the electron transfer to and from the analyte. Meanwhile, the reference electrode is used as a reference in measuring and controlling the working electrode and the auxiliary electrode provides all the current needed to balance the current observed at the working electrode. Addition of electrolyte in the system provides a conductive environment. Detection of analyte of interest is achieved by measuring current or voltage changes generated by the receptor-target interactions at the surface of the working electrode. Simple, fast and inexpensive sensing platform as well as simultaneous multi-analyte detection makes the electrochemical an attractive transducer. Electrochemical biosensors can be categorised into three main categories based on the type of measurement taken between analyte of interest and the electrode surface. The categories consist of amperometry which measures the current produced during oxidation or reduction with a constant voltage, voltammetry that measures the current with a constant or varying potential at electrode’s surface while potentiometry measures the potential of the electrolyte solution between the working and reference electrodes.

Enzymes or redox enzymes, inorganic or organic catalysts and nanoparticles are usually employed in this technique. Commonly used electroactive molecules are ferricyanide ([Fe(CN)₆]⁴⁻/³⁻) [29, 64, 65], ferrocene [66, 67], methylene blue (MB) [68, 69] and bis-anthraquinone-modified propanediol [70]. Electroactive-labelled electrochemical detection exploits the conformational change of receptor molecule structure upon binding to its target. Signal-on or signal-off architecture can be implemented for detection. As shown in Figure 2-6, in the signal “on” architecture, the electroactive end is distant from the electrode
surface, limiting the electron transfer. Electron transfer increases upon the target binding due to the conformational change of aptamer bringing the electroactive label to the surface. On the other hand, the signal “off” architecture yields a lower electron transfer upon target binding as the addition of a target disassociates the initially attached redox-tag.

Figure 2-6: Schematic representation of electrochemical detection with signal-on architecture (Reprinted (adapted) with permission from [68]. Copyright 2006, American Chemical Society).

In the work presented by Li et al. [64], small molecules such as adenosine are detected using part of the complementary aptamer strand in electrochemical impedance spectroscopy. This approach eliminates the dependence on conformational change of aptamers. Negatively charged adenosine-binding aptamer attached on the surface repels the negatively charged probe ([Fe(CN)₆]⁴⁻/₃⁻-anions. This causes high impedance that hinders the interfacial electron-transfer kinetics of redox probes. Impedance is reduced upon adenosine binding by releasing its negatively charged aptamer-target strand. The sensitivity of
electrochemical detection can be enhanced for measuring target protein down to femtomolar concentration by incorporating polymerase chain reaction as proposed in [71].

**Field-effect transistors**

Ion-sensitive field effect transistors, which were initially used for measuring pH [72, 73], have been applied to biomolecular interactions detection. ISFET is an attractive sensing device employed for biological detection due to its robustness, small size, and cost effectiveness. Electrical sensing using field-effect transistor is an example of label-free sensing approach. In this system, a reference electrode is exposed to the analyte solution. A thin insulating layer forms a channel that separates the source and drain deposited on silicon layer. The interfacial electron transfer resistance is controlled by the formation of ligand-receptor complex. Hence, target molecules are identified by measuring the gate potential variations. Figure 2-7 illustrates an example of an ISFET-based sensor employed in adenosine detection [9].

Instead of using external reference electrode in ISFET-based biosensor, Cid et al. [13] employed a network of single-walled carbon nanotube as the transduction layer. The use of CNTs in FET fabrication is favourable because it exhibits superior performance in terms of transconductance and sub-threshold slope. An biosensor based on aptamer-modified CNT-FETs for immunoglobulin E detection has been developed by Maehashi et al [74]. Linker molecules are used to covalently bind 5′-amino-modified IgE aptamers to the CNT. Alternately, So et al [11] modified the side wall of the CNT with carbodiimidazole (CDI)-Tween for
thrombin detection. Hydrophobic interactions bring Tween to the side walls while 3'-amine group of the thrombin aptamer is covalently bound to CDI moiety. The formation of aptamer-target complex shields the negative charges of aptamer, producing an increased height of Schottky barrier between the metal electrodes and the CNT channel and thus leading to a decrease in the electrical response.

Figure 2-7: Schematic diagram of an ISFET-based sensor (Reprinted (adapted) with permission from [9]. Copyright 2006, American Chemical Society).

**Impedance**

Electrical impedance is defined as the ratio of voltage to its corresponding current. The change of capacitance is commonly measured in impedance-based biosensors and it is known as capacitive biosensors. In capacitance-based biosensors, the dielectric properties change when target analyte bound to the bioreceptors immobilised on the electrode structures. A high-frequency impedance sensor was
reported by Lohndorf et al. [75, 76] for the detection of thrombin target using RNA α-thrombin aptamer. The sensor is 20×20 mm in dimension and its substrate is made up of borosilicate glass wafers to reduce electronic losses when operating at high frequency (1.28 GHz). It consists of 14 individual sensor elements whereby each sensor consists of eight small capacitors with a dimension of 2×6 µm and a 68 nm gap. In order to match the required impedance, four serial-connected capacitor pairs are connected in parallel. The nonspecific binding events and crosstalk from the impurity of the analyte and buffer are rectified using a reference sensor.

An integrated CMOS capacitive biosensor (see Figure 2-8) that is capable of detecting target molecules in sub-nM range was devised by Wang and Lu [77]. The capacitive biosensor consists of interdigitated electrodes with sub-micrometer gap covered with inter-metal dielectric layer and a buffer amplifier circuit. The electrodes have a thickness of 0.64 µm and the dielectric layer underneath the microelectrodes has a thickness of 4.4 µm acts as the sensing interface. When molecules bound to the sensing interface, the capacitance change occurred and was detected by the integrated continuous-time sensing circuit. The capacitance change was in the atto-farad range. A more detailed discussion on interdigitated array microelectrodes based impedance sensor was presented in ref. [78] A more extensive literature on impedance based sensing was reviewed in ref. [79].
Mass-sensitive

Mass-sensitive-based biosensor measures the changes in mass on the surface during formation of receptor-target complex. It operates based on piezoelectric effect and is effective in determining protein affinity on functionalised surfaces. One example of mass-sensitive-based sensing is the acoustics-based sensor, quartz crystal microbalance devised by Yao et al [80] to detect IgE. An electrical potential difference is generated between deformed piezoelectric surfaces when a pressure is exerted on a small piece of quartz. A detection limit of 2.5\(\mu\)g/l IgE in 5 min is achieved. To improve the sensor sensitivity, Lee et al [81] increased the amount of analyte molecules bound to the surface by implementing zinc oxide nanorod-grown QCM. Surface acoustic wave is another example of mass-sensitive-based sensor. Interdigital transducers are employed to produce and detect acoustic waves at the guiding layer at the surface of the substrate. Detection of biomolecular binding is performed by measuring the frequency or phase.
change that corresponds to the mass on the surface. SAW love-wave sensor implements shear horizontal waves guided in a layer on the sensor surface to reduce energy dissipation of the acoustic wave in order to increase surface sensitivity. This detection method is employed in the detection of thrombin binding [82].

**Micromechanical**

Micromechanical-based sensing is very promising in chemical sensing and biological sensing applications as it offers exquisite mass resolution, posses good force responsivity due to reduction of its dimensions and fast response time [83].

Microcantilever sensor [84] or also known as surface stress sensor is also categorised under micromechanical-based sensor. The microcantilever can be operated in two different modes: static [85, 86] and dynamic [87]. The microcantilever can generate various responses such as frequency and amplitude change in dynamic mode and change of beam displacement in static mode upon target molecule detection. In the static mode, one side of the microcantilever is functionalised with bioreceptors for detection of analyte of interest. Surface stress is generated when target analyte adsorbs onto the functionalised surface. The difference in surface stress between the top and bottom surface of the microcantilever produces bending. The bending can be upwards (positive) or downwards (negative) depending on the type of molecular interactions involved. Meanwhile, in the dynamic mode, one side or both sides of the microcantilever beam can be immobilised with biomolecules for target molecule detection. When
target molecules bind to the immobilised surface, the mass on the sensor surface will increase leading to the change in frequency.

Table 2-2: A summary of the existing aptamer-based biosensor performance.

<table>
<thead>
<tr>
<th>Transduction Mechanism</th>
<th>Target analyte</th>
<th>Receptors</th>
<th>Reporter Molecule</th>
<th>Limit of Detection</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical</td>
<td>Thrombin</td>
<td>ssDNA aptamer</td>
<td>Cationic polythiophene</td>
<td>2×10⁻¹⁵ mol</td>
<td>[62]</td>
</tr>
<tr>
<td></td>
<td>Thrombin</td>
<td>DNA aptamer</td>
<td>Molecular beacon</td>
<td>50 pM</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>L-argininamide</td>
<td>DNA aptamer</td>
<td>Molecular beacon</td>
<td>-</td>
<td>[38]</td>
</tr>
<tr>
<td></td>
<td>C-reactive protein</td>
<td>RNA aptamer</td>
<td>N/A</td>
<td>0.005 ppm</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td>Cocaine</td>
<td>DNA aptamer</td>
<td>Quantum dots and AuNPs</td>
<td>120 μM</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>Adenosine</td>
<td>DNA aptamer</td>
<td>Quantum dots and AuNPs</td>
<td>50 μM</td>
<td></td>
</tr>
<tr>
<td>Electrochemical</td>
<td>Cocaine</td>
<td>DNA aptamer</td>
<td>Methylene blue (MB)</td>
<td>10 μM</td>
<td>[68]</td>
</tr>
<tr>
<td></td>
<td>Theophylline</td>
<td>RNA aptamer</td>
<td>Ferrocene (Fc) redox probe</td>
<td>0.2–10 μM</td>
<td>[88]</td>
</tr>
<tr>
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<td>Thrombin</td>
<td>DNA aptamer</td>
<td>Ferrocene (Fc) redox probe</td>
<td>2 nM</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td>Thrombin</td>
<td>DNA aptamer</td>
<td>Ferrocene (Fc) redox probe</td>
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<td>[67]</td>
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<td>Ferrocene (Fc) redox probe</td>
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<td>Methylene blue (MB)</td>
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<td>[91]</td>
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<tr>
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<td>Thrombin</td>
<td>DNA aptamer</td>
<td>Guanine-rich secondary aptamer</td>
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<td>Thrombin and lysozyme</td>
<td>DNA aptamer</td>
<td>Quantum dots (CdS and PbS)</td>
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<td>[92]</td>
</tr>
<tr>
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<td>Thrombin</td>
<td>DNA aptamer</td>
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<td>10 nM</td>
<td>[11]</td>
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<td>Anti-thrombin aptamer</td>
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<td>330 pmol/l</td>
<td>[93]</td>
</tr>
<tr>
<td>Transduction Mechanism</td>
<td>Target analyte</td>
<td>Receptors</td>
<td>Reporter Molecule</td>
<td>Limit of Detection</td>
<td>Ref.</td>
</tr>
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<td>-------------------</td>
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</tr>
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<td>IgE</td>
<td>DNA aptamer</td>
<td>N/A</td>
<td>250 pM</td>
<td>[74]</td>
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<td>DNA thrombin aptamer</td>
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<td>72 ±11 pg/cm²</td>
<td>[82]</td>
</tr>
<tr>
<td></td>
<td>Rev peptide</td>
<td>RNA aptamer</td>
<td>N/A</td>
<td>77 ± 36 pg/cm²</td>
<td>[82]</td>
</tr>
<tr>
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<td>IgE</td>
<td>DNA aptamer</td>
<td>N/A</td>
<td>2.5 µg/l</td>
<td>[80]</td>
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<td>NS3 RNA aptamer</td>
<td>N/A</td>
<td>Nanomolar</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>Thermus aquaticus (Taq) DNA polymerase</td>
<td>DNA aptamer</td>
<td>N/A</td>
<td>-</td>
<td>[94]</td>
</tr>
<tr>
<td></td>
<td>HCV helicase</td>
<td>RNA aptamer</td>
<td>N/A</td>
<td>100 pg/ml</td>
<td>[95]</td>
</tr>
</tbody>
</table>

**2.2.3 Discussion**

Based on the presented literature, label-based biosensors are found to be inapt for point-of-care or clinical diagnostics. This is because the receptor molecules or target molecules require labelling with secondary molecules before detection. This additional step is cumbersome, involves additional cost, requires extra sample preparation steps, and is time consuming. Furthermore, the addition of labels to either the receptor or target molecules could alter the molecules binding affinities and their respective structure. This can modify the molecule’s original behaviour, functionality, and subsequently affect the stability. Sample lost can also occur during sample preparation which is undesirable particularly when the sample amount is limited.

As such, label-free approach is more suitable to meet the demands of high-throughput analysis in real-world applications. There are various label-free sensing options such as optical, electrochemical, impedance, mass-sensitive, and
micromechanical transduction method. Impedance and electrochemical techniques are simple and can easily include on-board electronics and circuitry using standard CMOS processes. The limit of detection of electronics-based sensing techniques is however not comparable to optical methods. Optical transduction methods are on the other hand too bulky to be employed for point-of-care diagnostics as additional equipment are required to convert the optical signals into electrical signals.

Microcantilever-based sensors are considered the ideal sensing platform due to several key advantages [96]. The advantages include small overall dimensions that increase the sensor portability, low-power consumption especially in static mode operation, low cost, ease of mass production using standard silicon processing technologies, highly sensitive and capable of parallel processing via microarray format which leads to rapid analysis. Therefore, the microcantilever is chosen as the transduction mechanism in this work. The following section presents a detailed description of state-of-the-art microcantilever-based sensors and the various read-out methods.

2.3 Microcantilever based sensor

Microcantilever sensors convert biological signals into a mechanical motion. The advantages of this sensing technique include simplicity, low sample volume, ease of integration with microfluidic devices, high sensitivity, and cost-effectiveness.
2.3.1 Modes of operation

The principle of detection is based on the transduction of the adsorption of target molecules on the immobilised receptor layer into the deflection of the beam (static mode) or resonant frequency shift (dynamic mode) (see Figure 2-9). In dynamic mode, resonance frequency [95, 97, 98] of the beam is measured. Meanwhile, the microcantilever displacement is detected in static mode. Resonance frequency, $f$ of an oscillating microcantilever beam can be written as:

$$f = \frac{1}{2\pi} \sqrt{\frac{k}{m}}$$  \hspace{1cm} (2.1)

where $k$ is the spring constant of the microcantilever beam and $m$ is the effective mass of the microcantilever beam. In the dynamic mode, the resolution of the system is determined by the quality factor [99]. Quality factor is defined as the ratio between mechanical energy accumulated and dissipated per vibration cycle.

In liquid environment, the quality factor reduced significantly due to damping effect of the viscous surrounding. This subsequently reduces the overall sensitivity of the sensing device.

Each of the methods has its own advantages and drawbacks. Static mode operation often produces better sensory performance compared to the dynamic mode in liquid environment due to the damping effects [100] of liquid on the beam vibration frequency. Apart from that, the dynamic mode sensing is often limited by the weight of the target molecules. Target molecules with very low molecular weight might not result in any frequency change in the sensing system
The static deflection of the microcantilever beam is caused by a gradient of mechanical stress generated in the beam. This stress is originated from the biomolecular interactions arisen during the binding/adsorption of target molecules on the coated surface of microcantilever. The biomolecular interactions may involve van der Waals interaction, electrostatic, hydrogen bonding, hydrophobic forces or steric hindrances. Depending on the type of interactions involved, compressive or tensile surface stress is generated causing the beam to bend downwards (compressive) or upwards (tensile). The relationship between the differential surface stress, $\Delta \sigma$ and cantilever deflection, $\Delta z$ is depicted in Stoney’s equation [102]:

$$\Delta \sigma = \frac{E t^2}{3(1 - \nu) l^2} \Delta z \quad (2.2)$$

whereby $E$ is the Young’s modulus of the cantilever, $t$ is the thickness of the microcantilever, $\nu$ is the Poisson ratio and $l$ is the cantilever length.
2.3.2 Readout methods

The displacement of the static beam is detected by using readout techniques such as the optical, piezoresistive, capacitive or MOSFET methods. The details of each read-out method are presented in the following sub-sections. The performance of the existing microcantilever-based sensors is summarised in Table 2-3.
Optical

Optical read-out system mainly consists of a laser and a photodetector unit. The optical read-out method is widely applied in detecting microcantilever deflection. This read-out method can be divided into two categories: optical lever and optical interferometry. The optical lever method [104-109] (see Figure 2-10) involves a laser diode focused at the free-end of the microcantilever beam. The reflected light is sensed by a four-segmented photodiode or a position-sensitive detector which translates the light energy into electrical signals. The displacement of the microcantilever free-end induces a change in the position of the reflected light beam, and subsequently changes the level of light intensity on the photodiodes or PSD. Therefore, the magnitude deflection of the beam can be determined. This detection method is advantageous as it is able to detect extremely small changes in the microcantilever bending but it is bulky, expensive and the alignment involves is complex and tedious. Differential measurements can be implemented by using a reference cantilever which is chemically inert. This is to eliminate interference from non-specific binding and environmental effects.

