Fall 1-31-2009

Aptamer-based nano-scale dielectric sensor for protein detection

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ABSTRACT

APTAMER-BASED NANO-SCALE DIELECTRIC SENSOR FOR PROTEIN DETECTION

by
Teena James

The specific detection and precise quantification of protein molecules play an essential role in basic discovery research as well as in clinical practice. In this research work, a novel protein detection mechanism based on nanoscale dielectric sensor functionalized with aptamer probes is developed. This work has been done in collaboration with Rational Affinity Devices LLC. The use of aptamer based detection offers several advantages over the traditional labor intensive antibody based immunosensing. In the initial phase of the work, the binding affinities of rationally designed oligomers towards a specific protein molecule (IgG) was studied and optimized under varying conditions of ionic strength and pH using fluorescence based methods. Also, various immobilization strategies for aptamer probes including agarose gel based biomimetic surfaces and self assembled monolayers were studied. In the second phase of the sensor development, two separate transduction mechanisms to produce a measurable signal from the immobilized aptamer-protein binding events were analyzed. Initially, a piezoelectric sensing mechanism utilizing the mass of the protein molecules was developed. Although found to be sensitive, this mechanism suffered from viscous damping in liquid phase measurements. Therefore, a novel nanoscale dielectric sensor was developed capable of monitoring biomolecular recognition events in liquid phase with high sensitivity. The use of this sensor for attaining highly sensitive label-free detection of alpha thrombin using immobilized aptamer probes is demonstrated in this work.

APTAMER-BASED NANO-SCALE DIELECTRIC SENSOR FOR PROTEIN DETECTION

by
Teena James

A Thesis
Submitted to the Faculty of
New Jersey Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Biomedical Engineering

Department of Biomedical Engineering

January 2009

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APTAMER-BASED NANO-SCALE DIELECTRIC SENSOR FOR PROTEIN DETECTION

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1. Teena James, Manu Sebastian Mannoor, Dentcho V. Ivanov, Les Beadling and Bill Braunlin "Debye Capacitive sensor for biomolecular detection" NSTI Nanotech, Boston, MA, June 20008. (Poster presentation)

2. Teena James, Manu Sebastian Mannoor, Dentcho V. Ivanov, Les Beadling and Bill Braunlin "NEMS Capacitive Sensors for Highly Sensitive Label -Free Nucleic-acid Analysis" Springer-Verlag Lecture Notes of the Institute for Computer Sciences, Social-Informatics and Telecommunications Engineering, 3rd International Conference on Nano-Networks, Boston, MA Sept. 2008. (Oral presentation)

3. Teena James, Manu Sebastian Mannoor, Dentcho V. Ivanov "Active MOS Capacitive Sensor Array for Lab-On-a-Chip Applications" 35th annual conference of Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Reno, NV, Sept. 2008. (Oral presentation)

4. Manu Sebastian Mannoor, Teena James, Dentcho. V Ivanov, Les Beadling and Bill Braunlin " Ultra Sensitive Debye Capacitive Sensors with Nano-scale Electrode spacing for Label-free Nucleic Acid Analysis " 5th International Congress of Nano-Bio Clean Tech, San Francisco, CA, Oct. 2008. (Oral presentation)

5. Manu Sebastian Mannoor, Teena James, Dentcho. V Ivanov, Les Beadling and Bill Braunlin "Nano-scaled Debye Capacitive Sensors for Highly Sensitive, Label-free, Nucleic Acid Analysis" Materials Research Society (MRS) Fall meeting, Boston, MA, Dec. 2008. (Poster presentation)

6. Teena James, Manu Sebastian Mannoor, Dentcho. V Ivanov, Les Beadling and Bill Braunlin "Active Field Effect Capacitive Sensor Array for High-Throughput Biomolecular Screening." Materials Research Society (MRS) Fall meeting, Boston, MA, Dec. 2008. (Poster presentation)

7. William Braunlin, Les Beadling, Manu Sebastian Mannoor and Teena James, Tunable Affinity Ligands for the Separation of Proteins and Biomacromolecular Complexes, Biophys. J. 2008 94. (Poster presention)

8. Manu Sebastian Mannoor, Teena James, Dentcho. V Ivanov, Les Beadling and Bill Braunlin "Label-free affinity based geno-sensors for point-of-care diagnostic applications" SPIE symposium on MOEMS-MEMS, San Jose, CA, Jan. 2009. (Oral presentation)

9. Les Beadling, Manu Sebastian Mannoor, Teena James and William Braunlin,"Tunable Affinity Ligands: A new approach to Affinity Chromatography" Biomaterials in Medicine and Personal Care, NewBrunswick, NJ , May 2008.

10. Teena James, Manu. S Mannoor and Dentcho. V Ivanov, "BioMEMS- Advancing the Frontiers of Medicine" Sensors, vol 8, no.9, pp 6077-6177, 2008.

Book Chapter

1. Manu Sebastian Mannoor, Teena James and Dentcho V. Ivanov "BioMEMS - Integrating Micro/Nano fabrication with Biomolecular Technologies" in Standard Hand Book of Biomedical Engineering & Design. Myer Kutz (Editor), McGraw-Hill (2008 in press)

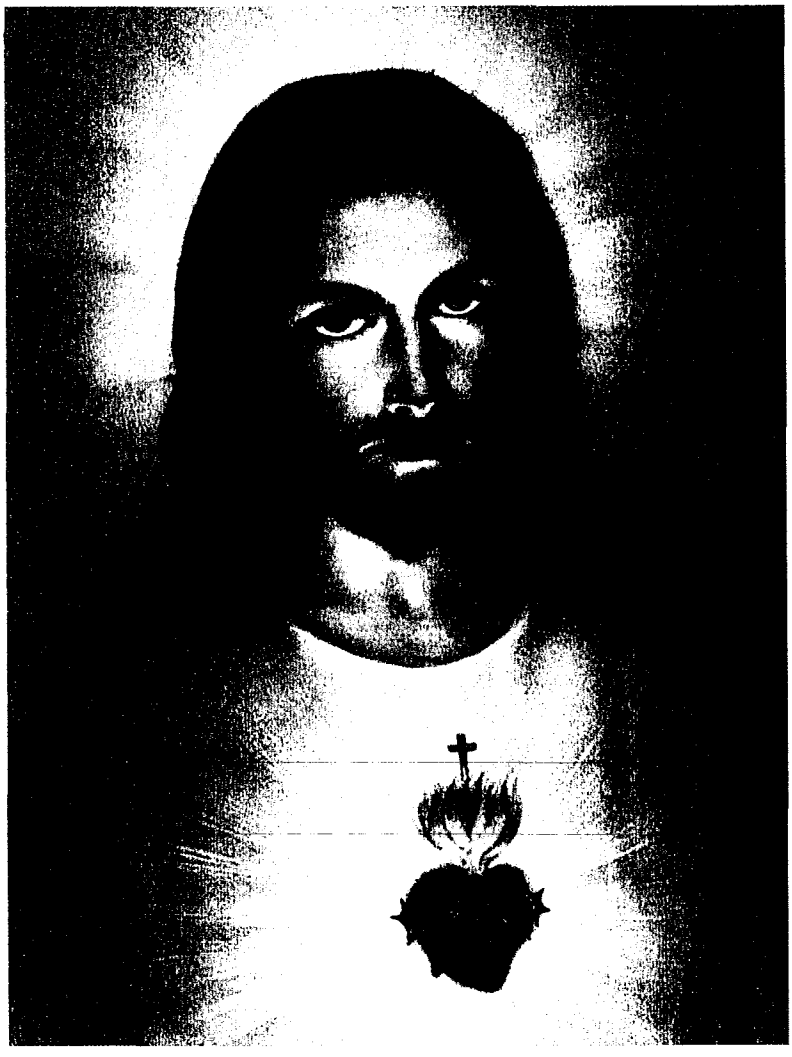
Invited Speaking events

1. NanoTX'08 - International Nanotechnology Conference and Trade Expo, Oct 2-3, 2008, Dallas, Texas. Session: Electronics & Materials.

Invention Disclosures and Patents

1. Active Dual Parametric Capacitive Biosensors, US Provisional Patent application filed, August, 2008.

Dedicated to my dear Lord Jesus



"Trust in the LORD with all your heart and lean not on your own understanding; in all your ways acknowledge Him, and He will make your paths straight."

(Proverbs 3:5-6)

ACKNOWLEDGEMENT

First, I thank my advisor Dr. Dentcho V. Ivanov, for his continuous support in my Masters thesis. I am greatly indebted to him for introducing me to the field of MEMS. I also thank Dr. Les Beadling, Dr. Bill Braunlin and Dr. Roger Cubicciotti of Rational Affinity Devices LLC under whose guidance I explored ideas and learned about the new class of molecules known as aptamers. I thank them for the support they gave me during the course of this work. I would also like to thank my thesis committee members Dr. William Hunter and Dr. Brian Pfister who took the time to review my thesis and gave me insightful comments on my work. I thank Dr. Raj Jarwal, process engineer at MFC, for helping me with the microfabrication processes. Special thanks go to Manu Sebastian Mannoor, dear friend and mentor, without whose help this work would have been impossible. He helped me develop ideas and vision of the enormous possibilities in the field of biosensing. Last but not least, I thank my family, for allowing me to embark on this journey to pursue my interest. Finally, I would like to say a big 'thank you' to Jesus, my Lord and Savior for being with me and for guiding me in every step of my way.

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CHAPTER 1

INTRODUCTION

Understanding the signaling information from cells is of utmost importance in various areas of medicine especially with regards to clinical diagnosis. This information is primarily carried in the form of protein molecules; therefore, their study is fundamental to understand the metabolism, life cycle, and inner working of cell. Due to this reason, many clinical tests rely on the accurate detection and quantification of proteins, as they are clear indicators of the physiological conditions of cell and body at large. Studies have focused on the development of tools and analytical techniques to understand and study these molecules in detail. Although standardized procedures have existed for detection of proteins and for studying their conformational changes and dynamics, this area is currently in a state of transition as the analytical technologies are undergoing rapid change. In the case of clinical diagnosis, the proteins of interests are often found in extreme low abundance and the traditional analytical methods are often limited in their sensitivity. Current methods used clinically are of either low sensitivity, or time-consuming and complicated, requires expensive instrumentation and additional labeling. Exploring improved alternatives for immunosensing has therefore become an area of great interest.