Detection of deflection of microarray of microcantilevers using the optical lever method has also been demonstrated. The serial time multiplexed optical beam method has been implemented for DNA hybridisation [110], protein assay [111] and detection of antibiotic vancomycin-mucopeptide binding [24]. In the work reported in ref. [110, 111], an array of eight identical silicon microcantilevers with 250 µm pitch is mounted at angle 11° toward the incoming laser beam. An adjustable mirror is used to redirect the beam to the detector.
To measure the deflection of all eight microcantilevers, a combination of time-multiplexed vertical-cavity surface-emitting lasers with regulated power supply and adjustable optics was used. Beam deflection for each cantilever was detected by a linear position-sensitive detector. On the other hand, Kim et al. [5] implemented a parallel charge-coupled device camera-based detection system for measurement of an array of silicon nitride microcantilevers for antibody-peptide interaction application. By defocusing the laser beam with respect to the microcantilever plane, several microcantilevers can be illuminated by using only one laser source. The employed CCD camera has an active area of $6.47 \times 4.83 \text{ mm}^2$ which can be divided into several regions of interests. An algorithm then calculates the position of intensity centroid of the beam residing inside each region of interest.
Interferometry [112, 113] is an alternative optical detection method that is highly sensitive which is capable of measuring deflections as small as 0.01 Å. This technique is based on constructive and destructive interferences between a reference laser beam and the laser beam reflected off the microcantilever. There are different types of optical interferometry configurations found in the literature such as diffraction gratings [113, 114], phase shifting interferometry [115, 116], and Fabry-Perot [117]. An example of the diffraction interferometry approach is the work by Savran et al. [113]. They developed two silicon nitride microcantilevers (1 µm thick) that are placed side by side and supported by a 10 µm thick L-shaped structures. Interdigitated fingers (shown in Figure 2-11 (a)) were placed between the supports and microcantilevers to form diffraction gratings. To prevent microcantilever warping and reduce bending of the fingers, the sections at the end of the microcantilevers where the fingers were positioned were made thicker than the flexible part of the microcantilevers. When a laser beam was directed to the fingers, the reflected light forms a pattern composed of several modes whose intensities depend on the vertical distance between the two finger sets. By measuring the intensity of a single mode using a photodetector, the microcantilever displacement could be determined.

Recently, a curved semitransparent SU-8 cantilever (see Figure 2-11(b)) was used in phase shifting interferometry [116]. Interference pattern in microscope image of the microcantilever device was analysed to measure the microcantilever displacement. The interference pattern was formed by laser light reflected off the cantilever and laser light reflected off the substrate. The distance
of the microcantilever from the substrate determines the number and position of interference fringes. A horizontal fringe shift will occur when the microcantilever beam deflects, and this deflection was detected and calculated via an automated algorithm. However, this technique suffers poor temporal resolution due to the slow acquisition of digital images.

Figure 2-11: Example of the interferometry optical read-out. (a) Diffraction grating formed by interdigitated fingers between the supports and microcantilevers (Reprinted with permission from [113]. Copyright 2003, American Institute of Physics.). (b) Schematics showing: (i) phase shifting interferometry using curved SU-8 cantilever, (ii) interference pattern, (iii) microscope image of interference pattern and (iv) intensity profile measured from cantilever image (solid line) and a least squares curve fit with $R^2 = 0.94$ (dashed line) (Reprinted from [116] Copyright 2010, with permission from Elsevier).
The advantages of the optical read-out method include: high sensitivity, absolute displacement is obtainable and linear response. However, the method requires elaborate optical setups in addition to tedious alignments. In optical level method, the laser source needs to be carefully aligned to the tip of the microcantilever and the photodiode needs to be aligned to the reflected laser beam.

**Piezoresistive**

The piezoresistive read-out method involves the embedding of piezoresistive material onto the cantilever during its fabrication. During microcantilever bending, the piezoresistive layer was subjected to tensile or compressive stress. This change of stress will cause a strain to the piezoresistor leading to a change of resistance that can be measured using a dc-biased Wheatstone bridge (see Figure 2-12 (a)). The output voltage from the Wheatstone bridge is expressed as:

\[ V_{out} = \frac{1}{4} \frac{\Delta R}{R} V_{in} \]  

Typically, a pair of microcantilever that is placed side by side is used during measurement. One of the microcantilevers is coated with bioreceptors for target molecule recognition while the other is blocked to prevent non-specific binding serving as a reference cantilever. This differential measurement setup is used for noise cancellation. This read-out method has been employed in ssDNA immobilisation [118], DNA hybridisation [119], antigen-antibody binding experiment [20], detection of trimethylamine (an indicator of food freshness) [120] as well as sugar detection [121].
Both doped single crystalline silicon and doped polysilicon exhibit excellent piezoresistive coefficient, and they are the commonly used in fabrication of piezoresistor in the CMOS process. A piezoresistive microcantilever (see Figure 2-12 (b)) fabricated by Wee et al. [20] consisted of five layers with boron doped polysilicon as the piezoresistive layer. A SiO$_2$ layer was used as an insulation layer while a silicon nitride layer was deposited on the microcantilever topside and backside to act as the encapsulation layer. A thin layer of gold deposited on top of the microcantilever served as the immobilisation layer for target-receptor binding. Meanwhile, Yang et al. [120] used a boron doped single crystalline silicon as the piezoresistive material sandwiched in-between two silicon dioxide layers for their microcantilever design. The bottom SiO$_2$ layer functioned as an insulation layer while the top layer functioned as a passivation layer. SiO$_2$ was deposited using plasma enhanced chemical vapour deposition, and was used to increase the sensor sensitivity due to its low stiffness. To further improve the sensitivity, SU-8 could be used in place of silicon as SU-8 has a lower Young’s modulus compared to silicon [122]. Besides that, it also has the advantages of simple fabrication steps and a cheaper alternative to optical detection.

The microcantilever geometry plays a role in determining the sensor sensitivity. Low aspect ratio piezoresistive microcantilevers good for surface-stress loading in chemical and biological applications [123, 124]. The piezoresistive read-out method has the advantage of ease of integration of readout
electronics on chip but suffers from poor sensitivity, non-linearity in piezo
response and thermal drifts [99].

Figure 2-12: Examples of microcantilever sensor with piezoresistive read-out. (a) The
left-hand side figure shows the arrangement of four integrated piezoresistors placed in
a Wheatstone bridge configuration. The right-hand side shows the cross-section view
of the microcantilever and substrate (Reprinted from [118] Copyright 2003, with
permission from Elsevier).  (b) The left-hand side figure shows an image of a
micromachined piezoresistive thin-film self-sensing microcantilever array sensor,
while right-hand side shows the integration of the microcantilever sensor in a
polydimethylsiloxane (PDMS) microfluidic cell (Reprinted from [20] Copyright 2005,
with permission from Elsevier).

Capacitive

The microcantilever acts as one of the parallel conductor plates of a capacitor in
the capacitive read-out method. The change of capacitance that occurs during
microcantilever bending is measured. Upon the microcantilever displacement, the
gap distance between the two plates is changed. This results in a change of the capacitance.

Britton et al. [125] applied the capacitive read-out method for chemical sensing of hydrogen and mercury vapour using a microcantilever sensor. For hydrogen detection, the microcantilever is selectively coated with palladium while microcantilever was gold coated for mercury sensing. Each cantilever was Π-shaped with two legs, 150 μm (long) × 100 μm (wide) × 2 μm (thick), joined to a capacitive cross plate, 112 μm (long) × 500 μm (wide), and anchored at the base of the legs 2 μm above the baseplate. Hydrogen levels down to 100 ppm can be detected. On the other hand, Satyanarayana et al. [126] (see Figure 2-13(a)), Cha et al. [127] and Tsouti et al. [128] used membrane instead of a free-end microcantilever with capacitive read-out for biomolecule detection. Membrane design is more suitable for applications in biological solution because it is able to seal the capacitor electrodes from the biological liquid. This then prevent faradaic current at the electrodes and eliminate the variations in the dielectric constant in the medium.

To increase sensor sensitivity, Sivaramakrishnan et al. [129] developed electrically stretched capacitive membranes to be used in their capacitive stiffness sensor (see Figure 2-13(b)). In this work, the membrane response to stiffness changes is enhanced by using an electrostatic actuation to induce a curvature on the membrane. The presence of the target analyte would change the membrane stiffness leading to a change of membrane curvature. This change in curvature can be monitored by measuring the change in capacitance. Electret microphones
membrane that is capable of trapping and storing charges was used in their sensor fabrication. It was coated with a single-walled nanotube film forming the recognition layer for detection of carbon dioxide. The advantage of this read-out method is its high sensitivity, absolute displacement, simple electronics circuitry, and compliant with CMOS technology which makes it easy to integrate in MEMS devices. However, it suffers from the interference problem such as variation in dielectric constant in different medium. This can be alleviated by performing differential measurement using a reference microcantilever.

Figure 2-13: Examples of microcantilevers with capacitive read-out. (a) Parylene membrane used for chemical sensing (Reprinted from [126] Copyright 2006, with permission from Elsevier). (b) Gas sensing using electrets membrane (Reprinted from [129] Copyright 2008, with permission from Elsevier). (c) Microcantilever with a square-shaped end to increase capacitance used for humidity sensing (Reprinted from [130] Copyright 2002, with permission from Elsevier). (d) 16 × 16 capacitive array matrix with ultra thin silicon membrane for DNA and chemical sensing (Reprinted from [131] Copyright 2008, with permission from Elsevier).
Metal oxide semiconductor field effect transistor

Similar to the piezoresistive read-out method, the MOSFET fabrication also involves embedding a component onto the microcantilever during its fabrication. In this technique, the transistor is embedded onto the microcantilever. In this read-out method, the beam displacement is detected by measuring the change of current between the source and drain. A fixed bias voltage is supplied to the gate and the source-drain region of the transistor. Upon the adsorption of the target analyte, surface stress is generated resulting in an increased in the channel resistance underneath the gate region. Subsequently, this alters the channel mobility of the transistor which leads to modulation of channel current.

In the work of Shekhawat et al. [132] the microcantilever was detected by embedding a MOSFET in the high-stress region of the microcantilever. The microcantilever was fabricated from silicon-on insulator while the embedded n-type MOSFET transistor was fabricated on each microcantilever by standard CMOS approach. The transistor was located at the rear of the microcantilever where it has the highest surface stress. The microcantilever surface was coated with a gold layer for the probe molecules immobilisation and a second uncoated microcantilever was used as a reference for differential measurement. The transistor and its contact pads were passivated with a thin coating of silicon nitride to shield them from any environmental influence.
Figure 2-14: Examples of the MOSFET read-out. (a) Operation of the embedded MOSFET cantilever system. The gold-coated cantilever bends upon target-receptor binding. When cantilever bends, the channel below the gate region is stressed resulting in the change of current due to the conductivity modulation (adapted from [132]. Reprinted with permission from AAAS). (b) SEM micrograph of a pair of identical MOSFET microcantilevers (Reprinted from [133] Copyright 2010, with permission from Elsevier).

The same read-out technique was also implemented [133] for force measurements in biomolecular detection. However, in this work, the microcantilever was fabricated on the non-standard (1 0 0) crystallographic direction of silicon to maximise the stress response of the transistor. This also resulted in a lower spring constant due to lower Young’s modulus of silicon in this direction. Unlike other
read-out methods, the MOSFET is less susceptible to noise. The MOSFET signal-to-noise ratio can be improved by reducing unwanted noise due to carriers via selecting localised doping regions on moderately resistive silicon microcantilevers, precisely controlling the doping region thickness, width and carriers to optimise mobility have a sharp dopant profile as well as designing a large gate area to suppress noise. It also has a better performance due to its sensitivity to local stress at channel surface.
Table 2-3: Summary of existing microcantilever-based sensors

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Ligand-Receptor/Chemical</th>
<th>Density</th>
<th>Beam Geometry (L×W×H) (µm)</th>
<th>Material Properties</th>
<th>Performance: Deflection* (Concentration)</th>
<th>Read-out Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>[107]</td>
<td>Biotin-streptavidin</td>
<td>(biotin-HPDP): 113.5 nm² per streptavidin (biotin-PEG): 68.7 nm² per streptavidin (biotin-SS-NHS): 118.5 nm² per streptavidin</td>
<td>220×20×0.6 (V-shaped)</td>
<td>SiNx $E=1.8 \times 10^{11}$ N/m², $ν = 0.25$, $k=0.03$ N/m</td>
<td>-150 nm (biotin-HPDP coated with 10 nM streptavidin) 0 nm (biotin-PEG coated with 10 nM streptavidin) 30 nm (biotin-SS-NHS coated with 10 nM streptavidin)</td>
<td>Optical</td>
</tr>
<tr>
<td>[110]</td>
<td>12-base DNA target</td>
<td>1.5×1010 DNA probe strands with 10% hybridisation efficiency</td>
<td>500×100×1 (rectangular)</td>
<td>Si, $E=168.5$ GPa, $ν = 0.25$, Spring constant, $k=0.02$ N/m</td>
<td>Differential measurement: -9.8 nm (500 nM)</td>
<td>Optical</td>
</tr>
<tr>
<td>[105]</td>
<td>Anti-PSA antibody</td>
<td>-</td>
<td>200×20×0.5 (V-shaped)</td>
<td>SiNx $E=1.8 \times 10^{11}$ N/m², $ν = 0.3$</td>
<td>-52 nm (60 ng/ml fPSA) -20 nm (6 ng/ml fPSA)</td>
<td>Optical</td>
</tr>
<tr>
<td>[104]</td>
<td>DNA</td>
<td>$6×10^{12}$ chains/ cm² with 60%-80% hybridisation efficiency</td>
<td>200×20×0.5 (V-shaped)</td>
<td>SiNx $E=1.8 \times 10^{11}$ N/m², $ν = 0.25$</td>
<td>-32 nm (immobilisation 50 ng/µl or 3.2 µM ssDNA (50-nt long)) Δ+15 nm after hybridisation with complementary ssDNA of 20-nt long)</td>
<td>Optical</td>
</tr>
<tr>
<td>Ref.</td>
<td>Ligand-Receptor/Chemical</td>
<td>Density</td>
<td>Beam Geometry (L×W×H) (µm)</td>
<td>Material Properties</td>
<td>Performance: Deflection* (Concentration)</td>
<td>Read-out Method</td>
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</tr>
<tr>
<td>[21]</td>
<td>Myoglobin-monoclonal antibodies</td>
<td>-</td>
<td>190×20×0.6 (V-shaped)</td>
<td>SiN, $E=1.8 \times 10^{11}$ N/m², $v=0.25$, $k=0.03$ N/m and 0.12 N/m</td>
<td>Differential measurement: -8 nm (85 ng/ml myoglobin)</td>
<td>Optical</td>
</tr>
<tr>
<td>[111]</td>
<td>Myoglobin and creatin kinase-antibodies</td>
<td>-</td>
<td>500×100×0.5 (rectangular)</td>
<td>SiO₂, $E= 70$ GPa</td>
<td>Differential measurement: -45 nm (50 µg/ml myoglobin) -50 nm (50 µg/ml creatin kinase)</td>
<td>Optical</td>
</tr>
<tr>
<td>[24]</td>
<td>Antibiotic vancomycin-mucopeptide (DAla/DLac)</td>
<td>$1 \times 10^{11}$ DAla per cantilever with a single footprint of 44 Å²</td>
<td>500×100×0.9 (rectangular)</td>
<td>Si, $E=168.5$ GPa, $v=0.25$,</td>
<td>Differential measurement: -8 nm (10 nM of Van) -29 nm (100 nM of Van) -114 nm (1000 nM of Van)</td>
<td>Optical</td>
</tr>
<tr>
<td>[108]</td>
<td>Physisorption Hydrogen bonding Chemisorption</td>
<td>$1.2 \times 10^{15}$ molecules/cm²</td>
<td>180×25×1 (V-shaped) Cantilever surface area = $8 \times 10^{-5}$ cm²</td>
<td>Si, $E=N/m^2$, $v=0.25$, $k=0.26$ N/m</td>
<td>-240±50 nm (Physisorption) -1000 nm (Hydrogen bonding) -4470 nm (Chemisorption)</td>
<td>Optical</td>
</tr>
<tr>
<td>Ref.</td>
<td>Ligand-Receptor/Chemical</td>
<td>Density</td>
<td>Beam Geometry (L×W×H) (µm)</td>
<td>Material Properties</td>
<td>Performance: Deflection* (Concentration)</td>
<td>Read-out Method</td>
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</tr>
<tr>
<td>[109]</td>
<td>Thiolated-ssDNA immobilisation</td>
<td>-</td>
<td>Si$_3$Ni$_4$: 200×20×0.5 (rectangular) SU-8 200×20×1.6 (rectangular)</td>
<td>Si$_3$Ni$_4$, $E$=190 GPa, $\nu$ = 0.25, $k$= 0.03 N/m SU-8, $E$=2.3 GPa</td>
<td>Si$_3$Ni$_4$: -400 nm (2 µM of ssDNA) SU-8: -2500 nm (2 µM of ssDNA)coated with 10 nM streptavidin)</td>
<td>Optical</td>
</tr>
<tr>
<td>[5]</td>
<td>Anti-myc-tag antibody with Myc-tag decapptide</td>
<td>-</td>
<td>200×40×0.5 (rectangular)</td>
<td>Si$_N$x</td>
<td>325 nm (13 nM of anti-myc-tag antibody)</td>
<td>Optical</td>
</tr>
<tr>
<td>[118]</td>
<td>ssDNA immobilisation</td>
<td>-</td>
<td>144×?×0.464 Thickness: Au-0.04 Si$_3$Ni$_4$-0.054 Si p++-0.15 Si$_3$Ni$_4$-0.22 (rectangular)</td>
<td>-</td>
<td>Minimum detectable surface stress = $1 \times 10^{-3}$ N/m</td>
<td>Piezoresistive</td>
</tr>
<tr>
<td>[122]</td>
<td>6-mercapto-1-hexanol</td>
<td>-</td>
<td>215×280×5 (rectangular)</td>
<td>SU-8, $E$=4.5 GPa</td>
<td>-2 µm (60 µl of 6-mercapto-1-hexanol)</td>
<td>Piezoresistive</td>
</tr>
<tr>
<td>Ref.</td>
<td>Ligand-Receptor/Chemical</td>
<td>Density</td>
<td>Beam Geometry (L×W×H) (µm)</td>
<td>Material Properties</td>
<td>Performance: Deflection* (Concentration)</td>
<td>Read-out Method</td>
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<tr>
<td>[121]</td>
<td>D-(-)-Fructose and 4-mercaptophenylboronic acid (4-MPBA)</td>
<td>-</td>
<td>120×35×0.5 (rectangular)</td>
<td>Si E=155.8 GPa ν=0.28</td>
<td>Sensitivity = 11.2 mN/m/mM</td>
<td>Piezoresistive</td>
</tr>
<tr>
<td>[119]</td>
<td>DNA hybridisation</td>
<td>$3 \times 10^{11}$ molecules/cm²</td>
<td>125×60×0.75 (rectangular)</td>
<td>-</td>
<td>Sensitivity = $3.5 \times 10^{-5}$ mN</td>
<td>Piezoresistive</td>
</tr>
<tr>
<td>[127]</td>
<td>16-mer DNA Thrombin-thrombin aptamer</td>
<td>$3 \times 10^{13}$-4×$10^{13}$ probes/cm²</td>
<td>Diameter: 500 (membrane)</td>
<td>PDMS: E=1 MPa, ν=0.5; Au: 78 GPa, ν=0.44</td>
<td>Central deflection: -20 nm, ΔC = 6fF (1 μM of DNA)</td>
<td>Capacitive</td>
</tr>
<tr>
<td>[126]</td>
<td>IPA and toluene vapours</td>
<td>-</td>
<td>Diameter: 300 (membrane)</td>
<td>Parylene: E=3.2 GPa, ν=0.4; Au: 80 GPa, ν=0.3</td>
<td>Hydroxyl terminated device show ΔC of 1.5% decrease (IPA) ΔC of 7% decrease (toluene)</td>
<td>Capacitive</td>
</tr>
<tr>
<td>Ref.</td>
<td>Ligand-Receptor/Chemical</td>
<td>Density</td>
<td>Beam Geometry (L×W×H) (µm)</td>
<td>Material Properties</td>
<td>Performance: Deflection* (Concentration)</td>
<td>Read-out Method</td>
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</tr>
<tr>
<td>[132]</td>
<td>Biotin-streptavidin Immunoglobulin G</td>
<td>-</td>
<td>250×50×1.5 (rectangular)</td>
<td>SiNₓ</td>
<td>5 nm</td>
<td>MOSFET</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sensitivity: 0.1-0.2 mA/nm</td>
<td></td>
</tr>
<tr>
<td>[133]</td>
<td>Force measurement for biomolecular detection</td>
<td>-</td>
<td>200×24×0.34 (rectangular)</td>
<td>Si E=130 GPa SiO₂ E=75 GPa Al E=70 GPa Si₃N₄ E = 250 GPa</td>
<td>Sensitivity: 25 µV/pN Resolution: 56 pN</td>
<td>MOSFET</td>
</tr>
</tbody>
</table>

* Downwards deflection is given as negative (compressive surface stress) whereas upwards deflection is indicated as positive (tensile surface stress)
2.3.3 Discussion

The microcantilever-based biosensor is a label-free biosensing approach that translates the biomolecular recognition event into mechanical signals. The generated mechanical signals are dependent on the microcantilever operation mode. The static mode produces absolute deflection of the microcantilever beam while the dynamic mode produces a change in resonance frequency upon target molecules recognition. In liquid environment, the static mode microcantilever is more sensitive compared to the dynamic mode microcantilever. This is because the dynamic mode suffers from damping effect that reduces its quality factor and hence the microcantilever sensitivity.