1.1 Objective & Goals

The advancement of medical research is highly dependent on the both advancements in engineering technology as well as molecular research. In this work, such a combined achievement is reported. This thesis describes the work done in the development of an immunosensor coupled with the optimization of Tunable Affinity Ligands (TALs), the patented product of Rational Affinity Devices LLC. In this work, a new electrochemical sensing mechanism has been developed for protein recognition. In addition, studies relating to configurational changes of aptamer molecules in relation to buffer condition have also been performed. Along the course of this research, other experiments relating to formation of biomimetic layer and piezoelectric sensing of proteins were also performed.

1.2 Overview

In this thesis, a novel label-free immunosensor utilizing the specific advantages of the aptamer based recognition system and improved sensitivity offered by the nanoscale dielectric signal transduction mechanism is demonstrated.

Aptamer molecules, being more stable and having better shelf life were found to be most suited for this application as compared to the conventional antibody based sensing. These molecules were linked to the gold sensor surfaces through thiol covalent linking. The strong affinity of these molecules for gold surfaces was exploited for coating the gold electrodes. This coating procedure using of alkanethiol requires minimal modification in the sensor, and therefore proved to be very compatible with the microfabrication process sequence.

In the first part of the sensor development, a sensing platform, using Quartz Crystal Microbalance, which uses piezoelectric change for detecting protein molecule, is presented. When an oscillating potential is applied to the QCM a mechanical oscillation is produced in the disk due to its piezoelectric nature whose frequency of vibration is sensitive to any mass changes. Although this technique is advantageous as a label free sensing mechanism, the damping effect of liquid on the surface in the measurement posed to be a major problem. For real time monitoring of binding events, a system capable of giving a more sensitive signal in liquid environments was therefore necessary. This led to the investigation of the dielectric biosensing platform.

The microfabricated capacitive sensors presented in the current work offers many advantages which include small sample volumes, easy sensor array fabrication, better multiplexing possibilities and portability in contrast to the macro-scale setups. The sensor was found to be extremely sensitive to alkanethiol immobilization and to protein binding. Alpha thrombin (test protein) sensing experiments were performed with sensors functionalized with aptamers and the sensors showed almost 35% decrease in permittivity for specific binding as compared to 4.32% and about 0.23% change observed for nonspecific binding (the interaction between alpha thrombin aptamer and lysozyme and scrambled aptamer and alpha thrombin).The sensor was capable of detecting alpha thrombin at 0.05uM concentration at 0.5xSSC salt solution.

The work is organized as follows: Chapter 2 gives an overview of current methods and techniques available for biosensing. The concept of label-free immunosensing is introduced and a short review of the various label-free immunosensing is presented. Chapter 3 gives an overview of the various recognition elements that can be

used for protein recognition. The focus of this work, aptamers, are introduced in this chapter, SELEX process for aptamer generation is briefly explained. A brief comparison of aptamer recognition to antibody based recognition is also given. Investigation of salt concentration and pH on aptamer-protein interactions is also given in this section. In Chapter 4, a discussion of the immobilization strategies used in the development of the sensor is presented along with the investigation on the formation of biomimetic layers. In the first part of Chapter 5, the development of piezoelectric immunosensor is presented. Due to its limitations, a new electrochemical sensing mechanism was developed and tested. This is discussed in the second part of the Chapter 5. The experimental data is summarized and recapitulated and future research plans are outlined in the last Chapter.

CHAPTER 2

LITERATURE REVIEW

2.1 Immunosensors

The fundamental concept that forms the basis of both analytical immunoassay in solution and immunosensors on solid phase is the specificity of molecular recognition between an antigen and an antibody. Monitoring the product formation that occurs due to this specific reaction is the underlying principle behind these analytical methods. The principle of immunoassay was first published by Yalow and Berson in 1959 [1]. Since then this field has grown rapidly and clinical laboratories has greatly benefited from this technique. The idea of immunosensor as a heterogeneous immunoassay system was conceived as a result of an increased interest in the trace analysis for clinical sciences.

As science and technology progressed over the years, diagnostics have also been subjected to continuous improvement. Currently, much research and development are put into the development of personalized diagnostic tools that are highly sensitive and capable of early detection of diseases. The idea of downsizing diagnostic tools was made a reality by the engineering advances in surface and material science. Today, many molecular based diagnostics are emerging that enable identification of susceptibility to diseases long before the actual symptoms are manifested [2]. Protein expression of cells can reveal a great amount of information which can be useful in innumerable ways for medical purposes. Therefore, much research has been done in this area for developing sensors capable of identifying specific protein molecules.

In this chapter the current state-of-the-art immunosensing technologies are discussed, many of which have greatly benefited from the advances in the MEMS

technology. Immunosensors can be broadly classified under two main headings; label-based and label-free. In label-based (indirect) mechanisms, a signal generating label is incorporated into the system to monitor the formation of the complex. In the case of label-free (direct) detection, the sensors detects the formation of the immune complex by monitoring the physiochemical changes that occur during the process. Label-free technologies offer a number of distinct advantages over traditional label-based methods as they are non-invasive and require minimal manipulation of reaction components. In addition they do not suffer from potential assay artifacts such as compound auto fluorescence or quenching as no fluorescent dye or label is involved. Another advantage is that there is no requirement for target modification thus label-free technologies can be used to investigate biological processes, such as certain enzymatic reactions and ion channel gating, for which label-dependent assay formats are currently limited or unavailable.

2.2 Principle of Biosensing

The concept of biosensing was born during the 1960s as a result of the phenomenal work carried out by Updike and Hicks [3]. Since then, this field has undergone rapid advancements. The primary driving factor for this rapid growth is due the fact that these systems are envisioned to play significant role in medicine, food safety, homeland security and environmental monitoring. Protein biosensors are defined as chemical sensors with a recognition system utilizing a biochemical mechanism for detection of protein analyte molecules. These sensors contain usually two basic components

connected in series: a biochemical (molecular) recognition system (receptor) and a physico-chemical transducer [4].

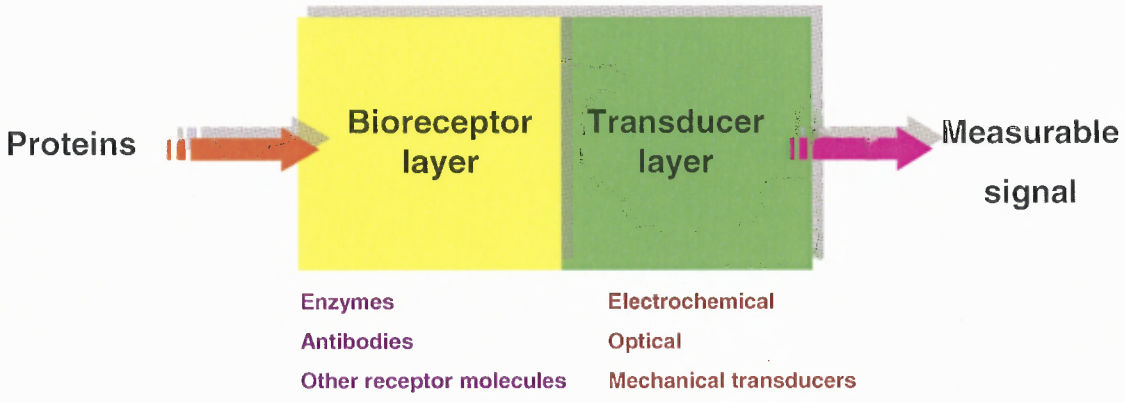

Figure 2.1 Schematic representation of a protein biosensor.

The bioreceptor layer translates information from the biochemical domain (protein analyte concentrations) into a physio-chemical output signal. The degree of selectivity towards the analyte is highly dependent on the specificity of this bioreceptor layer. The transducer part of the biosensor serves to transfer this physio-chemical signal from the bioreceptor layer, mostly to the electrical domain. Over the years, a number of transducing mechanisms have been tried for various biosensing applications. Of these, the most popular ones are optical (by fluorescence spectroscopy) and electrochemical methods. The following section briefly describes the basic sensing principles used in the various biosensing structures. Most of the biosensors are *affinity-based*, which uses a biorecognition layer (probe molecules) immobilized on a transducer surface to bind to the analyte molecules selectively. Microfabricated biosensors utilizing electrical, mechanical, piezoelectric and acoustic signal transduction mechanisms have been developed over the years.

2.3 Optical Techniques

The modulation of the properties of light in response to the biochemical changes that occurs within the system forms the basis of these techniques. Examples of such properties include refractive index, absorbance, reflectance, scattering, interference, fluorescence, polarization etc. In the case of certain type of sensors, the biomolecules themselves carries their own signal. These are known as intrinsic sensors.

Figure 2.2 Schematic of label-based technique for immunosensing.

2.3.1 Fluorescence

Figure 2.2 shows the principle used in fluorescence detection. In this method, the probe molecules (capture antibodies) are immobilized onto a solid surface. The sample containing a heterogeneous mixture containing targets is then allowed to interact with this surface. These bound targets (such as antigens) can either be detected by flourophores which are directly attached or in two steps by first being tagged with a reagent which can then be detected in a second step using a labeled affinity reagent as shown in the Figure. Although this process is one of the most commonly used methods due to their high sensitivity and selectivity, these techniques are complicated and time consuming.