A read-out system is required to convert the mechanical displacement into measurable electrical signals in the case of static mode microcantilevers. There are various read-out methods reported in the literature including piezoresistive, capacitive, MOSFET, and optical. Each of the methods has its own advantages and disadvantages. The piezoresistive has the advantage of ease of integration with electronic circuitries but suffers from poor sensitivity, non-linearity in piezo response and thermal drifts. On the other hand, the capacitive read-out method has high sensitivity, simple electronic circuitry, capable of absolute displacement measurement but suffers from interference problems such as variation in dielectric constant in different medium. The optical read-out method such as the optical lever has high resolution, linear response, and is simple and produces absolute displacement measurement. These advantages are the motivating factors for this chosen read-out method to be employed in this work. However, the heat from the
laser could cause the microcantilever beam to deflect producing false positive deflection. To eliminate the error generated, a reference microcantilever can be employed.

2.4 Summary

Biosensors are widely applied in point-of-care diagnostics, drug screening, proteomic, and genetic analysis. The requirement to detect different molecules at increasingly low concentrations has motivated the miniaturization of these sensors to reduce sample volume.

Antibodies were previously the receptor molecules of choice until the development of aptamers. Aptamers are gaining widespread acceptance due to better binding specificity and binding affinity compared to antibodies. Aptamers are highly regarded as the emerging class of receptor molecules due to the stability, ease of production, and cost-effectiveness which leads to the development of aptasensors. There are many different types of sensing principles that have been proposed and developed. They can be categorised according to their sensing principle such as optical, electromechanical, impedance, mass-sensitive and micromechanical. Each has their own advantages and disadvantages.

Microcantilever-based sensors have been garnering a lot of interests due to their superior sensitivity and ease of fabrication compared to other sensing methods. However, existing microcantilever-based sensors are yet to be applied in real world applications as the system performance is limited by deflection detection mechanism. To improve this sensitivity, microcantilever sensor
geometries should be optimised and novel materials should be use for the
collection of the sensor. Further description of the working principles and
fabrication steps are discussed in the later chapters.
CHAPTER 3
THEORETICAL CONCEPTS

3.1 Background

The origin of surface stress is still not fully understood and remains a hot debate topic among researchers. Based on different studies carried out so far, the origin of surface stress is due to intermolecular forces [110, 134-136] occurring (hydrophobic, hydrophilic, steric hindrance, van der Waals, hydrogen bonding and electrostatic forces) between the molecules and/or surface reconstruction [137], or the covalent bond between the surface metal atoms and the linker molecule [138], or configurational entropy [104], or change of electronic charge density [139] or flexoelectric effect [140]. The type of intermolecular forces involved is dependent on the type of the molecule under investigation.

The measured information such as the beam deflection needs to be associated with the change in surface stress and hence, a theoretical model on the bending of thin beam caused by surface stress needs to be developed. However, the theoretical description of the beam bending mechanism in microcantilever sensors is lagging behind the experimental developments. This is collectively influenced by the complexity of the transducer system and the sensing molecules involved. The contributing factors include the material used for sensor fabrication, surface morphology and cleanliness of the immobilised surface as well as the
immobilisation procedures that affects the receptors’ orientation and density at the grafting layer [7]. Furthermore, the sensing molecules used for detection of target molecules may include some or all of the following activities: conformational changes, surface charge change or change of molecular density in the sensing layer which will affect the change of surface stress and subsequently the amount of deflection of the beam. In order to optimise the performance of the surface stress-based sensor, a robust model is needed. Thus, it is pertinent to investigate the underlying forces involved during molecular binding or in molecules adsorption on the sensor surface that governs the beam deflection and present them under a general mechanical framework.

The next section of this chapter summarises the findings of the origin of surface stress that causes the microcantilever displacement after molecular adsorption or target-receptor binding on its surface. Theoretical model of the microcantilever sensor is then given in the following section.

3.2 Related works

Stoney’s [102] experimental work in 1909 is the pioneer in the analysis of surface stress induced bending. To measure the residual stress in metallic thin films deposited by electrolysis, Stoney proposed the bending plate method. He observed that a metal film deposited on a thick substrate was in a state of compression or tension without any external loads applied to the system. This consequently strains the substrate to bend it. His method is based on the concept that a differential interfacial stress applied to the faces of an elastic plate will cause
deformation. His technique has become the basis in the measurement of surface stress by relating the curvature and stress in a film-substrate system. Since then, microcantilever beams have been extensively studied and used as a platform in chemical sensing [108, 136, 139, 141, 142] as well as biomolecular reactions [110, 134, 143, 144]. The work on this thesis involves protein detection and therefore the rest of this section focuses on the surface stress generated by biomolecular reactions.

Origin of surface stress in microcantilever deflection was investigated for DNA hybridisation. Fritz et al. [134] covalently immobilised synthetic 5′ thiol-modified 12-mer/16-mer oligonucleotides on the gold-coated side of a microcantilever. Injection of complimentary oligonucleotides into the liquid cell caused the microcantilever to bend. This was due to the difference in surface stress between the functionalised gold surface and non-functionalised Si surface. The increase of the number of charges in the molecular layer from the sugar-phosphate backbone of the oligonucleotides and the increase in the surrounding counterions during DNA hybridisation caused both repulsive electrostatics and steric interactions. This subsequently led to a compressive surface stress in the cantilever. McKendry et al. [110] suggested that the electrostatic repulsion after hybridisation are relatively small and the dominant component of the micromechanical bending signal is due to steric hindrance as the probe density on the surface is high.

The same compressive stress was also observed in the DNA hybridisation experiment conducted by Hagan et al. [143] and Stachowiak et al. [144].
However, both authors described that the repulsive force was a resultant from the hydration forces between the DNA molecules and not from the electrostatic forces or conformational entropy. Stachowiak et al. further specified that the surface stress was dependent on the hybridisation density whereby the hybridisation density brings together the impact of ionic strength and chain length which relates to the grafting density. At low ionic strength, the osmotic pressure of counterions dominates the intermolecular forces whereas in the medium or high ionic strength regime, hydration forces dominate as the grafting density is independent of the ionic strength.

Unlike the previous work whereby a compressive surface stress was generated after hybridisation, Wu et al. [104] observed an upward motion of the microcantilever beam (tensile surface stress) working under high ionic strength buffer. They explained that the origin of the surface stress was the interplay between changes in configurational entropy and intermolecular energetic induced by specific biomolecular interactions. They also demonstrated that by controlling the change in configurational entropy (hybridisation at low ionic strength buffer), the bending direction could be reversed to downwards (compressive surface stress). Based on the reported works presented here, we can conclude that the mechanism of surface stress generated by DNA hybridisation is not completely understood and comprehensive models are still lacking.

Moulin et al. [145] studied the adsorption of immunoglobulin G (IgG) and bovine serum albumin (BSA) on a gold surface in a buffer solution. In their experiment, 10 µl of IgG or BSA was injected into the liquid cell and the
cantilever deflection was observed up to 11 hours. The microcantilever showed a compressive stress response after the injection of IgG while injecting BSA causes a tensile surface stress. According to the authors, the compressive stress in IgG experiment originated from the protein unfolding caused by protein-surface interaction. The protein tried to “spread” (expand) on the surface upon unfolding and since they were confined within the monolayer compressive surface stress occurs. Another reason given was the attractive hydrophobic forces between adsorbed proteins causing the protein to rearrange, and since they are relatively immobile within the monolayer surface, the biomolecules were deformed by flattening causing a build up of compressive surface stress. In the case of BSA adsorption, the surface-protein interaction was weak causing the protein having some mobility on the surface. Attractive hydrophobic interaction caused the molecule to pack together. These resulted in tensile stress as the proteins contracted.

Investigation of biotin-streptavidin binding interactions was performed by Shu et al. [107] using the microcantilever sensor. The investigation was carried out on 3 different biotin modified surfaces: (i) \(N-(6-(\text{biotinamido})\text{hexyl})-3'-(2'\text{-pyridyldithio})\text{-propionamide}\) (biotin-HPDP) (ii) biotin-polyethylene glycol disulfide (biotin-PEG) (iii) biotin-disulfide-N-hydroxysuccinimide ester (biotin-SS-NHS). Injecting streptavidin onto the biotin-HPDP coated surface generated a downward deflection while biotin-PEG coated surface did not produce any bending signal. Upwards bending was observed in the biotin-SS-NHS coated microcantilever. Based on the surface characterization study, the authors found
that there was a low surface density of streptavidin protein adsorbed on the biotin modified gold surfaces. Therefore, they felt that short range interaction such as the steric hindrance was unlikely to play a significant role in the cantilever response. Since the experiment was conducted at pH 7.0 while the streptavidin had an isoelectric point at pH 5.0. This made the protein negatively charged. Hence, they suggested that the electrostatic interactions between the adsorbed protein molecules and the biotin-modified surface induced the surface stress.

Kiselev et al. [146] studied the catalytic growth of amyloid fibrils from chemically immobilised liozoyme molecules on gold or silicon using a microcantilever. Hen-egg lysozyme was immobilised onto one side of the cantilever (either gold or silicon surface) while the other side was treated with a buffer solution of tris-(hydroxymethylaminomethane) to block unreacted aldehyde groups. When the immobilised cantilever was immersed in an acetate buffer with pH 4.5, no bending was observed. However, when the cantilever was immersed in glycerine buffer with pH 3.0, bending signal was generated. This was because at pH levels lower than 3.8, lysocide undergoes a conformational transition causing protein aggregation on the surface. This led to an increase in surface tension in the film surface resulting in the cantilever bending towards the lysozyme-coated gold surface.
Table 3-1: Existing work on investigation of origin of surface stress.

<table>
<thead>
<tr>
<th>Author</th>
<th>Experiment</th>
<th>$\Delta \sigma_i$</th>
<th>Origin of $\sigma_i$ (dominant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moulin et al. [145]</td>
<td>Protein adsorption (IgG and BSA)</td>
<td>(BSA) Tensile</td>
<td>Weak surface-protein interaction caused protein to have some mobility on surface. Hydrophobic attraction caused molecules packing together.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(IgG) Compressive</td>
<td>Protein-surface interactions or attractive hydrophobic</td>
</tr>
<tr>
<td>Shu et al. [107]</td>
<td>Biotin-streptavidin binding</td>
<td>biotin-HPDP-</td>
<td>Electrostatic forces between the adsorbed protein molecules and the surface</td>
</tr>
<tr>
<td></td>
<td></td>
<td>streptavidin (Compressive) 88.7 mN/m</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>biotin-PEG-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>streptavidin (No bending) 17.8 mN/m</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>biotin-SS-NHS (Tensile)</td>
<td></td>
</tr>
<tr>
<td>Fritz et al. [134]</td>
<td>DNA hybridisation (12-mer oligonucleotide)</td>
<td>Compressive 5 mN/m</td>
<td>Repulsive force due to electrostatic and steric interactions.</td>
</tr>
<tr>
<td>McKendry et al. [110]</td>
<td>DNA hybridisation</td>
<td>Compressive 2.7 mN/m</td>
<td>Steric hinderance effect</td>
</tr>
<tr>
<td>Wu et al. [104]</td>
<td>DNA hybridisation</td>
<td>Tensile and compressive</td>
<td>Due to configurational entropy depending on buffer ionic strength</td>
</tr>
<tr>
<td>Hagan et al. [143]</td>
<td>DNA hybridisation</td>
<td>Compressive</td>
<td>Strong repulsive force as a result of hydration forces between DNA molecules.</td>
</tr>
<tr>
<td>Stachowiak et al. [144]</td>
<td>DNA hybridisation</td>
<td>Compressive</td>
<td>In low-ionic strength-osmotic pressure of counterions dominates intermolecular forces. In medium/high ionic strength, hydration forces dominate.</td>
</tr>
<tr>
<td>Wu et al. [105]</td>
<td>Bioassay of PSA</td>
<td>Compressive</td>
<td>Change in surface free energy of one surface of the cantilever</td>
</tr>
<tr>
<td>Author</td>
<td>Experiment</td>
<td>$\Delta \sigma_s$</td>
<td>Origin of $\sigma_s$ (dominant)</td>
</tr>
<tr>
<td>----------------------------</td>
<td>-------------------------------------------------</td>
<td>--------------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Ndieyira et al. [24]</td>
<td>Detection of antibiotic-mucopeptide binding</td>
<td>Compressive 34.2±5.9 mN/m</td>
<td>Repulsive steric forces. A critical number of target-receptor binding events are required to yield observable stress and demonstrates a local short range transduction mechanism.</td>
</tr>
</tbody>
</table>

### 3.3 Theoretical modelling

Conversion from microcantilever static displacement to surface stress in most reported works [147] is based on Stoney’s equation [102] (refer to Equation (2.2)). The fundamental assumption made in Stoney’s equation is that the whole deformed structure has a uniform curvature and it was originally derived for unconstrained circular plates along their edges using elasticity theory. Therefore, this formula might be found to be an inaccurate representation of rectangular microcantilever beam which has one of its edges fixed. Improvements on original Stoney’s equation were carried out to improve the accuracy of calculated theoretical value. Instead of using Young’s modulus in the formula, it was suggested that the biaxial modulus was used [148]. Sader [149] proposed a new model with the assumption that the loading of the microcantilever by surface stress is equivalent to the loading of microcantilever by moments per unit length. The theoretical model was presented by using finite element analysis of governing plate equation, and the energy minimisation technique was used to derive the analytical formula.

On the other hand, Zhang et al. [150] derived the relationship between surface stress and beam displacement at the continuum level using three models
under different loading conditions to represent the surface stress on the microcantilever beam. The models include: (i) a corresponding concentrated moment at the beam free end, (ii) a corresponding concentrated moment at the beam free end in addition to a corresponding concentrated axial load at the beam free end, and (iii) a corresponding uniformly distributed axial stress in addition to bending moment per unit length along the beam span. The modelling equation was developed using principle of virtual work. The model developed in [150] was modified by Tsai et al. [151] to include both tangential and normal surface stresses in the model.

Pinnaduwage et al. [108] described the relationship of microcantilever binding and adsorbate-induced surface stress using the energy conservation approach. The bending of the microcantilever was claimed due to the increased surface free energy where binding occurred and thus leading to an increased in surface stress. The microcantilever beam deflection magnitude is dependent on the adsorption process type. Physisorption generated the lowest bending signal, a few hundred nanometer and the adsorption process is reversible. The highest beam deflection was the chemisorption process which was in a few thousand nanometer range and the adsorption process is irreversible.

Other reported methods used to quantify the surface stress from the microcantilever deformation are the classic Kirchhoff hypothesis by Ibach et al. [152] for cubic crystalline plate, finite element analysis applied by Dahmen et al. [153] to study the model of a bending plate made of cubic crystal with (1 0 0) or (1 1 1) surfaces clamped along at an edge, a continuum-based point-defect model.
with adatom energy depending on the surface strain by Kukta et al. [154], and atomistic modelling in [155-158] to describe the surface stress induced by adsorbate-adsorbate and adsorbate-substrate interactions.

As the deformation of the microcantilever occurs at the continuum level and the interactions between the adsorbates occur at molecular level, it is necessary to relate these two in the theoretical model. Based on the works of Dareing and Thundat [155] and Yi and Duan [156], the relations between the surface stress at the continuum level and the adsorption interaction at atomic or molecular level is explored and derived in this section. From here, the energy method is applied to find the deflection of the microcantilever induced by the surface stress as it is a scalar quantity and easier to manipulate as well as relate the two models.