2.3.2 Chemiluminescence

Enzyme-linked immunosorbent assay (ELISA), is the most common example of this category. In this method, the primary antibody is first immobilized on the solid support, next the antigen (target) is captured on the primary antibody. In the next step, a secondary antibody, linked to an enzyme is added to bind to the antigen. This is then followed by the addition of a substrate which is converted to the detectable colored product by the bound enzyme. As discussed earlier, this method also is complicated and time consuming.

2.3.3 Surface Plasmon Resonance (SPR)

When gold- or silver-coated surfaces are exposed to monochromatic p-polarized light above the critical angle of incidence, a sharp reduction (SPR minimum) in the amount of reflected light is observed due to the resonant transfer of the energy from the incoming light to surface plasmons generated at the metal/glass interfaces. The specific angle (or wavelength) at which this occurs is extremely sensitive to the local optical properties of the interface. Therefore, this method can be used to detect biomolecular complexes formed at the interface due to biomolecular recognition events[5, 6]. The major challenge with the use of SPR for biosensing is the difficulty to multiplex and hence, this technology does not lend itself to be used in an array format for enhancing sensor selectivity.

2.4 Electrochemical Immunosensing

In electrochemical immunosensing, the specificity of biorecognition process is used together with the electrochemical technique for identification of specific protein

molecules. This class of biosensors can be classified into two main categories namely, amperometric and potentiometric. Amperometric immunosensors measures the current flow generated by an electrochemical reaction at constant voltage[7, 8] whereas potentiometric methods detects the change in the potential between a working electrode and a reference electrode. Field effect sensors are examples of potentiometric sensors. The main advantage of potentiometric sensors is its simplicity of operation and possibility of miniaturization, but they suffer from problems of sensitivity and nonspecific effects of binding or signaling (influences from other ions present in the sample) [9]. In the case of amperometric immunosensors, however, better sensitivity is obtained, but the current is generated by oxidation or reduction of redox species at the electrode surface. Therefore, they require electrochemically active labels (directly or as products of an enzymatic reaction) for the electrochemical reaction of the analyte at the sensing electrode.

2.5 Acoustic Sensors

These sensors rely on the changes in the measurable acoustic wave properties of the sensor for measuring biomolecular recognition events. The basic operations of these sensors are based on the propagation of acoustic shear waves in the substrate of the sensor. When biomolecular complexes are formed at the surface of the sensor due to biomolecular recognition events, the phase and velocity of the propagating acoustic waves are altered. A number of such acoustic wave devices have been developed and demonstrated for biosensing applications. These sensors are configured in different ways such as Transverse Shear Mode (TSM) devices (Quartz Crystal Microbalance), Surface

Acoustic Wave (SAW) devices and Flexural Plate Wave (FPW) devices. These devices consist of a piezoelectric substrate and converts electrical energy into mechanical energy in the form of acoustic waves.

2.5.1 Quartz Crystal Microbalance

In Quartz crystal microbalance, the principle of the sensing mechanism is the resonant frequency changes of an AT-cut quartz crystal which is directly proportional to the mass loading on its surface. When the target-probe complex formation on surface due to the molecular recognition layer, a mass change occurs, this is shown as a change in its resonant frequency[10]. For a typical TSM device fabricated on quartz crystal (AT-cut) operating at 10 MHz, a mass change of 1 ng produces a frequency change of 1 Hz.

2.5.2 Surface Acoustic Wave Devices

The SAW device is made of relatively thick plate of piezoelectric materials (Zinc oxide or lithium niobate) with interdigitated electrodes to excite the oscillation of the surface wave. The SAW is stimulated by applying an AC voltage to the fingers of the interdigitated electrode on the piezoelectric crystal surface. Changes in the properties of the piezoelectric crystal surface due to biomolecular interaction affect the propagation of these surface acoustic waves.

2.6 Surface Stress Biosensor

The most common example of surface stress biosensors are microcantilevers. The general idea behind a micro cantilever biosensor is that biological stimuli can affect mechanical characteristics of the structure in such a way that the resulting changes can be measured

using electronic, optical or other means. Microfabricated cantilevers are shown to be capable of measuring extremely small displacements and extremely small mechanical forces[11]. These structures are coated with bioreceptor molecules and biorecognition events takes place on the surface of these sensors[12]. They can operate in two modes-static and dynamic. According to the mode of operation, the cantilever deflections upon mass loading can be detected by optical methods, resonant frequency changes, modulation of electric magnetic fields or by changes in thermal residual stress. Microcantilevers are extremely sensitive to surface stress changes, can be easily microfabricated, selectively functionalized and possibly implemented in large arrays for biosensing applications[13]. However, they have limitations due to the bulky size of the optical detection system, long term drift from non-specific adsorption on non-sensing side, loss of signal due to severe bending of the cantilever, and limited dynamic range.

Micro membrane is another configuration of a surface stress biosensor[14]. Unlike microcantilevers, they use of low stiffness materials like polymers instead of silicon based materials. This can solve the problem of sensitivity, but lack of suitable polymers that are microfabrication compatible, chemically inert and mechanically stable has made this solution infeasible till recent times.