3.3.1 Continuum model

The model system implemented for the continuum modelling consists of a beam fixed to one side as depicted in Figure 3-3. The length, width and thickness of the cantilever beam are represented by $l$, $w$ and $t$. Below are some of the assumptions made for the model formulation:

i. The cantilever material is homogenous, isotropic and obeys Hooke’s law.

ii. The adsorbed molecules on the surface are modelled as a layer with a finite thickness.

iii. The length of the beam is much greater than its cross-sectional dimensions.
iv. The microcantilever follows Euler-Bernoulli beam theory whereby shear deformation and rotary inertia are negligible.

v. Misfit strain state in-plane is equi-biaxial and spatially constant over the plate system stress.

Figure 3-1: Diagram illustrating the microcantilever model with its dimensions.

The total elastic strain energy, $U_{\text{elastic}}$ stored in the microcantilever can be written as

$$U_{\text{elastic}} = U_{\text{bulk}} + U_{\text{surface}}$$  \hspace{1cm} (3.1)
whereby $U_{\text{bulk}}$ is the bulk strain energy and $U_{\text{surface}}$ is the surface strain energy.

Based on the work by Dahmen et al. [153] surface stress can be decomposed into a pure torque and surface stress of equal sign acting on both surfaces. In the context of the linear elasticity theory, the deformation of the microcantilever due to one-sided surface loading (see Figure 3-2) is identical to the deformation of the microcantilever beam resulting from the combination of two loads: equal stress on top and bottom and pure torque. Equal stress on the top and bottom surface result in elongation or extension of the beam while pure torque load give rise to bending.

Figure 3-2: Surface stress description. Surface stress loading (a) can be decomposed into (b) torque loading and (c) equal stress loading on top and bottom surface.
Hence, the bulk strain energy is modelled to include strain energy due to axial loading and pure bending. The axial strain energy stored in a stressed microcantilever in Figure 3-2(c), $U_a$ can be expressed as

$$U_a = \int_0^l \frac{F^2}{2E_{bulk}A} \, dx$$  \hspace{1cm} (3.2)

where $F$ is the axial force acting on the beam and $A$ is the cross-section areas of the bulk beam. $E_b$ denotes the biaxial modulus and is given as $E_{bulk} = E/(1 - \nu)$. Based on the assumption made in (i), equation (3.2) can be written in terms of the normal strain in the $x$-direction, $\varepsilon_x$

$$U_a = \frac{A}{2E_{bulk}} \int_0^l E_{bulk}^2 \varepsilon_x^2 \, dx$$

$$= \frac{1}{2} AE_{bulk} \int_0^l \varepsilon_x^2 \, dx$$  \hspace{1cm} (3.3)

Meanwhile the bending strain energy due to pure torque loading, $U_b$ can be presented as

$$U_b = \int_0^l \frac{M^2}{2E_{bulk}I_{bulk}} \, dx$$  \hspace{1cm} (3.4)

where $M$ is the moment acting on the beam and $I_{bulk}$ is the second moment of area of the microcantilever beam. Based on Euler-Bernoulli beam theory for small deflections, the radius of curvature, $R$ can be calculated via the formula below:
\[
\frac{1}{R} = \frac{M}{EI} \quad (3.5)
\]

where \( E \) is Young’s modulus and \( I \) is second moment of area. Therefore, substituting (3.5) into (3.4) yields

\[
U_b = \frac{1}{2} E_{\text{bulk}} l_{\text{bulk}} \int_0^l \frac{1}{R^2} \, dx \quad (3.6)
\]

Stress in the surface layer can be denoted as

\[
\sigma_s = \sigma_0 + E_s \epsilon_s \quad (3.7)
\]

where \( \sigma_0 \) is the intrinsic surface stress while \( E_s \) is the surface modulus and strain in the surface is denoted as \( \epsilon_s \).

The elastic strain energy for the surface is

\[
U_{\text{surface}} = \frac{1}{2} E_s l_s \int_0^l \frac{1}{R^2} \, dx + \frac{1}{2} A_s E_s \int_0^l \epsilon_s^2 \, dx \quad (3.8)
\]

where \( A_s \) is the cross-section area of the surface. Hence, the total elastic strain energy of the microcantilever can be written as
\[ U_{\text{elastic}} = \frac{1}{2} E_{\text{bulk}} l_{\text{bulk}} \int_0^l \frac{1}{R^2} \, dx + \frac{1}{2} A_{\text{bulk}} E_{\text{bulk}} \int_0^l \varepsilon_x^2 \, dx \]

\[ + \frac{1}{2} E I_s \int_0^l \frac{1}{R^2} \, dx + \frac{1}{2} A_s E_s \int_0^l \varepsilon_x^2 \, dx \]

\[ = \frac{1}{2} E I \int_0^l \frac{1}{R^2} \, dx + \frac{1}{2} A E \int_0^l \varepsilon_x^2 \, dx \]

(3.9)

where \( EI \) and \( AE \) refers to the bending stiffness and longitudinal or axial stiffness for the entire microcantilever. Bending stiffness, \( EI \) is expressed as

\[ EI = E_{\text{bulk}} l_{\text{bulk}} + E_s l_s \]

\[ = \frac{E_{\text{bulk}} \, wt^3}{(1 - v) 12} + S \left[ \frac{wh(4h^2 + 6ht + 3t^2)}{6} \right] \]  

(3.10)

where \( h \) is the thickness of the adsorbate monolayer and the area moments of the inertia for the bulk and surface layer are equivalent to

\[ l_{\text{bulk}} = \frac{wt^3}{12} \]  

(3.11)

\[ l_s = 2 \left( \frac{wh^3}{12} + wh \left( \frac{t + h}{2} \right)^2 \right) \]

\[ = \frac{wh(4h^2 + 6ht + 3t^2)}{6} \]  

(3.12)

Axial stiffness, \( AE \) is expressed as
\\[ AE = A_{\text{bulk}} E_{\text{bulk}} + A_s E_s \]

\\[ AE = \frac{E_{\text{w}} t}{(1 - v)} + S w \]  \hspace{1cm} (3.13)

whereby \( v \) is the Poison ratio and \( S = h \left( \frac{E_s - E_{\text{bulk}}}{(1-v)} \right) \) is the surface modulus.

### 3.3.2 Atomistic model

The model system implemented for the atomistic modelling consists of a microcantilever with adsorbed molecules on the top surface [156, 159] depicted in Figure 3-3.

i. The surface of the cantilever is chemically homogenous and the molecules are distributed uniformly over the entire cantilever surface which forms a monolayer. The mean interspacing distance between the adsorbates is represented by \( b \).

ii. The interaction in the first monolayer of the adsorbates is the main contributing force of surface stress, and the forces in the second and above adsorbate layers are negligible [155].
Upon adsorption, interaction can occur among the adsorbates on solid surface or via the substrate [160]. Referring to the review in Section 3.2, the molecular interactions can involve either one or a combination of forces such as electrostatic forces, van der Waals interactions, hydrophobic interactions, steric hindrance as well as hydrogen bonding. The van der Waals interaction and electrostatic interaction are found to be the main driving force for physisorption. The former describes the interactions involved between adsorbates while the latter is mainly for adsorption of charged molecules.

**Van der Waals Interaction**

The resultant van der Waals force, $U_{vdw}$ between the substrate atom and biomolecules interaction is derived from Lennard-Jones (LJ) potential energy function (refer to Figure 3-4).

---

*Figure 3-3: Schematic illustrating uniformly distributed adsorbed molecules on the cantilever surface. Atom 1, 2 and 3 are substrate atoms whereas atom 4 and 5 are adsorbates or molecules adsorbed on the surface. The origin of the coordinate system is located on the midplane and z-axis is perpendicular to the midplane.*
\[ U_{\text{vdw}}(r_{ij}) = \xi \left[ \left( \frac{r_0}{r_{ij}} \right)^{12} - 2 \left( \frac{r_0}{r_{ij}} \right)^{6} \right] \]  

(3.14)

\( \xi \) is defined as the depth of potential well, \( r_0 \) is the finite distance at which the potential energy is minimum and \( r \) is the distance between the atoms. The term \( r^{-12} \) describes the Pauli repulsion at short ranges due to the overlapping electron orbital while the term \( r^{-6} \) describes the attraction at long ranges. The LJ energy equation can be also written as:

Figure 3-4: Graph showing the Lennard-Jones potential function and its derivative force function.
whereby both $A_n$ and $B_n$ are Lennard-Jones constant. The subscript $n = 0$ represents the constant for interaction between adsorbate and substrate while $n = 1$ represents the constant for interactions between adsorbates.

Referring to Figure 3-3, the distance between atom 2 and atom 4 ($r_{24}$) as well as between atom 2 and atom 5 ($r_{25}$) can be found using the theorem of Pythagoras;

$$r_{24} = r_{25} = \sqrt{a^2 + \left(\frac{r_{45}}{2}\right)^2}$$  \hspace{1cm} (3.16)

whereby

$$r_{45} = b \left[1 + \varepsilon_b(z)\right]$$

$$= b \left[1 + \varepsilon_b \left(a + \frac{t}{2}\right)\right]$$

$$= b \left[1 + \kappa \left(a + \frac{t}{2}\right)\right]$$ \hspace{1cm} (3.17)

The function $\varepsilon_b(a + t/2)$ is the bending strain at $z = a + t/2$ and $\kappa$ is the beam curvature. Total potential energy due to van der Waals interactions between the adsorbates over the length of the cantilever is given as

$$U_{vdw}(\kappa) = \theta_w \int_0^1 U(r_{24}) + U(r_{25}) + U(r_{45})dx$$ \hspace{1cm} (3.18)
Here, $\theta$ is the adsorption density and is related to $b$, distance between adsorbates via the formula

$$b = \sqrt{1/\theta} \quad (3.19)$$

Since atom 2 and 4 have the same distance value as atoms 2 and 5, $U(r_{25}) = U(r_{24})$.

$$U(r_{24}) = \frac{A_0}{\left(\sqrt{a^2 + \left(\frac{r_{45}}{2}\right)^2}\right)^{12}} - \frac{B_0}{\left(\sqrt{a^2 + \left(\frac{r_{45}}{2}\right)^2}\right)^6} \quad (3.20)$$

Interaction energy between adsorbates, atom 4 and atom 5 can be written as

$$U(r_{45}) = \frac{A_1}{\left(b \left[1 + \kappa \left(a + \frac{t}{2}\right)\right]\right)^{12}} - \frac{B_1}{\left(b \left[1 + \kappa \left(a + \frac{t}{2}\right)\right]\right)^6} \quad (3.21)$$

The total potential energy due to van der Waals interaction is
The total potential energy of the whole system is expressed as:

\[
U_{\text{tot}} = U_{\text{elastic}} + U_{\text{vdw}}
\]

\[
= \frac{1}{2} EI \int_0^l \frac{1}{R^2} dx + \frac{1}{2} AE \int_0^l \varepsilon_x^2 dx + \theta w \int_0^l U(r_{24}) + U(r_{25}) + U(r_{45}) dx
\]

(3.23)

If the thickness of the cantilever is larger than 2 nm, the extension of the beam is very small which makes little contribution to the beam deformation [156]. Then, the elastic strain energy due to axial loading can be neglected. The equation can be reduced to
\[ U_{total} = U_{elastic} + U_{vdw} \]

\[ = \frac{1}{2} EI \int_0^l \frac{1}{R^2} dx + \theta w \int_0^l U(r_{24}) + U(r_{25}) + U(r_{45}) dx \]

(3.24)

Substituting (3.22) into (3.24) yields

\[ U_{total} = \frac{EIl}{2} \kappa^2 + \theta w \left\{ \left[ \frac{2A_0 l}{b^2 (\kappa \left( a + \frac{t}{2} \right) + 1)^2 + a^2} \right]^6 - \frac{2B_0 l}{b^6 (\kappa \left( a + \frac{t}{2} \right) + 1)^6} \right\} + \frac{A_1 l}{b^{12} (\kappa \left( a + \frac{t}{2} \right) + 1)^{12}} \]

(3.25)

In the equilibrium state, the total potential energy of the whole system should be stationary. Applying minimum principle of energy, \( \frac{\partial U_{total}}{\partial \kappa} = 0 \).
Using trigonometry (see Figure 3-5), the relationship between the microcantilever curvature and displacement $\Delta z$ can be expressed as

$$\Delta z = \frac{1}{k}[1 - \cos(\kappa)]$$  \hspace{1cm} (3.27)

To obtain the displacement, $\Delta z$, $\kappa$ value that satisfies equation (3.26) is substituted in (3.27).
3.4 Summary

The origin of surface stress that causes the displacement of the microcantilever was explored and identified as the intermolecular interactions due to the van der Waals interaction, electrostatic interaction, hydrogen bonding, hydrophobic interactions as well as steric hindrance. The effect of adsorption-induced surface stress on the microcantilever beam was modelled by deriving the continuum mathematical model of the system from the atomistic model describing the adsorbates interactions. The van der Waals interaction force was included in the atomistic modelling as it is the main driving force in physisorption.
CHAPTER 4

APTASENSOR DESIGN

4.1 Background

The proposed SU-8 microcantilever-based aptasensor is designed using the finite element method. The aim of this exercise is to study the influence of cantilever parameters on its deflection. This chapter describes the conceptual design of the device followed by a report of different simulations conducted. Discussions on the obtained results are presented in the subsequent section.

4.2 Conceptual design

The proposed aptasensor design consists of a beam that is fixed to one side. The top side of the cantilever is immobilised with biorecognition elements for target sensing. Meanwhile, the bottom side is coated with a layer of gold so that a laser beam could be deflected off its surface for beam deflection measurement via an optical lever read-out method during the biomolecules detection.

4.3 Modelling and simulations

Modelling of the proposed sensor design prior to realization of the actual device helps to optimise the design parameters for better sensitivity. The modelling and simulation of the sensor is performed using the finite element method. Coventorware, a MEMS multiphysics software is used as the software tool to aid
in our studies. The beam model consists of three layers. The bulk layer was the silicon layer, and a thin layer was deposited on top of the bulk layer to serve as the adsorbate layer. A thin layer of gold was deposited on the bottom side of the beam to serve as a reflective surface to reflect the laser beam during optical lever detection. Based on the literature, typical surface stress generated by the biomolecular interactions is 10 mJ/m². This was the value used to simulate the surface stress in all the studies.

Surface stress ($\sigma_s$) is defined as the work done per area necessary to strain an existing surface. The SI unit for surface stress is N/m. It is the integral of the normal stress or bulk stress ($\sigma_m$) in the monolayer over its thickness, $t_m$, shown in the equation below:

$$\sigma_s = \int_0^{t_m} \sigma_m \, dz$$  

(4.1)

To simulate the surface stress, a normal stress of 10 MPa is applied biaxially in the x-direction and y-direction in the thin layer. The value of the normal stress was calculated using Equation (4.1) with an assumption of 1 nm thick adsorbate layer forming on the microcantilever surface. All the simulated cantilever beam models are meshed using the Manhattan parabolic mesh.
4.3.1 Investigating the effect of microcantilever material

Polysilicon is the most common material used for microcantilever fabrication. Other materials such as silica and silicon nitride are also used for cantilever fabrication. Recently, polymer such as SU-8 has garnered a lot of interest in MEMS fabrication due to its ease of fabrication. In addition, polymers have lower Young’s modulus compared to their silicon counterparts. The low Young’s modulus means low stiffness which leads to more bending and subsequently larger displacement. The stiffness or spring constant, \( k \), of the cantilever beam is depicted in the formula below:

\[
k = \frac{Ewt^3}{4l^3}
\]  

(4.2)

where \( E \) is the Young’s modulus, \( w \) is the microcantilever width, \( t \) is the thickness of the microcantilever and \( l \) is the length of the microcantilever. The impact of the different material used for cantilever beam fabrication on the beam’s displacement is examined in this section. Table 4-1 describes the mechanical properties of the materials utilised in the simulation studies. A microcantilever beam with the
dimensions of 600×200×2 µm is implemented for the simulation studies in this section.

### Table 4-1: Mechanical properties of different simulated materials.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Polysilicon</th>
<th>Silica</th>
<th>Si₃N₄</th>
<th>SU-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>E (GPa)</td>
<td>160</td>
<td>73</td>
<td>315</td>
<td>4.4</td>
</tr>
<tr>
<td>ν</td>
<td>0.22</td>
<td>0.17</td>
<td>0.27</td>
<td>0.22</td>
</tr>
<tr>
<td>ρ (kg/m³)</td>
<td>2230</td>
<td>2200</td>
<td>3184</td>
<td>1190</td>
</tr>
<tr>
<td>k (N/m) (calculated values)</td>
<td>0.02</td>
<td>7.85×10⁻³</td>
<td>0.03</td>
<td>4.32×10⁻⁴</td>
</tr>
</tbody>
</table>

#### 4.3.2 Investigating the effect of microcantilever geometry

To investigate the effect of geometrical dimensions of the microcantilever beam on the sensor sensitivity, FEM modelling and simulations were conducted for various conditions. The first simulation studied the role of the length of cantilever beam in influencing the microcantilever’s sensitivity. A SU-8 rectangular beam with a constant width of 50 µm and thickness of 2 µm and a varying length was modelled. The length of the beam was varied by assigning a trajectory variable using the parametric study function available in Coventorware.

The effect of the thickness of the microcantilever beam on the sensor sensitivity was also investigated. In this study, the length and width of the beam were kept constant at 100 µm and 50 µm while the thickness of the beam was varied using the parametric study function in Coventorware. The displacement of the beam with the varying thickness of the beam was observed.

Also the role of different shapes on the sensitivity of the cantilever operating in static mode was investigated in the simulation studies. Other than the conventional rectangular-shaped microcantilever, rectangular beams with uniform holes distributed across their lengths, a trapezoidal-shaped microcantilever beam,
a triangular-shaped microcantilever beam, a U-shaped microcantilever beam, and a rectangular-shaped with a hollow centred microcantilever beam were tested. Sensitivity analysis relating the microcantilever deflection and surface stress was also carried out using the final chosen beam dimensions.

4.4 Results and discussions

4.4.1 Effect of microcantilever material on aptasensor sensitivity

Based on the graph shown in Figure 4-2, the cantilever made of SU-8 polymer generated the highest displacement value followed by silica, polysilicon and silicon nitride. The SU-8 cantilever with the lowest spring constant resulted in a more sensitive response than those materials with higher spring constant upon target binding.

![Figure 4-2: Graph illustrating the displacement for various cantilever materials.](image)

Figure 4-2: Graph illustrating the displacement for various cantilever materials.
SU-8 polymer produces the highest displacement for the same amount of surface stress applied across the beam applied to other materials.

4.4.2 Effect of beam geometry on aptasensor sensitivity

Figure 4-3 to Figure 4-5 illustrate different lengths of cantilever beam and their respective displacements when the beam experienced a differential surface stress of 10 mJ/m². A graph was plotted for the displacement of the beam against its length. Based on the presented graph in Figure 4-6, it can be observed that as the length of the microcantilever beam increases, larger displacements are generated. However, in the second simulation study, increasing the thickness of the beam caused a decrease in the microcantilever displacement (see Figure 4-7). Therefore, the displacement of the beam is proportional to its length but inversely proportional to its thickness.

Figure 4-3: Simulated displacement for a 100 µm long beam.
Figure 4-4: Simulated beam displacement for a 200 µm long beam.

Figure 4-5: Simulated beam displacement for a 400 µm long beam.

Figure 4-6: Graph showing the relationship between the displacement of the cantilever beam versus its length.
Figure 4-7: Graph showing the relationship of the displacement of the cantilever beam with respect to its beam thickness.

The results for the simulated shapes are depicted in Figure 4-8 to Figure 4-12. Based on the observed results (refer Figure 4-14), all the shapes produced the same displacement for the same X-Y stress. This showed that the examined shapes do not notably contribute to the microcantilever sensitivity in the static mode. Essentially, the shapes presented actually differ in width. Based on the published literature [102], the microcantilever sensitivity is independent of the width of the beam, thus the simulated result was consistent with the literature. Therefore, the conventional rectangular-shaped beam was chosen as the final design for ease of fabrication.
Figure 4-8: Manhattan-meshed rectangular-shaped microcantilever with uniformly distributed square holes (Shape A).

Figure 4-9: Manhattan-meshed trapezium-shaped microcantilever (Shape B).
Figure 4-10: Tetrahedron-meshed triangular-shaped microcantilever (Shape C).

Figure 4-11: Manhattan-meshed U-shaped microcantilever (Shape D).
Figure 4.12: Manhattan-meshed rectangular-shaped microcantilever with a hollow centre (Shape E).

Figure 4.13: Manhattan-meshed rectangular-shaped microcantilever (Shape F).
4.4.3 Sensitivity analysis

Based on the results presented in Section 4.4.1 and 4.4.2, the final dimensions of the microcantilever used in this work was set to 1000 µm × 200 µm × 2 µm. Since the width of the microcantilever does not play a role in the microcantilever sensitivity, the beam was designed as 200 µm wide so that the microcantilever could be easily fabricated using a transparency mask. This is further elaborated in Section 5.4.1. Referring to Figure 4-7, the sensitivity of the proposed microcantilever aptasensor obtained via simulation studies was 82.87 µm/ N/m.
Figure 4-15: Graph showing the relationship of the deflection of the SU-8 microcantilever beam (1000 µm × 200 µm × 2 µm) versus surface stress.

4.5 Summary

In summary, this chapter described an investigation of the design parameters that affect the sensitivity of aptasensor using the finite element method. Simulations of all the designs were conducted using Coventorware. From the simulation results, it can be concluded that the material used for beam fabrication affects the deflection sensitivity. SU-8, which is a negative photoresist polymer, was found to generate deflection of one magnitude higher than its silicon counterpart. Apart from that, the length and thickness of the beam also played a role in the aptasensor sensitivity. The beam deflection magnitude increased with increasing length of the
beam while increasing the thickness of the beam resulted in the decrease of
deflection. Meanwhile, the deflection magnitude was independent of the beam’s
width. Based on the conducted studies, the chosen beam geometry for this work
was 1000 µm long, 200 µm wide and 2 µm thick. The sensitivity of the proposed
microcantilever beam was 82.87 µm/ N/m.
CHAPTER 5

APTASENSOR FABRICATION

5.1 Background

There exist various methods for fabrication of polymeric microcantilever structures. The procedures involved are dependent on the type of desired polymer. In this work, SU-8 polymer is used for the fabrication of the aptasensor prototype. SU-8 is a negative photoresist epoxy-acrylate polymer [161]. The chemical composition of SU-8 includes: (i) EPON epoxy resin, a multifunctional glycidyl ether derivative of bisphenol-A novolac used to provide high-resolution patterning for semiconductor devices, (ii) organic solvent cyclopentanone (SU-8 2000 series), whereby its amount determines the final thickness of the film, and (iii) photoinitiator that generates strong acid upon exposure to 365 nm UV light. The acid facilitates the cross-linking of the polymer during post exposure bake. SU-8 is highly transparent in the UV region, biocompatible, has low Young’s modulus compared to silicon and has excellent chemical resistance. Moreover, SU-8 fabrication technology is compatible with standard silicon processing. These make them an excellent choice as the fabrication material for the proposed aptasensor.

Applications of SU-8 photoresist are popular in MEMS development but there are no standardised fabrication conditions. The spin coating and development of SU-8 is very much dependent on the equipment and also the
geometries of the fabricated structure. In this chapter, the protocols developed for the aptasensor fabrication are described. Firstly, the chapter introduces various existing fabrication techniques applied for fabrication of SU-8 microstructures. Also, various methods used for lift-off of the final structure from the silicon substrate are reviewed. This is followed by a description of the protocols developed and optimised for the fabrication of a multi-layer microcantilever structure using negative photoresist, SU-8. Different methods for releasing 2-µm thick SU-8 structures from the silicon substrate are investigated and discussed.

5.2 Related works

5.2.1 Fabrication of multi-layer SU-8 structure

Frederick et al. [162] investigated two different methods to produce multi-layer freestanding structures using SU-8. The first method utilised two different UV exposure doses to create thin freestanding parts. The high absorption of short UV wavelength in the SU-8 resist was exploited in this method. The fabrication process began by spinning a single layer of SU-8 on the substrate, baked and exposed the SU-8 down to substrate with UV light of 365 nm wavelength through a mask. Subsequently, the top part of SU-8 was exposed using a 313 nm wavelength UV light using another mask. Post exposure bake occurs after the exposure. The uncross-linked SU-8 was developed leaving a freestanding structure. This method is simple and faster in terms of fabrication but suffers from bending stress at the top layer due to the uneven exposure at different depths.
leading to uneven cross-linked polymer. This problem is more prominent in single clamped beams.

The second method was the buried mask method whereby a metal layer was deposited after spin coating, softbake, exposure and post exposure bake of the first SU-8 layer before spin coating the second SU-8 layer. The deposited metal layer will prevent UV penetration into the lower SU-8 layers. In this method, care need to be taken when depositing the metal layer. Sputtering technique was deemed unsuitable as this method produces too much UV light and heat that will crosslink the unexposed SU-8. Hence, resistive heating evaporation method was implemented by the authors. In this method, the softbake steps for all SU-8 layers except for the bottom layer was replaced by vacuum treatment to prevent wrinkle formation due to unwanted cross-linking in the unexposed part caused by the elevated temperature. This method generates zero bending stress but the vacuum treatment step time is very long for thick structures (3 hr for 30 μm thick structure).

Alternatively, multiple layer SU-8 microstructures could be achieved by using multiple coatings and exposure steps with a single developing step at the end of the fabrication process. This method is simple as it utilises SU-8 as the structural material as well as sacrificial material. This had been demonstrated by Mata et al. [163] for the development of six level SU-8 microstructures with overall thickness of up to 500 μm and minimum feature size of 10 μm. However, this method has its challenges as it only works well if the thin layer is coated over the thick SU-8 layer as unwanted cross-linking of SU-8 could occur at the bottom
layer during the top layer exposure. Apart from that, different thickness of SU-8 layers could cause non-uniform exposure due to different UV light absorption.

5.2.2 Structure release from substrate

In cases where SU-8 polymer is used as device structure material, the final device is required to be released from the substrate for further applications. However, cross-linked SU-8 polymer is very difficult to be stripped or released. Based on the literature, the techniques used to strip/release SU-8 can be categorised into dry release and wet chemical release methods.

In the dry release method, a thin layer of fluorocarbon film with low free surface energy was deposited on the silicon wafer before spin coating of the SU-8 structure layer [164, 165]. The fluorocarbon layer was deposited using plasma in an advanced silicon etch device. The etch device operates by alternating between etching and passivation cycle to plasma etch deep trenches into silicon. For the deposition of the fluorocarbon layer, the etch cycle in the device was disabled and one passivation cycle was set to 90s at a coil power of 300 W, electrode power of 20 W and the flow rate of fluorocarbon at 120 sccm. To release the final SU-8 structure from the substrate, a weak mechanical force was applied perpendicularly to the substrate surface. The weak adhesion of SU-8 to the fluorocarbon layer enables easy lift-off of the device. Microcantilevers with thickness as low as 2 µm could be released using this method.

Meanwhile, in the wet chemical release method, a sacrificial layer is deposited on the wafer before deposition of the SU-8 structure layer. The
sacrificial layer is then removed using specific solvent or etchant at the end of the fabrication process for releasing the final structure. The sacrificial material used to release SU-8 structures ranging from metals to polymers. A few hundred nanometers of metal sacrificial layer such as Cr/Al [166], and Cr/Au/Cr [87, 122] were reported in the literature. For proper structure release of millimeters square area, using copper, the deposited thickness of the copper layer required to be around 5-10 µm thickness [167].

Luo et al. [168] demonstrated the release of SU-8 membrane with an area of 50 cm² and thickness of 9, 13, 25, 100 and 200 µm using polystyrene as the sacrificial layer. Solid polystyrene was dissolved in toluene solution and spin coated onto a glass substrate followed by a baking step to cure it. Then the SU-8 membrane layer was spin coated on top of the sacrificial layer. After performing all the SU-8 processing steps, the polystyrene layer was dissolved in a toluene bath, and the SU-8 membrane was removed from the substrate.

A comparative experiment involving Cr, Cr/Au, Cu, Al, PMMA, polyimide and polystyrene as sacrificial materials for releasing a 12-µm thick SU-8 structure was conducted by Psoma et al. [120]. Amongst the metals, for a given similar thickness of 100nm, copper offered the fastest release rate followed by gold, aluminium and lastly, chromium. Sacrificial materials using polymers demonstrated a faster release time compared to the metal layers but suffered from a poor adhesion between SU-8 and polymer sacrificial layers which caused small structures detached from the walls, and they were damaged as soon as they were placed in the acetone. This was evident clearly in polystyrene. However, this
problem was not observed for larger structures. Optimum thickness for structure release was found to be 1.6 mm for a polystyrene layer and 250 nm for a PMMA layer. Polyimide was found not compatible with SU-8 as the resist reacted with SU-8.

An alternative sacrificial material used for the release of SU-8 structures is Omnicoat produced by MicroChem. It is from the line of PMGI resist with proprietary solvent blends with high thermal stability properties, optically transparent acting as an adhesion promoter for SU-8. A SU-8 structure of 2 mm × 0.5 mm × 65 µm [169] and a SU-8 structure of 160 µm thickness [170] were released using Omnicoat. The processing steps for Omnicoat are relatively simple. They include spin coating of Omnicoat layer followed by curing at 200 °C for 1 min. Remover PG was used to release the final structure from the substrate. SU-8 structures as thin as 5 µm were successfully lifted-off using this method [171].

From the literatures, there exist various techniques involving different sacrificial materials for removal of SU-8 from different types of substrates. In the following sections, conducted experiments to explore these techniques are reported and the best approach to release a SU-8 microcantilever with 2 µm thickness and 1000 µm length is presented.

5.3 Materials and reagents

5.3.1 Substrate cleaning

Sulphuric acid (H₂SO₄ 96%), and hydrogen peroxide (H₂O₂ 30%) were purchased from Sigma Aldrich. The percentages are the dilutions as they arrive in bottle and
used as they are. Isopropanol (IPA) solvent and deionised (DI) water were used for substrate cleaning. Nitrogen air for drying.

5.3.2 Photoresists and developers

Negative photoresists: SU-8 2002 for thin structure (~1.5-3 µm) and SU-8 2050 for thick structures (~40-180 µm) were purchased from MicroChem.

Positive photoresists: AZ4562 was purchased from Microchemicals while polymethyl methacrylate (PMMA) was purchased from Microchem.

Adhesion promoter/release layer: Omnicoat was purchased from Microchem

Developers: SU-8 developer, propylene glycol methyl ether acetate (PGMEA) was purchased from MicroChem. Meanwhile AZ400K was purchased from Microchemicals and was used in 1:4 dilutions. For removal of PMMA, acetone was used. Remover PG (N-Methyl Pyrolidinone) from MicroChem was used for stripping of SU-8.

5.3.3 Tools and equipments

Cleaning: Ultrasonic bath, wafer tweezers, tweezers, and 1 litre beakers × 4

Photomasks: Transparent film, Kapton tape, quartz substrate

Substrate material: Silicon wafer (any silicon wafer with one sided polished surface)

Photolithography and measurement tools: Laurell technologies spin coater, EVG620 mask aligner, Plasmalab 100 ICP380 deep reactive ion etcher,
profilometer, scanning electron microscope, optical microscope, programmable hotplates, sputter coater, and disposable pipettes.

*Personal protective equipments:* Safety glasses, nitrile gloves, and protective face screen (for acid handling).

## 5.4 Experimental procedures

### 5.4.1 Mask fabrication

Mask patterns of the microcantilever structure were drawn using Eagle layout editor. Eagle layout editor is commonly used for printed circuit board design. It was discovered that this software tool was sufficient to aid in the device pattern design. It is important to take note that SU-8 is a negative resist, hence the mask should be designed in such a way that the desired features are transparent areas to enable the UV light to pass through. This would polymerise the SU-8. This type of mask is known as dark field or negative mask and three mask designs were developed in this work due to the multiple layer structure. One for the alignment marks layer (see Figure 5-1), one for the cantilever layer (see Figure 5-2) and the other for the body layer (see Figure 5-3).
Figure 5-1: Alignment marks mask design.

Figure 5-2: Microcantilever layer mask design.
Initially, the mask used in UV exposure was fabricated by printing the desired patterns on a transparency sheet using a laser printer. However, the quality of the mask (see Figure 5-4) was poor whereby the black areas were not fully opaque and the edges of the lines were not straight. Hence the resultant microcantilever structure (see Figure 5-5) had very uneven edges. To overcome this issue, masks were printed on a transparent film using an imagesetting service. The minimum mask feature for imagesetting mask is around 25 µm which is adequate for the fabrication of the device as the minimum feature is 200 µm.

Printed mask films were cleaned with IPA and dried using nitrogen. Acetone should not be used for cleaning the transparent film masks as it would remove the printed ink. To load the mask onto the mask aligner, the mask film needs to be placed onto a quartz substrate. The mask was cut to fit onto the square
quartz substrate. The transparent film mask was centred and taped to the quartz substrate using Kapton tapes with the emulsion side facing up.

Figure 5-4: Comparison of the captured images of the mask patterns. Image (a) and (b) were fabricated using imagesetting on a film while image (c) and (d) were fabricated using a laser printer and a transparency sheet.

Figure 5-5: Comparison of the fabricated microcantilevers using the imagesetting masks (a and b) and laser printed transparency masks (c and d).
5.4.2 Aptasensor fabrication

The proposed microcantilever structure was fabricated using SU-8 polymer due to its lower Young’s modulus compared to silicon. The surface micromachining technology was implemented. The steps involved are listed as follows:

i. Silicon substrate cleaning

A silicon wafer was used as the substrate material and it was cleaned using piranha solution prior to fluorocarbon deposition. Piranha solution was made by mixing equal parts of H₂SO₄ 96% and H₂O₂ 30% in a beaker. Silicon wafer was immersed in the piranha solution for 20 min, followed by DI water rinse for 5 min. The rinsing step was repeated for three cycles, with each cycle using fresh DI water.

ii. Alignment marks patterning

Alignment marks layer is necessary for multi-layer structure fabrication as it facilitates the features to be aligned properly during UV exposure. To pattern the alignment marks, a lift-off technique (see Figure 5-6) using positive photoresist, AZ4562 as the sacrificial material was implemented. The positive photoresist was spin coated on the cleaned wafer at 3000 rpm for 30 sec. Then it was baked at 110°C for 2 min. The wafer was then loaded onto the mask aligner with the alignment marks photomask placed emulsion side down. The alignment marks on the wafer was patterned under constant UV exposure of 180 mJ/cm². After exposure, the wafer was developed in a solution consisting of 1:4 ratio of AZ400K developer and DI water for 1 min, followed by DI water rinse to remove
non-crosslinked photoresist. The whole wafer was then sputtered with a 100 nm thick layer of gold. The gold that was deposited on the crosslinked photoresist layer was removed using acetone in an ultrasonic bath.

Figure 5-6: Mask patterning using lift-off technique. (a) Positive photoresist AZ4562 was spun onto the wafer. (b) Negative mask was used for the alignment marks. The photoresist was exposed with UV after baking process. (c) The photoresist layer was then developed to remove the areas that were exposed to UV light. (d) A layer of gold was sputtered onto the wafer. (e) The wafer was immersed in a beaker filled with acetone solution and placed in an ultrasonic bath to dissolve the photoresist layer on the wafer.

iii. Polymer or sacrificial layer deposition for structure release

In this step, three different approaches were examined. The procedures varied according to the release method implemented. The procedures for the dry release of microcantilever via fluorocarbon layer deposition and wet chemical release of the microcantilever structure using Omnicoat and PMMA photoresist are described in the following.