CHAPTER 3

PROTEIN RECOGNITION SYSTEM

3.1 Receptor Elements

Receptor elements are major players in the development of any biosensing device. These elements were initially isolated from the living system, but now many of them can be synthesized in the laboratory. Receptor preparations for biosensing applications have been a major area of study from 1980s onwards [9]. Antibodies represent a major class of molecules that has been utilized as recognition elements for protein detection. This has been the case as they are the most popular class of molecules that can be created to almost any antigenic structure due to their high diversity at the binding region. For this reason, they have also made substantial contributions towards the advancement of diagnostic assays and have become indispensable in most diagnostic tests that are used routinely in clinics today[15].

The development of the Systematic Evolution of Ligands by EXponential enrichment (SELEX) process, however, made possible the isolation of oligonucleotide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity [15]. These oligonucleotide sequences, referred to as "aptamers", are beginning to emerge as a class of molecules that rival antibodies in both therapeutic and diagnostic applications[15].

3.2 Aptamer based Recognition

Aptamers are oligonucleotides, designed and engineered such that they have specific binding sites to certain types of molecules. The aptamer molecules are isolated from random nucleic acid libraries by "in-vitro selection and amplification technique"[16, 17] (termed SELEX: systematic evolution of ligands by exponential enrichment). Aptamers, although predominantly unstructured in solution, fold upon associating with their ligands into molecular architectures in which the ligand becomes an intrinsic part of the nucleic acid structure. The folding of these molecules to well-defined secondary and tertiary structure due to their self annealing process results in a number of conformations .Thus these molecules are considered to recognize the target molecules by shape or conformation [18].

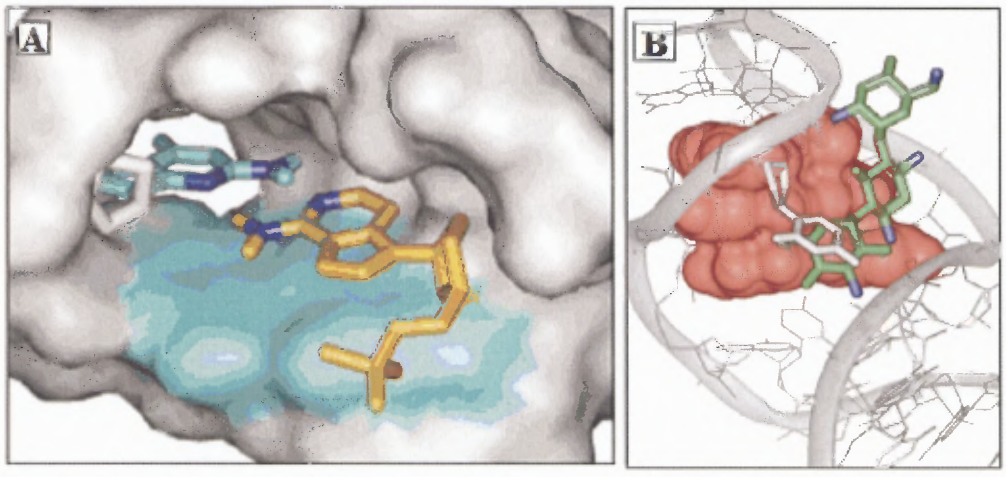

Figure 3.1 Molecular recognition of ligands by aptamers.

The recent studies on these molecules on their use as ligand binding molecules have been shown useful for the detection of protein analytes [19-22], nucleic acids [23-26] and even small molecular targets such as metal ions, dyes [27-29] etc. As discussed,

the most commonly used diagnostic method for protein detection is the antibody based immunoassay, and many studies have focused on the development of biosensor based on this technique. As an alternative to antibodies, aptamers are being used for protein detection as they are more specific and sensitive. Aptamers raised against specific targets including cellular proteins can be linked to fluorescent labels and used as superior and inexpensive substitutes for antibodies.

Table 3.1 Examples of Nucleic Acid Aptamers

Ligand	Nucleic Acid	References
Theophylline	DNA aptamer	[30]
Thrombin	DNA aptamer	[31, 32]
Flavin mononucleotide	RNA aptamer	[33]
Adenosine monophosphate	RNA & DNA aptamer	[34, 35]
Arginine	RNA & DNA aptamer	[20, 36]
Citrulline	RNA aptamer	[20]
Tobramycin	RNA aptamer	[37]
Neomycin B	RNA aptamer	[38]
HIV-1 Rev peptide	RNA aptamer	[39]
HTLV-1 Rex peptide	RNA aptamer	[40]
MS2 coat protein	RNA aptamer	[41]