(a) Deposition of fluorocarbon layer

To enable easy release of the microcantilever structure at the end of fabrication, a thin fluorocarbon layer was deposited on the silicon wafer to modify its surface
properties. The deposition step is based on the work in [164, 165]. Deposition of fluorocarbon layer was achieved using DRIE equipment. Etching cycle was disabled in DRIE and only the fluorocarbon passivation cycle was activated. Firstly, silicon substrate was cleaned with oxygen plasma. The parameters used are 800 W coil power, 20W electrode power and gas flow of 45 sccm at a pressure of 45 mTorr for 5 min. Subsequently, fluorocarbon is deposited with 300 W coil power, 20 W electrode power, fluorocarbon flow rate of 120 sccm and 90 sec deposition time. The pressure recommended in [165] during deposition was set to 60 mTorr. However, this value was not compatible with our equipment and process. Photoresist was observed sliding off the silicon wafer during spin coating of the low viscosity SU-8 2002 (see Figure 5-7). Hence, experiments were conducted to optimise the pressure for the deposition as the pressure affects the hydrophobicity of the silicon surface. 70 mTorr was found to be suitable from the conducted experiments. Short argon plasma was applied for declamping. The parameters were 400 W coil power, 0 W electrode power, gas flow of 50 sccm and pressure of 50 mTorr for 10 secs.
Figure 5-7: Photoresist sliding off the wafer surface during spin coating.

Figure 5-8: After optimization of deposition parameters. Photoresist managed to coat the whole wafer surface once C₄F₈ deposition parameters were optimised.

(b) Omniccoat as the release layer

4ml of Omniccoat was dispensed onto the wafer. The wafer was spun at 500 rpm for 5 sec with acceleration of 100 rpm/s. Then the spin speed was increased to 3000 rpm for 30 sec with acceleration of 300 rpm/s. The wafer was then baked on
a hotplate for 1 min at 200 °C. The measured resultant thickness was around 13 nm.

**(c) PMMA as the sacrificial layer**

After alignment marks patterning, a sacrificial layer made of a positive photoresist was spin coated on the wafer. 5 ml of PMMA A6 was dispensed onto the wafer and spin coated at 500 rpm for 2 min at acceleration of 100 rpm/s. Subsequently, the wafer was baked at hotplate at 170 °C for 5 min. The measured resultant thickness was about 700 nm.

**iv. Fabrication of microcantilever layer**

After the polymer or sacrificial layer deposition, 4 ml of SU-8 2002 was spun onto the silicon wafer to fabricate the microcantilever layer. The spin coater was set to an acceleration of 100 rpm/s and a spin speed of 500 rpm for 5 sec. Then the acceleration was increased to 300 rpm/s and a spin speed of 1500 rpm for 30 sec. Using a profilometer, the resulting thickness of the film was measured and had an average of 2.16 µm. A softbake was performed on a digital programmable hotplate ramping from room temperature to 65°C at 120°C/hr and held for 1 min. The temperature was further ramped up to 95°C at 120°C/hr and held for another 1 minute. The wafer was left to cool to room temperature prior UV exposure. The microcantilever layer film mask that was taped to the quartz substrate was loaded onto the mask aligner with the ink side of the mask facing towards the SU-8 coated silicon wafer. The wafer was exposed with a constant dose of 80 mJ/cm².
A post exposure bake followed immediately at 65°C for 1 min and 95°C for another minute with the same ramping rate 120°C/hr.

After cooling the wafer to room temperature, the wafer was developed by immersing it in SU-8 developer for a minute followed by IPA rinsing to remove the non-crosslinked parts. The wafer was left to dry in at ambient temperature. Blow drying the wafer would lead to undesired deformation of the microcantilever on the wafer due to the strong pressure from the air gun.

Figure 5-9: After development of SU-8 2002 layer. Uncross-linked polymer is removed using PGMEA solution before spin coating of the body layer.

v. Fabrication of body layer

A second layer of SU-8 was spun onto the wafer to produce the body layer of the structure. The purpose of this layer is for ease of handling and transporting of the thin microcantilevers in real-world applications. 4 ml of SU-8 2050 was dispensed
onto the wafer and the acceleration spin speed was set to 100 rpm/s and 500 rpm spin speed for 5 sec. This was then followed by increasing the acceleration speed to 300 rpm/s and spin speed of 1500 rpm for 30 sec. The mean thickness of the microcantilever was 2 µm. Similar to SU-2050, a softbake step followed directly after spin coating. The softbake parameters were 5 min at 65°C with a ramping rate of 120°C/hr from room temperature, followed by 21 min baking at 95°C at 120°C/hr. Body layer photomask taped onto the quartz substrate was loaded on the mask aligner with the emulsion side facing down. The wafer was then exposed to UV with a constant dose of 240 mJ/cm². Pose exposure bake parameters were 1 min at 65°C with a ramping rate of 120°C/hr from room temperature and 11 min at 95°C at 120°C/hr.

Finally the substrate was soaked in SU-8 developer for 15 min and another 15 min in fresh developer followed by rinsing with IPA to remove the uncross-linked SU-8 for the body layers. Figure 5-10 shows the fabricated microcantilever structure. The whole fabrication procedures are summarised in the flowchart given in Figure 5-12 with the aid of diagram illustrations in Figure 5-13.
vi. **Sputtering of gold layer**

As optical lever detection method is employed in this work, a thin layer of gold was sputtered onto one side of the microcantilever surface to enable the laser beam reflects off the microcantilever surface. The sputtering of the gold layer could be done before the microcantilever was released from the wafer surface or after the release of final device.
Figure 5-11: Photograph showing the gold-coated microcantilever before device release.

Figure 5-12: Flowchart overview of the proposed aptasensor fabrication process.
Figure 5-13: Diagram illustrating the steps involved in the fabrication of the proposed microcantilever.
(a) Deposition of sacrificial material. (b) Spin coating of the SU-8 2002 photoresist layer on top of the deposited sacrificial layer. (c) After softbake, the microcantilever was formed by subjecting the wafer to UV exposure with the mask aligned to the alignment marks patterned on the wafer. (d) Regions that were not exposed by UV light were developed. (e) Thicker SU-8 2050 photoresist was spun on the wafer. (f) After softbake, the SU-8 layer was exposed to UV light to form the body layer of the microcantilever by aligning the marks in the mask to the marks on the wafer. (g) Uncross-linked regions of the photoresist were developed. (h) Microcantilever was released from the wafer either by using a mechanical tweezers (using the dry release technique) or by developing the sacrificial layer (wet release technique).
5.5 Results and discussions

5.5.1 Dry release method

After developing the wafer, the wafer was left to dry at ambient temperature. Microcantilevers were mechanically lifted off from the wafer substrate using a pair of tweezers. For the body layer which was thicker, there was no problem lifting them off the wafer surface, but for the thinner microcantilever structure, it was very hard to release them. Hence, the yield of successful lift off was very low at 40% as the microcantilevers tend to break during lift-off due to their thin structure. For those microcantilevers that had been successfully lifted off the silicon surface, some of the beams were deformed and they were rolled up. This may be due to the mechanical stress caused during lift-off as well as thermal stress.

Figure 5-14: Image taken by optical microscope after lift-off.
Residual stress in the microcantilever caused the beam to bend and warped after lift-off.

5.5.2 Wet chemical release via Omnicoat layer

It is widely known that SU-8 is very difficult to remove from a surface. To aid in removal of cross-linked SU-8 structures, a layer of Omnicoat was spin coated between the silicon substrate and SU-8 layer. To release the microcantilever structures, the wafer was immersed in Remover PG solution for 2 hours whereby the Remover PG solution was replaced at half-time. Subsequently, the wafer was rinsed in IPA solution, followed by DI water rinse. Although it is suggested in the chemical datasheets that ultrasonic agitation could be used to increase the removal rate, it was not suitable for this work as the microcantilevers were very thin and fragile. There were some curves observed in the microcantilever after the release process due to the thermal stress. Only 25% yield was obtained using this release method. This was lower than the previous dry release method. Remover PG works by swelling the Omnicoat layer and eventually lifting-off the structure. It mainly strips the SU-8 rather than releasing the layer. This method works better for thicker structures, for example the microcantilever body layer. However, due to the long and thin (thickness ≈ 2 µm) nature of the microcantilever, it was very difficult to obtain a straight final structure as the surface of the microcantilever tended to wrinkle due to the swelling mechanism during lift-off.
5.5.3 Wet chemical release via PMMA sacrificial layer

A layer of PMMA, a positive photoresist was spin coated onto the wafer before depositing the microcantilever layer for easy release of the final device. The sacrificial layer using positive photoresists is easily removed or dissolved using solvents such as acetone. Cross-linked SU-8 has a very good chemical resistant therefore it is compatible with acetone. Figure 5-16 depicts the dissolution of the PMMA sacrificial layer after soaking the wafer in acetone solution. Total time taken to release the whole microcantilever structure was 30 min. Compared to the previous two methods, this approach produced the highest yield of 90%. It also took less time to release the final structure compared to the Omnicote approach. Similar to the previous methods, there was a slight curvature at the microcantilever due to the thermal stress. This problem was alleviated by doing a hard bake before structure release. A detailed description is provided in the subsequent section.
Figure 5-16: Optical images showing the dissolution of PMMA in acetone. (a) Dark regions at the microcantilever were the PMMA sacrificial layer. (b) PMMA layer was dissolved by immersing the wafer in acetone solution.

5.5.4 Troubleshooting

Referring to Figure 5-17, a microcantilever curvature was observed after lift-off which was caused by the residual stress due to different coefficient thermal of
expansion of the different SU-8 layer. The curvature was more prominent on the longer microcantilever. To reduce the residual stress, the temperature of the softbake and post exposure bake for the body layer was lowered to 50 °C and baked for 10 hours. In addition, after developing non-crosslinked parts of the body layer, the wafer was hard baked at 90 °C for 1 hr and let to cool down to room temperature at 2 °C/min to minimise the thermal stress before the microcantilever structures were released.

![Figure 5-17: SEM image of the 1mm long microcantilever. Thermal stress caused the curvature on the microcantilever.](image)

### 5.6 Summary

The proposed device was fabricated using SU-8, a negative photoresist via surface micromachining technique. The main fabrication steps involved: (i) alignment marks patterning due to the multi-layer structure of the device, (ii) sacrificial layer
deposition for final structure release, (iii) deposition and development of the microcantilever layer, (iv) deposition and development of the body layer, (v) sputtering a layer of gold on the microcantilever and (vi) release of the final device. A long and low bake temperature was implemented to reduce the residual stress in the microcantilever.

Three different release methods were evaluated. Mechanical lift-off using tweezers was very tedious and required careful handling as a slight force could break or bend the microcantilevers. It was easy to incorporate a sacrificial layer in the device fabrications process to release SU-8 structures from the wafer. The wet method was more convenient than the dry method as the deposition of the sacrificial layer was rapid and simple with no additional equipment required. The wet release method using PMMA as the sacrificial layer was found to generate the highest yield of 90%.
CHAPTER 6

APTASENSOR SURFACE IMMobilisation

6.1 Background

Immobilisation of receptor molecules of target analyte is necessary in order to achieve high specificity in biosensors. Receptor molecules can be attached to a wide range of substrates including metal, carbon nanotube and polymer surfaces. Surface immobilisation affects the binding affinity, sensitivity and specificity of the sensing device.

During immobilisation, it is crucial to ensure that there is a good linkage between the sensor substrate surface and immobilised receptor molecules so that they are not easily displaced by biological medium. It is also important to be able to control the orientation of the receptor molecules during surface immobilisation so that the active sites of the immobilised receptor molecules face the biological medium and easily accessed by target analyte. It is essential that immobilised receptor molecules maintain their biological activity while retaining their native 3D structure and possessing good stability.

In this chapter, an overview of surface immobilisation techniques is presented. The functional tail groups that are employed to anchor the receptor molecules onto the aptasensor surface are described. The approach implemented to introduce functional groups onto the aptasensor surface is described as well.
The protocols for aptamer immobilisation and their associated results are also presented.

### 6.2 Related works

A variety of surface immobilisation techniques that use molecular recognition elements which including antibodies, nucleic acids, peptides, proteins and aptamers, have been reported in the literature [172-174]. Aptamers are oligonucleic acids which can be either DNA or RNA that are synthesised via in vitro combinatorial strategy of selection known as systematic evolution of ligands by exponential enrichment (SELEX) [175]. They can be synthesised in large quantities with high purity. Aptamers are valuable molecular recognition agents as they have high binding affinity with targets of dissociation constants ranging from picomolar to milimolar [176]. This feature enables them to recognise the target molecules with high selectivity compared to antibodies.

Besides that, anchoring of aptamers on the sensor surface is simple as the functional moiety can be easily attached to the 5'-end or 3'-end of the nucleic acid structure via simple chemical reactions. Aptamers have high thermal stability and can be regenerated as well. In the following, a summary of the commonly used techniques for immobilisation of aptamers on different substrates are presented.

Gold is the common substrate employed for aptamers immobilisation. Functional groups were usually introduced on the surface via thiol-Au coupling [53, 67, 177, 178]. In the work reported in [178], the gold surface was modified via self-assembled monolayer (SAM) of 11-mercaptopundecanoic acid. In this
scheme, 50 mM of N-hydroxysuccinimide (NHS) and 200 mM N-(3-dimethylaminopropyl)-N-ethylcarbodiimide were used to activate the carboxyle groups. Immobilisation of RNA/DNA aptamer was achieved via coupling of amino linker at 5′ terminus of DNA/RNA aptamer with the activated SAM. Radi et al. [89] implemented the SAM technique for immobilisation of ferrocene-labelled aptamer thiol on gold electrode. After the immobilisation, an electrode was immersed in 50 µl of 1.0 M KH₂PO₄ buffer solution containing 0.1 M 2-mercaptoethanol (2-ME) for 10 min to block the surface forming a mixed monolayer to prevent nonspecific binding of substrate. Subsequently, an electrode was placed in 1.0 M HCl under a stirring environment for 10 min and followed by a cleaning with deionised water to remove any trace of counterions.

A method based on specific interactions via biotin-streptavidin/avidin/neutravidin was used for immobilisation of aptamers on gold surface. The orientation of the immobilised molecules could be controlled using this technique. Ostatná et al. [53] implemented the biotin-streptavidin/avidin/neutravidin immobilisation technique for the attachment of aptamers to gold. The gold surface of a SPR chip was initially cleaned with piranha solution (1:3 mixtures of 30% hydrogen peroxide and sulphuric acid) and deionised water, and subsequently dried under nitrogen stream.

The chip was then immersed in alkanethiols solution containing 7:3 mixtures of C₁₁OH (provide a stable non-fouling background) and C₁₆COOH (used for immobilisation of streptavidin/avidin/neutravidin) at room temperature in a dark room for 12 hr. Subsequently, it was rinsed with ethanol, dried with nitrogen
followed by rinsing with deionised water and dried with nitrogen. Activation of carboxylic group was achieved by dissolving N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate in 1 mg/ml dimethylformamide for 4 hr. After the activation step, the chip was cleaned with water and dried using nitrogen. A layer of streptavidin, avidin or neutravidin was formed by flowing streptavidin, avidin or neutravidin in sodium acetate buffer over the pre-activated surface at flow rate of 30 µl/min for 20 min. Nonspecific adsorption was removed using a solution of high ionic strength. Biotinylated DNA aptamer was immobilised on the surface at a flow rate of 30 µl/min for 30 min. Meanwhile, Bini et al. [51] further modified the carboxylated dextran-coated SPR chip by first activating the surface with 50 mM NHS and 200 mM EDAC followed by streptavidin coating and surface blocking using 35 µl of 1 M ethaloamine. After thermal treatment at 90 °C for 1 min and 0 °C for 10 min, 100 µl of biotinylated aptamer in immobilisation buffer was injected and biotin was used to saturate the remaining free streptavidin sites to block the surface.

Aptamer immobilisation on carbon nanotube (CNT) usually involves surface modification before attaching the aptamer to the CNT surface via linker molecules. Aptamer immobilisation on single walled carbon nanotube substrate, demonstrated by So and colleagues [11], was achieved through initial pre-treating the CNT wall with carbodiimazole-activated Tween 20 followed by covalent immobilisation of 3'-NH₂ of thrombin aptamer to the carbodiimazole moiety. A different strategy was employed by Maehashi and colleagues [74] utilising 1-dimethylformamide as the linker. The reagent is adsorbed to the sidewalls of CNT
by incubating 5 mM of pyrenebutanoic acid succinimidyl ester in dry dimethylformamide solutions for 1 hr. IgE aptamers was then covalently attached to the CNT channels via the 5′-amino-modified terminus.

In the work by Yoon et al. [179], a conducting polymer nanotube was utilised as a substrate for thrombin detection. Surface immobilisation of thrombin aptamer involves surface modification by copolymerising pyrrole-3-carboxylic acid (P3CA) with pyrrole in a reverse microemulsion system to produce carboxylic acid-functionalised polypyrrole (CPPy) nanotubes. Afterwards, CPPy nanotubes were chemically tethered onto a (3-aminopropyl)trimethoxysilane (APS)-modified microelectrode substrate via coupling reactions between carboxyl groups of CPPy and the amino groups of APS. Amine-terminated thrombin aptamers were subsequently bound to the nanotube via covalent binding.

The earliest work of immobilisation of aptamer on a glass substrate was reported by Potyrailo et al. [180] in 1998. Anti-thrombin DNA aptamer was covalently attached onto a glass microscope cover slide. Firstly, the glass surface was partially converted to diol silica by suspending the cleaned glass slides in a 10% aqueous solution of (glycidoxypropyl)trimethoxysilane. After degassing for 10 min, the reaction proceeded at constant pH of 3.5 at 90 °C for 4 hr with occasional shaking. The slides were then rinsed with distilled water, acetone, and ether and dried in vacuum overnight at 100 °C. The slide surface was activated in 0.5 M 1,1'-carbonyldiimidazole in dry acetonitrile for 1 hr at 20 °C, followed by rinsing with acetonitrile and being dried. Coupling of aptamer was achieved by incubating 3′-amine-modified ssDNA aptamer in 50 mM sodium phosphate (pH
8) buffer on the activated surface at 20 °C in air at ~60% relative humidity. Unreacted aptamer was rinsed off with phosphate buffered saline (PBS) and ethanolamine solution was used to block unreacted surfaces.

Recently, Ho et al. [181] demonstrated aptamer immobilisation on glass and polydimethylsiloxane (PDMS) substrate via short DNA duplexes. DNA oligomer 1 was covalently attached to aldehyde functionalised glass via amine-modified 5′-terminus. Saline sodium citrate (SSC) containing 4% bovine serum albumin (BSA) was used to reduce non-specific binding. Cy3-modified oligomer 2 and 0.2 μM biotin-modified oligomer 3 were hybridised for 1 hr to form 1·2·3 complex. The PDMS coupled to the oligomer 3 bipartite aptamer via biotin-streptavidin method. PDMS 4×4 pillar pieces surface was activated in 12.5% HCl overnight and derivatised with (3-glycidoxypropyl)-trimethoxysilane to generate epoxide groups. NH₂-PEG-biotin was spotted on PDMS pillar followed by overnight incubation in argon atmosphere at 80 °C. Double-distillation water was used to remove excess polymers. Finally, PDMS was incubated with 1 µg/ml streptavidin in SSC and 0.4% BSA for 30 min, washed and gently dried with nitrogen gas. A summary of the reported aptamers immobilisation techniques is given in Table 6-1.
<table>
<thead>
<tr>
<th>Immobilisation method</th>
<th>Substrate</th>
<th>Aptamer</th>
<th>Linker/spacer</th>
<th>Ref.</th>
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<tr>
<td>Specific interaction (biotin-streptavidin)</td>
<td>Dextran-modified gold surface</td>
<td>44-mer RNA aptamer: 5′-biotin- TT TTT TTT TTT TTT GGCUGUAAGGU GGUCGGUGUGG CGAGUGUGUUA GGAGAGAUGUC-3′</td>
<td>Polytymine</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>44-mer RNA aptamer: 5′-biotin-TEG- GCCUGUAAGGU GG’UCG GU GUGGCGAGUGU GUUAGGAGAGA UUGC-3′</td>
<td>Triethylene glycol (TEG)</td>
<td></td>
</tr>
<tr>
<td>Specific interaction (biotin-streptavidin)</td>
<td>Magnetic beads</td>
<td>(i) 29-mer DNA aptamer: 5′-biotinylated- TTTTTTTTTTGT TGG TGTGTTGG-3′  (ii) 29-mer DNA aptamer extended with 21-mer G/C-rich sequence: 5′-GCCTCGCGATGG TGGACGTAGAGT CCGTGGT AGGGCAGGTTG GGGTGACT-3′</td>
<td>-</td>
<td>[71]</td>
</tr>
<tr>
<td>Specific interaction (biotin-streptavidin/avidin/neutravidin)</td>
<td>Gold</td>
<td>32-mer DNA aptamer: 3′-GGG TTT TCACTT TTG TGG GTT GGT GTG GTT GG-5’ (APTA)</td>
<td>-</td>
<td>[53]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20-mer DNA aptamer: 3′-CCA ACGGT TT GTG GTT GG-5’ (LOOP)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Immobilisation method</td>
<td>Substrate</td>
<td>Aptamer</td>
<td>Linker/spacer</td>
<td>Ref.</td>
</tr>
<tr>
<td>-----------------------</td>
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<td>------</td>
</tr>
<tr>
<td>SAM (thiol) Gold</td>
<td>19-mer DNA aptamer: 3'-SH-(CH$_2$)$_6$-GGTTGG TGTGGT TGG-5' (APTA-SH)</td>
<td>6 hydrocarbon-(CH$_2$)$_6$-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAM (amine) Gold</td>
<td>25-mer RNA aptamer: 5'-NH$_2$-(CH$_2$)$_3$-GGGAACAAAGCUGAAGUACUUACC C-3'</td>
<td>3 hydrocarbon-(CH$_2$)$_3$-</td>
<td>[178]</td>
<td></td>
</tr>
<tr>
<td>SAM (amine) Gold</td>
<td>15-mer DNA aptamer: 5'-NH$_2$-(CH$_2$)$_3$-GGTTGGTGTGGT TGG-3'</td>
<td>3 hydrocarbon-(CH$_2$)$_3$-</td>
<td>[178]</td>
<td></td>
</tr>
<tr>
<td>SAM (thiol) Gold</td>
<td>32-mer DNA aptamer: 5'-HS-(CH$_2$)$_6$-AGAGAACCTGGGGGAGTATTGC GGAGGAAGGT-3'</td>
<td>6 hydrocarbon-(CH$_2$)$_6$-</td>
<td>[177]</td>
<td></td>
</tr>
<tr>
<td>SAM (amine and thiol) Gold</td>
<td>15-mer DNA aptamer: 5'-NH$_2$-GGTTGGTGTGGT TGG-SH-3'</td>
<td>6 carbon spacers at each terminus</td>
<td>[67]</td>
<td></td>
</tr>
<tr>
<td>Surface modification CNT</td>
<td>15-mer ssDNA aptamer: 5'-GGTTGGTGTGGT TGG-NH$_2$-3'</td>
<td>CDI-Tween</td>
<td>[11]</td>
<td></td>
</tr>
<tr>
<td>Surface modification CNT</td>
<td>D17.4ext: 5'-NH$_2$-GCCGGGGCACGTTTATCCGTCCTCCTCCTAG TGGGTGCCCGCGC-3'</td>
<td>5'-GCCG-3'</td>
<td>[74]</td>
<td></td>
</tr>
<tr>
<td>Surface modification CPPy</td>
<td>15-mer ssDNA aptamer: 5'-GGTTGGTGTGGT TGG-3'</td>
<td>Primary aliphatic amino linker</td>
<td>[179]</td>
<td></td>
</tr>
</tbody>
</table>
According to the literature thus far, no work has been published on aptamer immobilisation on SU-8 surface. In addition, most work reported on surface functionalisation using wet chemical methods. There is no investigation conducted on surface functionalisation using plasma for aptamers immobilisation. To the best of our knowledge, the method presented in this chapter is the first reported work on plasma SU-8 surface modification for aptamer immobilisation. The next section outlines the protocols involved for SU-8 surface functionalisation via oxygen plasma.

6.3 Materials and reagents

6.3.1 Surface cleaning

Sulphuric acid (H_2SO_4 96%), and hydrogen peroxide (H_2O_2 30%) were purchased from Sigma Aldrich. The percentages are the dilutions as they arrive in bottle and were used as it is.
6.3.2 Surface immobilisation

**Surface functionalisation:** Oxygen gas

**Carboxyl activation:** Sodium phosphate buffer pH 6, hydrochloric acid, 1-[3-(dimethylamino)propyl]-3-ethylcarboiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS).

**Aptamer immobilisation:** 15 bases DNA thrombin aptamer with a T₅ linker, TBA15-1 (molecular weight: 6705.5 g/mol) with the sequence of 5'-GGT TGG TGT GGT TGG TTT TT-NH₂-3' and 15 bases control aptamer with a T₄ linker CR-1 (molecular weight: 6376.3 g/mol) with the sequence of 5'-GTG GTG GTT GTG GTT TTT T-NH₂-3' were purchased from Integrated DNA Technologies and diluted to the 0.1 µM concentration for each of the aptamer. 1 mg/ml bovine serum albumin (BSA), 0.1 M sodium phosphate buffer at pH 7 was purchased from Sigma Aldrich.

6.3.3 Tools and equipments

A plasma reactor system (a purpose built, inductively coupled with RF 13.56 MHz reactor), contact angle tester, tweezers, atomic force microscope, Eppendorf pipettes, and 500 µl vials.

6.4 Experimental procedures

6.4.1 Surface functionalisation using gas plasma treatment

Plasma treatment of the SU-8 surface was conducted using an in-house built reactor inductively coupled with RF 13.56 MHz (see Figure 6-1) [182]. Plasma
treatment was performed in a 30cm-long and 7.5 cm-radius tubular glass reactor enclosed in a Faraday cage. A specially designed antenna in the inlet of the chamber acts as the plasma source, leaving the rest of the chamber as the afterglow area. It could transfer more than 100% of RF power into the plasma using the incorporated RF matching network. Also, it could be operated either in a continuous wave or a pulsed mode.

Figure 6-1: Block diagram of the plasma system used for the microcantilever surface functionalisation (Reprinted (adapted) from [183]. Copyright 2009, with permission from John Wiley and Sons).

Figure 6-2: Photograph of the plasma system.
Microcantilever surfaces were cleaned before surface functionalisation. Microcantilever surfaces were dipped in piranha solution for 3 mins, followed by rinsing in DI water for 5 mins. The rinsing step was repeated thrice. Then, the microcantilevers were placed in the plasma source area and the chamber was evacuated to achieve a base pressure lower than $1 \times 10^{-3}$ mbar using a rotary pump.

Carboxyl functional groups were introduced to the surface via oxygen gas plasma treatment. A combination of continuous plasma and pulsed plasma mode was used. Argon plasma was applied for surface cleaning and activation prior to oxygen plasma treatment. Then, continuous wave plasma was applied to the microcantilever surface followed by a pulsed plasma treatment. The plasma parameters are listed in Table 6-2. After the plasma treatment, the gas flow was stopped and the aptasensor was left in the chamber under vacuum for 30 min to allow the surface to stabilise before exposing to air.

<table>
<thead>
<tr>
<th>Table 6-2: Plasma conditions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon plasma</td>
</tr>
<tr>
<td>Power, (W)</td>
</tr>
<tr>
<td>Pressure, (mbar)</td>
</tr>
<tr>
<td>Time, (s)</td>
</tr>
<tr>
<td>Duty cycle, (%)</td>
</tr>
</tbody>
</table>

6.4.2 Folding of aptamers

Aptamers were diluted to appropriate concentration in nuclease free water before use, refer to Section 6.3.2. Next, aptamers were heated at 85°C for 5 min. After heating, aptamers were allowed to cool to room temperature for 10 min. Aptamers were then incubated at 37°C for 15 min.
6.4.3 Activation of carboxyl groups

A total of 200 µl of 1:1 of 0.2 M of EDC and 0.05 M of NHS was mixed in PBS buffer. HCl was added to the activation solution to reduce the pH value to pH 6. The activation solution needs to be prepared fresh. The microcantilever was left soaking for 1 hr in the activation solution. After an hour, the microcantilever was rinsed with DI water and pure ethanol. The microcantilever was left to dry at ambient temperature.

6.4.4 Immobilisation of DNA aptamers

The activated surface was then immersed in solution of 0.1 M sodium phosphate buffer pH 7 (PBS) containing 0.1 µM of 3′-end amine modified DNA aptamer for 2 hr at room temperature. The surface was rinsed with 200 µl 1 mg/ml bovine serum albumin (BSA) solution in 0.1 M PBS buffer thrice for about 30 mins to remove physically adsorbed DNA aptamer and to block the remaining carboxyl. The blocked aptamer surface was soaked in 0.1 M PBS buffer to remove unbound BSA for 10 min, and was rinsed thoroughly with PBS. An overview of the immobilisation procedure is shown in Figure 6-3.

Figure 6-3: Flowchart illustrating aptamer immobilisation procedure.
6.4.5 Surface morphology

An Atomic Force Microscope from Veeco Instruments Inc. and a Dimension® Icon® Atomic Force Microscope were used to characterise the surface roughness of the aptasensor surface before and after plasma treatment, as well as after aptamer immobilisation. The tapping mode instead of the contact mode was used for the surface scanning to prevent the microcantilever tip from dragging the biomolecules on SU-8 surface. Due to the small dimensions of the microcantilevers, the characterisation had been performed on a larger SU-8 substrate which had been functionalised and immobilised in parallel with the microcantilevers.

Contact angle tester was used to examine the wettability property of SU-8 surface. DI water was utilised in the experiment.

6.5 Results and discussions

6.5.1 Surface functionalisation

Surface functionalisation refers to the introduction of functional groups on the sensor surface for biomolecules immobilisation. Plasma treatment uses gasses such as argon, oxygen, nitrogen, ammonia or tetrafluoromethane to introduce chemical functionalities onto the substrate surface or create radicals for cross-linking or subsequent surface grafting [184]. Compared to the conventional wet chemical method used for surface modification or functionalisation, gas plasma treatment offers the benefits of ease of implementation and it is a one-step process performed under dry environment.
Oxygen gas was used during the plasma treatment to produce functional carboxyl (-COOH) groups on the SU-8 surface. Free epoxy groups on the SU-8 surface were cleaved to form carboxyl groups (as shown in Figure 6-4 and Figure 6-5). However, oxygen plasma treatment also generated other functional groups such as hydroxyl group (-OH) and carbonyl group (-C=O) by opening the epoxy rings in the SU-8 chemical structure. Therefore, the plasma conditions were optimised to produce higher concentration of carboxyl groups.

There are two types of plasma treatment modes that could be used to functionalise the SU-8 surface, namely: continuous wave plasma and pulsed plasma. Plasma consists of reactive species such as charged particles, neutral particles, free radicals and UV photons. In continuous wave plasma, the plasma is constantly ‘ON’, having constant ion bombardment and UV radiation that result in cross-linking polymer structures on the surface and lowers the required surface functionalities.

On the other hand, pulsed plasma which has an average input power dependent on the applied duty cycle, DC \( (P_{\text{avg}} = P_{\text{peak}} \times DC) \). The duty cycle is calculated based on the plasma ‘ON’, \( (t_{\text{on}}) \) and ‘OFF’, \( (t_{\text{off}}) \) time.

\[
DC = \frac{t_{\text{on}}}{t_{\text{on}} + t_{\text{off}}} \tag{6.1}
\]

Modulating the plasma enables the control of free oxygen radical density on the SU-8 surface as it allows only free radicals to be attached to the surface without any dispersion by charged particles and UV photons during the ‘OFF’ time.
Besides that, pulsed plasma can minimise the etching of the SU-8 surface compared to continuous wave plasma. Pulses plasma however suffers from the stability issue due to the lower cross-linked polymer network. A combination of continuous wave and pulsed plasma treatment produced a surface layer with the advantages of high stability layer obtained from continuous wave plasma. Also, a high-density functional groups layer was obtained via pulsed plasma [185]. Therefore, surface functionalisation of the aptasensor was performed using oxygen plasma in two stages where the first stage was the continuous wave plasma and the second stage was the pulsed plasma.

3'-amine-terminated aptamers were immobilised on the surface via electrostatic interactions between the functional carboxyl group on the surface and functional amine group at the aptamer. However, the carboxyl functionalised surface needed to be activated using EDC and sulfo-NHS before it could react with the primary amine functional group attached to the aptamer. EDC converted carboxylic group into an unstable O-acylisourea ester reactive intermediate that was susceptible to amine attacks. The unstable intermediate could result in undesired products. Hence, sulfo-NHS was used in conjunction with EDC to assist the carboiimide coupling. The resultant product was a semi-stable sulfo-NHS ester which further reacted with the primary amine group attached to the DNA aptamer forming an amide bond. BSA was used to block the remaining surface of the aptasensor to prevent unspecific binding. Aptamers were heated at 85 °C prior to surface immobilisation to prevent the formation of intermolecular complexes [186, 187]. The thermal treatment unfolds the DNA strand making the
amine functional group at 3’end easily accessible for interaction with carboxyl functional groups on the aptasensor surface.

![Figure 6-4: Chemical structure of SU-8 polymer.](image)

6.5.2 Contact angle measurement

Contact angle measurement was carried out on a pristine SU-8 surface. Figure 6-6 and Figure 6-7 show the surface wettability of the SU-8 surface. A droplet of water was pipetted onto the surface of untreated SU-8 and based on the photograph shown in Figure 6-6, it is evident that the pristine SU-8 surface is

![Figure 6-5: Chemical structure of SU-8 after oxygen gas polymerisation.](image)
highly hydrophobic. Using a contact angle tester, the measured water contact angle was 89.4°. Immediately after oxygen plasma treatment, a DI water droplet was dispensed onto SU-8 using a pipette. Based on Figure 6-7, it observed that the surface wettability had increased significantly. The oxygen plasma treatment had modified the surface property of the SU-8 substrate from hydrophobic to hydrophilic with a measured water contact angle of 10.3°.

Figure 6-6: Water contact angle of pristine SU-8 surface.

Figure 6-7: Water contact angle after oxygen plasma of SU-8 surface.
6.5.3 Surface morphology

Figure 6-8 to Figure 6-10 show the surface morphology of the SU-8 for the untreated and the plasma treated SU-8 surface. The surface area of the images are 1 µm × 1 µm. Figure 6-8 shows the AFM image of the pristine SU-8 surface taken before the surface functionalisation. The surface was quite smooth with surface roughness RMS of 3.44 nm. Referring to Figure 6-9, after surface functionalisation using oxygen plasma, the surface roughness RMS had increased to 17.95 nm. This shows that the plasma treatment caused the SU-8 surface corrugation which was not observed in the pristine SU-8 surface. This is because, apart from modifying the surface chemistry of SU-8, oxygen plasma treatment also induced etching on the surface. Increased surface roughness also plays a role in changing the wettability properties of SU-8 which was reported in the previous section. From the image, it is also observed that a uniform nano-structured surface has been formed at the plasma treated sample.

Meanwhile, Figure 6-10 illustrates the morphology of the plasma treated SU-8 surface after aptamer immobilisation. Comparing the SU-8 surface shown in Figure 6-9 and Figure 6-10, the surface morphology after aptamer immobilisation was smoother with the surface RMS roughness reduced from 17.95 nm to 11.68 nm. The aptamer molecules are larger in size compared to the functional groups on the surface. Hence, amine functional groups on the aptamers interacted and attached to the carboxyl functional groups leading to a smoother surface morphology which was evident in the reduction of surface roughness value. This
demonstrates the binding of thrombin aptamer to the functionalised group on the SU-8 surface, and therefore the successful immobilisation of thrombin aptamers.

Figure 6-8: Image morphology of the pristine SU-8 surface.

Figure 6-9: Image morphology of the oxygen plasma treated SU-8 surface.
Figure 6-10: Image morphology of the oxygen plasma treated SU-8 surface after thrombin DNA aptamers immobilisation.