3.2.1 Advantages of Aptamer Sensing

Aptamers are equal to monoclonal antibodies in binding affinities but they hold major advantages that make them particularly attractive for biosensing. Aptamers are produced in vitro, whereas antibodies are produced in vivo. This allows the productions of

in vitro, whereas antibodies are produced in vivo. This allows the productions of aptamers against toxic proteins or nonimmunogenic proteins. The binding affinity, or the stability of the protein – ligand complex etc can be improved or modified by varying its environments or design. It is also possible to attach functional groups to them, thus allowing covalent or indirect immobilization on the substrate. In addition, aptamers are usually synthesized in large bulk using chemical synthesis resulting in homogenous qualities of aptamers, but in the case of antibodies, they vary from one organism to the other, and thus it is not possible to observe such homogeneity. Finally, the simple structure of the nucleic acids allows them to be more densely immobilized than antibodies on biosensors. They have larger shelf life than antibodies as they are more resistant to denaturation unlike antibodies which has to be handled very carefully. Denaturation of aptamers does occur, but the process is reversible and hence it is possible to regenerate them in short period of time. Another major advantage of aptamers molecules is their small size (30 to 100 nucleotides) which enables higher immobilization density resulting in better efficiency of biosensors. Biosensors which are based on aptamers have been studied using a wide range of signal transduction approaches like fluorescence [42, 43], quartz crystal microbalance[10, 44]etc.

3.2.2 The SELEX Method

This is the general process for aptamer generation. It is an iterative process of *in vitro* selection and amplification, by which large libraries of oligonucleotides are screened. As shown in Figure, the SELEX process starts with the synthesis of a random sequence of oligonucleotides. Normally, the starting round contains around 10^{15} individual sequences, thus giving a high probability of selecting an aptamer specific for the target of interest.

Each of these oligonucleotide sequences will have unique 3D structures due to their specific sequences. In order to find aptamers to a given target molecule, the target protein molecules are incubated together with the nucleic acids. Upon formation of the molecule-target complex, separation is done by filtration or other methods and another step of amplification is done by PCR to have a new pool enriched in target binding species and this will be used for the next selection/amplification cycle.

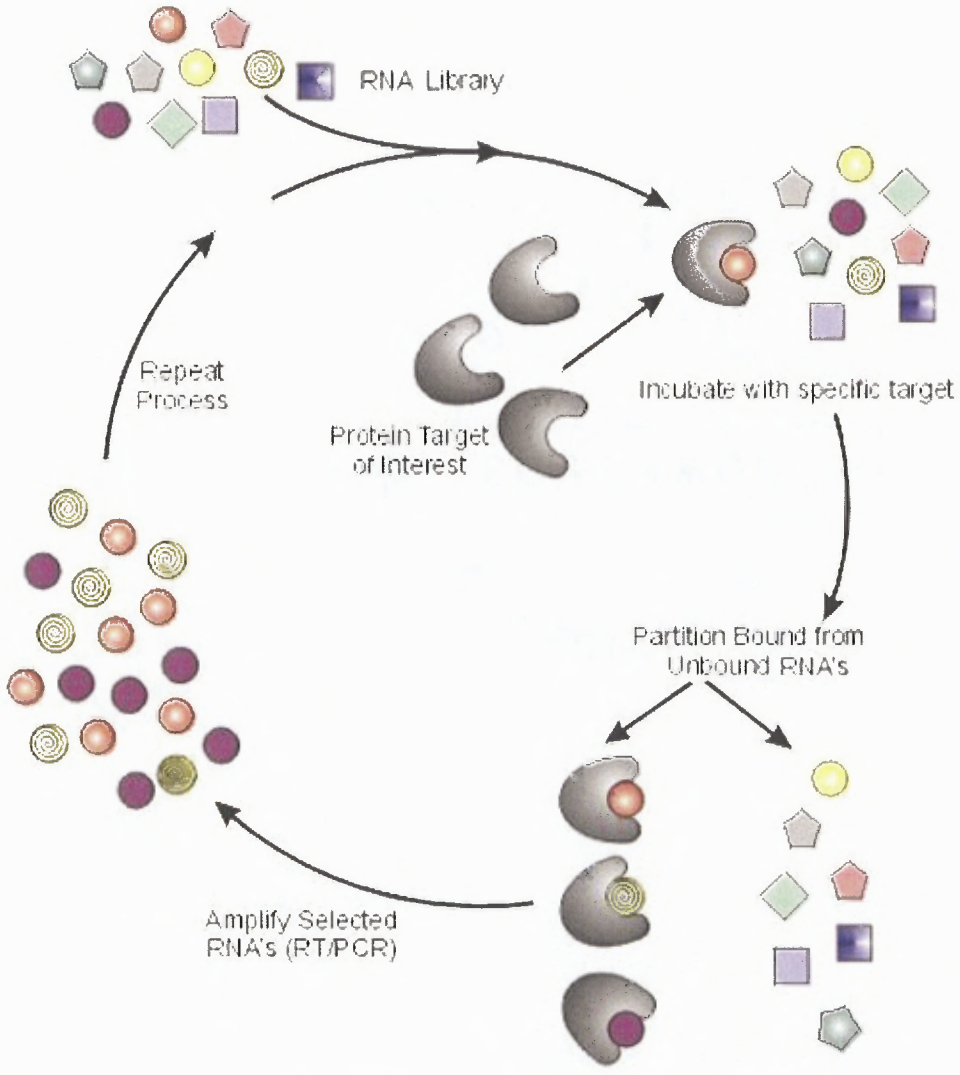

Figure 3.2 Schematic diagram for *in vitro* selection of aptamer.