6.6 Summary

Surface immobilisation affects the binding affinity, specificity and sensitivity of an aptasensor. Therefore, careful consideration is required when designing the immobilisation protocols. It is of paramount importance that the anchoring of the aptamers to the aptasensor surface is stable and possessed strong linkage so that they are not easily displaced in the biological medium. In this work, oxygen plasma was used to introduce carboxyl functional groups onto the non-reactive SU-8 surface of the aptasensor. A combination of continuous wave and pulsed plasma was implemented for the SU-8 surface functionalisation to achieve a stable polymer network layer with a high density of functional groups. Plasma treatment on the SU-8 surface caused its surface property to change from hydrophobic to hydrophilic. EDC/Sulfo-NHS was used to activate the carboxyl
functional groups before reacting with amine functional group that is attached to the thrombin DNA aptamers to form self-assembled monolayer. AFM analysis was conducted before and after surface functionalisation and immobilisation. Results showed that the devised immobilisation protocols were successful.
CHAPTER 7

APTASENSOR CHARACTERISATION AND BIOLOGICAL EVALUATION

7.1 Background

To demonstrate the proof-of-concept of the developed aptasensor biological testing was conducted. Thrombin molecule was chosen as the system model. It is reported that thrombin plays a role in many diseases including thrombosis, neuronal disease such as Alzheimer’s [188], pulmonary metastasis [189, 190], and pulmonary fibrosis [191]. Apart from that, thrombin is a major target for anticoagulation and cardiovascular disease therapy [192]. Thrombin-aptamer binding has been vigorously investigated and characterised. As such, demonstrating thrombin detection using the devised aptasensor is very relevant for point-of-care diagnostics or drug discovery.

Microcantilevers needed to be calibrated prior to biological testing in order to get accurate measurements. This chapter begins with a description of thrombin molecules, followed by the listing of the apparatus and reagents used in the experiments. Next, the methods used for characterisation of sensitivity and spring constant of the fabricated microcantilevers are described. Biological testing with thrombin molecule is conducted and the corresponding deflection measurement is
observed and recorded. The results obtained for both the microcantilever calibration and thrombin detection are also discussed.

7.2 Thrombin

Thrombin molecule is a serine protease that recognises multiple macromolecular substrates and has both procoagulant and anticoagulant functions. It is responsible for the blood coagulation by converting the soluble fibrinogen into insoluble strands of fibrin. Thrombin also plays a role in platelet aggregation regulation and endothelial cell activation [193].

Thrombin molecule has a pronounced uneven charge distribution that causes a high positive and negative electrostatic filed strength outside the thrombin surface [194]. Thrombin consists of several functional regions which include the active site, a polar binding site and two anion-binding exosites; anion-binding exosite I or also referred as fibrinogen-recognition exosite and anion-binding exosite II or also referred as heparin-binding exosite [195]. The fibrinogen-recognition exosite interacts with fibrin, hirudin, thrombin modulin, and thrombin receptor. Meanwhile, the stronger positive heparin-binding exosite binds with heparin, and prothrombin fragment F2. Temporal sequence of thrombin substrates is governed by the interplay of fibrinogen-recognition exosite and heparin-binding exosite. The TBA15-1 aptamer primarily binds to the fibrinogen-recognition exosite of thrombin with dissociation constant, $K_d$ of 100 nM [196].
7.3 Materials and reagents

7.3.1 Thrombin Binding

0.1M Tris-acetate pH 7.4, and human α-thrombin 50% glycerol/water (v/v) (molecular weight: 36,700 g/mol) was purchased from Haematologic Technologies and diluted to the appropriate concentration before use.

7.3.2 Tools and equipments

Tweezers, JPK NanoWizard® II atomic force microscope, BioCell™ coverslip based liquid cell, pipettes, glass slide were used.

7.4 Experimental procedures

7.4.1 Microcantilever sensitivity calibration and characterisation

To calibrate the sensitivity of the microcantilever, a force distance measurement on a hard clean surface i.e. glass slide was conducted. The microcantilever was calibrated and characterised using JPK NanoWizard® II AFM. The fabricated SU-8 microcantilever was fixed to a glass block holder with a spring using a pair of tweezers and mounted on the AFM head. Clean glass slide was placed at the sample holder situated on the AFM stage. The microcantilever was moved towards the glass slide surface by setting the z-length piezo movements. The force-distance curve was obtained via ‘Calibration Manager’ menu tab and the linear portion of the curve was used for sensitivity fitting.
After obtaining the sensitivity measurement, the spring constant of the microcantilever was obtained using a thermal noise measurement technique. The microcantilever was retracted from the glass slide surface and then oscillated. Its resonance frequency was obtained and fitted with Lorentz curve to calculate its spring constant value.

7.4.2 Biomolecules detection

System Setup

For the detection of thrombin molecules, the optical level read-out method was implemented. The fabricated microcantilevers were mounted onto the NanoWizard® II AFM using a pair of tweezers by clamping the microcantilevers to a glass block holder via a spring and attaching it to the AFM head. The deflection of the microcantilever was determined by reflecting a laser beam off the gold-coated surface of the microcantilever. A four-segment photodiode was used to detect the angle of deflection. The laser beam was adjusted so that the laser spot was at the end of the microcantilever (as shown in Figure 7-1) and the mirror as well as the detector were adjusted so that the laser beam was positioned at the centre of the detector in the laser alignment window shown in the SPM software developed by JPK. A BioCell™ coverslip based fluidic cell (from JPK Instruments AG, Berlin, Germany) with a volume capacity of 400 µl was mounted onto the AFM stage for the thrombin detection experiment in a controlled environment. The detection system setup is depicted in Figure 7-2.
Figure 7-1: Optical image showing the laser beam adjusted onto the end of the microcantilever.

Figure 7-2: Thrombin molecule detection system setup.

**Binding experiment**

The thrombin molecules solution obtained from the supplier was aliquot and diluted to the desired concentration before use. Prior to introducing the thrombin molecules into the fluidic cell, the microcantilevers were equilibrated in the buffer.
solution for an hour (0.1 M Tris-acetate pH7.4) to obtain a baseline of the
deflection of the microcantilevers. 1 µM of thrombin in binding buffer solution
was injected to the fluidic cell and incubated for 30 min. Next, the buffer solution
was introduced to remove unbound thrombin molecules on the microcanti lever
surface. The deflection of the microcantilever was monitored in-situ using the
real-time oscilloscope function in the SPM software developed by JPK. The
measurements were recorded in a static environment without the continuous flow
of the buffer solution. The binding experiment was conducted in a controlled
temperature of 27±0.2 °C. A microcantilever with immobilised control aptamer s
served as a control sample in the experiment to test nonspecific interactions.

7.5 Results and discussions

7.5.1 Sensitivity calibration

The photodetector measures the microcantilever deflection, \( z_v \) in Volts by
measuring the differential voltage between the four segments of the photodiodes.
To obtain the height value, \( z \) which is comparable to the scanner’s \( z \)-piezo height,
\( z_P \) a conversion factor is needed. This conversion factor is known as the sensitivity
\( S \) of the microcantilever:

\[
z = Sz_v \tag{7.1}
\]

From the conducted force spectroscopy analysis on a glass slide surface, a
characteristic force-distance-curve was recorded and shown in Figure 7-3. It is
noteworthy that the indentation of the substrate is negligible or zero as the SU-8
microcantilever is very soft and the vertical deflection of the microcantilever corresponds to the z-piezo height of the scanner. Then the sensitivity \( s \) is related to the constant slope in the repulsive range of the curve:

\[
S = -\frac{\Delta z_p}{\Delta z_v}
\]  

(7.2)

Hence, a sensitivity fit was performed only on the linear part of the curve shown in Figure 7-3 and the measured value was 1.378 \( \mu \text{m/V} \).

![Figure 7-3: Graph showing the force-spectroscopy of the SU-8 microcantilever. The red colour line represents microcantilever extends while the dark red line represents microcantilever retracts.](image)
7.5.2 Spring constant

The thermal vibrations of the microcantilever deflection were measured by the SPM software (JPK Instruments AG, Berlin, Germany). A frequency spectrum that illustrates a peak at the microcantilever’s resonance frequency was obtained and the area under the resonance peak was fitted. The spring constant was calculated using the fitting algorithm by applying the equipartition theorem (refer to Figure 7-4). The measured resultant spring constant for 1000 × 200 × 2 µm SU-8 microcantilever was 1.389 mN/m and the theoretical calculation of spring constant using Equation (4.2) is 1.6039 mN/m. The percentage error between the measured and calculated value is 13.39%. This value is one magnitude higher compared to the spring constant of the silicon nitride microcantilever [87]. This shows that the low Young’s modulus of SU-8 which is 40 times lower than its silicon counterpart produced a much more sensitive and flexible microcantilever beam for aptasensor applications.

To perform differential sensing, it is important to use identical microcantilevers as reference and sensing components. Therefore, the resonance frequency of the fabricated microcantilevers was evaluated via microcantilever tuning in SPM software to investigate the variations among them. The measured deviation resonance frequency was 1.402±0.051 kHz.
7.5.3 Thrombin molecules detection

To improve interaction and binding accessibility between DNA aptamers and thrombin molecules, detection of thrombin molecules was conducted in binding buffer solution (Tris-acetate pH7.4). This would favour the aptamer to fold into a G-quadruplex structure, a 3D structure configuration with intricate binding pockets that binds specifically to the thrombin binding domain. Figure 7-5 illustrates the bending of the microcantilever beam after thrombin molecules bind to the immobilised thrombin DNA aptamers on the aptasensor surface. Meanwhile, Figure 7-6 shows the aptasensor response when 1 µM of thrombin
was injected into the fluidic cell. The response curve showed an increase in the vertical deflection with a maximum displacement of 1900 nm when the microcantilever reached its steady-state. Using Stoney’s equation to find its corresponding surface stress, the microcantilever sensitivity was calculated and it was equal to 130.683 $\mu$m/ N/m. The microcantilever was immobilised with aptamers on its top side and mounted inverted on the AFM head. Therefore, a positive deflection value indicates that the aptasensor experience compressive surface stress while a negative deflection value means tensile surface stress. The obtained beam deflection value for aptasensor fabricated using SU-8 was of one magnitude higher than those made of silicon nitride. This means that the fabricated aptasensor was able to generate a larger displacement for the same amount of surface stress which leads to a higher aptasensor sensitivity. The drift and external noise such as thermomechanical and turbulence during injection were compensated by using a reference microcantilever which both side of its surfaces had been blocked using BSA.

Figure 7-5: When thrombin molecules bind to the immobilised thrombin DNA aptamers, the microcantilever will experience a change of surface stress between the top, immobilised surface, and the bottom, blocked surface. This leads to the bending of the microcantilever beam.
To evaluate the specificity of the aptasensor, control aptamer, CR-1 was immobilised onto the sensor surface and similar concentration of thrombin was injected and incubated for the same amount of time, 30 min in the binding buffer solution. It could be seen from the red line in Figure 7-6 that the aptasensor did not respond in any measurable deflection. This shows that the aptasensor has a high specificity.

![Graph showing deflection as a function of time](image)

**Figure 7-6:** Injection of 1 μM thrombin molecules in Tris-acetate buffer at pH 7.4. Graph shows the bending response of the aptasensor as a function of time.
During the thrombin binding experiment, the resonance frequency of the aptasensor was also measured to confirm the thrombin detection. The resonance frequency was found using the microcantilever tuning window found in SPM software. Referring to Figure 7-8, the resonance frequency of the aptasensor in liquid before thrombin injection was around 50 kHz. After thrombin injection into the fluidic cell, the resonance frequency peak shifted to the left. After incubating for 30 mins, the aptasensor surface was rinsed with binding buffer and it could be observed that its amplitude was reduced. After another 30 mins, its amplitude was reduced significantly and the resonance frequency was shifted to around 20 kHz. The change of resonance frequency indicates that the aptasensor experienced a change in mass which was caused by the binding of thrombin molecules on the aptamer immobilised aptasensor surface.
Figure 7-8: Resonance frequency response.

To verify that the thrombin molecules did indeed bind to the immobilised aptamers on the aptasensor surface, a comparison of the aptasensor surface topography before and after the thrombin binding experiment was conducted using AFM. Based on the left image in Figure 7-9, it is observed that initially the aptasensor surface had peaks and valleys which indicate that the surface is rough with RMS roughness of 11.68 nm. The image on the right which was scanned after the thrombin-aptamer binding shows that the surface is flatter and smoother with the RMS roughness value of 7.23 nm. This demonstrates that the thrombin molecules successfully bind to the immobilised aptamers on the aptasensor surface.
7.6 Summary

The fabricated aptasensor was characterised and calibrated before being utilised for protein detection. The measured calibration values were its sensitivity, 1.378 µm/V and its spring constant, 1.389 mN/m. The obtained spring constant was very close to the theoretical calculation of 1.6039 mN/m with a percentage error of 13.39%. The aptasensor capability to perform label-free detection of biomolecules was also demonstrated using an optical lever read-out method. Injection of 1µM thrombin molecules into the fluidic cell containing the aptasensor generated a beam displacement of 1900 nm. A positive displacement value signifies that the interaction between the thrombin molecule and the thrombin aptamer generated a compressive surface stress. This value is of one magnitude higher than the aptasensor made from silicon nitride. All the measurements for the beam displacement were compensated for drift and external noise via a reference microcantilever. Through the surface topography analysis, binding of thrombin molecules to the immobilised aptamers layer was verified. The specificity of the
aptasensor was demonstrated by using a control aptasensor that was immobilised with control aptamer. No significant response was observed for the control aptamer when thrombin molecules were injected into the fluidic cell.
CHAPTER 8

CONCLUSION AND FUTURE OUTLOOKS

8.1 Conclusion

This thesis reported the design, development, and surface immobilisation of a surface-stress-based microcantilever aptasensor using SU-8 as the fabrication material. SU-8 is a negative photoresist epoxy-acrylate polymer that is highly transparent in the UV region, is biocompatible, has low Young’s modulus, and is chemically resistant. Furthermore, the SU-8 fabrication technology is compatible with standard silicon processes and this leads to the ease of integrating it with circuits.

The underlying principle of surface-stress-based microcantilever aptasensor was described. The source of surface stress that caused the deflection of the microcantilever was explored and identified. The fabricated aptasensor consists of a beam with one fixed end. The bottom side of the SU-8 microcantilever was coated with a thin layer of gold that serves as a reflective surface for the laser beam in optical lever read-out method. The dimensions of the developed aptasensor were optimised using the finite element method.

Surface micromachining was carried out for the aptasensor fabrication. The microfabrication process was explored, developed and optimised to improve the fabrication yield. Three different methods of releasing the final aptasensor
structure from the silicon substrate were investigated. The release methods include dry release technique using hydrophobic fluorocarbon layer, wet chemical release using Omnicoat, and wet chemical release using PMMA photoresist as the sacrificial layer. Results showed that the wet chemical release using PMMA produced the best yield percentage of 90%. The wet chemical release method using Omnicoat produced the lowest yield. The fabricated aptasensor was characterised using AFM and its spring constant was 1.389 mN/m. This value is of an order of a magnitude higher than that of silicon nitride based microcantilever.

Detection of thrombin molecules using the fabricated aptasensor was carried out for proof-of-concept. Thrombin DNA aptamers, which are nucleic acid ligands, were immobilised on the aptasensor surface. Aptamers offer several advantages over antibodies as they can be synthetically synthesised, posses high binding affinity, have good storage life, and be regenerated. This thesis is the first reported work on the immobilisation of aptamers on SU-8 surface. A novel plasma treatment technique that utilised a combination of continuous wave plasma and pulsed plasma modes was used to create reactive species on the aptasensor surface for aptamer immobilisation. Oxygen gas was used in the plasma treatment to produce carboxyl function groups on the aptasensor surface. Surface morphology of the treated and immobilised surface were characterised using AFM.

An optical lever read-out using AFM was implemented to measure the displacement of the microcantilever aptasensor. Thrombin molecules were
injected into the fluidic chamber and a real-time beam displacement was recorded. The measured deflection was 1900 nm for 1 µM thrombin molecules. The calculated microcantilever sensitivity was 130.683 µm/N/m. Thrombin molecules were verified bound to the immobilised surface via examination of the surface morphology before and after thrombin immobilisation using AFM. The drift and noise in the system was compensated using a reference microcantilever. Specificity of the fabricated aptasensor was also evaluated using an aptasensor immobilised with control aptamers. The output signal generated showed no significant bending for the control aptasensor. Essentially, the fabricated aptasensor could be used for other proteins or nucleic acids detection as long as its surface is immobilised with ligands that recognise the analyte of interest.

8.2 Future outlooks

A microcantilever-based aptasensors is a very promising sensing platform for point of care diagnostics, drug discovery, and genetics analysis. It is a label-free approach, capable of batch processing, is cost-effective, and most importantly offers rapid analysis via the microarray format.

To further improve its sensitivity performance for low concentration protein detection, a closed-loop feedback sensing system using PID controller could be incorporated. This can be achieved using an active microcantilever by incorporating an actuator and a sensing microcantilever into one sensing system. The microcantilever beam will act as an actuator by depositing a thin layer of conductive polymer on the surface opposite of the immobilised surface.
Conductive polymers are deemed as a very promising actuating material as they have low operating voltage, ease of fabrication and low weight. In addition, they are biocompatible, can be produced in biodegradable form, can be doped with biomolecules and have been extensively studied as biosensors [197-200].

The system continuously measures the displacement and compares it to its input. The error between the two values will generate the corresponding feedback voltage to be supplied to the conductive polymer actuator to minimise the error and bringing it to zero level (as shown in Figure 8-1). All these are processed by the PID controller. This will ensure that the microcantilever is kept at the equilibrium position at all times. The amount of voltage required to balance the microcantilever is proportional to the surface stress caused by the molecular interactions on the immobilised surface of the microcantilever. This force balancing mechanism can improve the sensor’s stiffness, eliminate drifts and increase sensitivity. The active component of the sensing device can also help compensate the residual stress in the microcantilever after releasing it from the substrate.
Apart from that, integrating the aptasensor with microfluidics and packaging it with integrating circuits for on-board processing can reduce noise caused by its surrounding environment, and increase its portability.
REFERENCES


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