CHAPTER 4

BIOFUNCTIONALIZATION

The surface structure of a biosensor has to be optimized well as the sensitivity of the device is based on the characteristics of its bioreceptor layer. In most cases, the bioreceptor molecules are immobilized on or placed in close proximity to the transducer surface. For the above reason, immobilization procedure and such biofunctionalization techniques play a major role in the development of biosensors. In general, biofunctionalization methods can be classified into two major categories; methods in which no control over the orientation of the receptor molecules are exercised and methods that avoid random orientation. A great amount of work has been done in this area for ensuring the recognition molecules remain attached to the surface, and also for ensuring that their natural orientations are not disturbed.

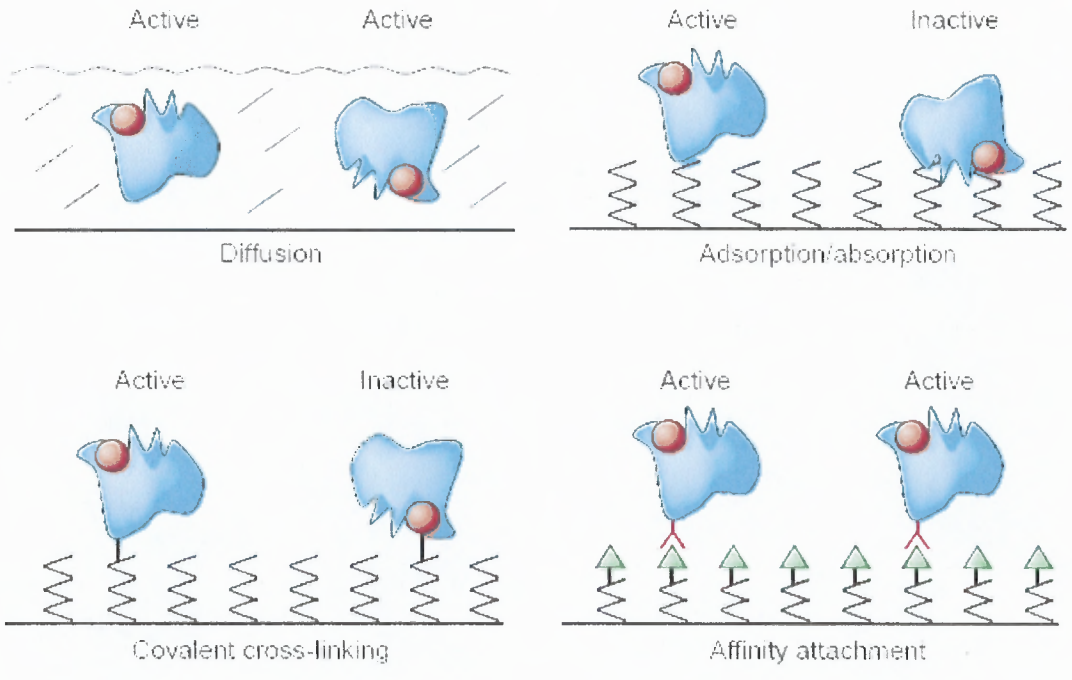

Figure 4.1 Various kinds of protein attachment to surfaces [45].

Figure 4.1 shows the various methods of receptor molecule attachment. They can be attached to different surfaces using diffusion, adsorption, absorption, covalent linkage and affinity interaction. In the case of proteins, most of the methods, except affinity interaction results in random orientation, which may make them inaccessible to targets [45]. However, in the case of aptamers attached to surface through covalent linkages, every molecule is uniformly attached to the surface, thus maintains their native conformations making target molecules accessible to them.

4.1 Biomimetic Surfaces

In order for the recognition elements to be attached on the sensor, the surface of the sensor has to be modified to achieve maximum binding capacity. One of the common methods includes coating the sensor surface with nitrocellulose or poly-lysine so that the receptor molecules can be adsorbed to the sensor surface through non-specific interactions. As shown in Figure 4.1, the attached molecules may have random orientations and thus may not remain in the active state for biomolecular recognition. These types of attachment also tend to have higher noise level as they tend to lay on the surface of the sensor.

Table 4.1 Various Types of Biomimetic Surfaces

Surfaces	Attachment chemistry	Limitations
Agarose	Diffusion	Tough to fabricate, miniaturize
Nitrocellulose	Adsorption & Absorption	High non-specific binding
Poly-lysine coated	Adsorption	Non-specific adsorption
PVDF	Adsorption & Absorption	Non-specific attachment

4.1.1 Objective

In this experiment, the use of agarose as a biomimetic surface to aid in the immobilization of probe molecules was investigated. In order to understand the optimum concentration and minimally achievable thickness for biomolecule immobilization, the following experiment was performed.

4.1.2 Protocol

Various concentrations of agarose solutions were made up in 10mM water solution of $NaIO_4$. This mixture was heated to 80 degrees C to make a solution of the melted agarose (time ½ hr.). The melted agarose was pored over the treated glass slide (2 ml/slide) and spun on with various rotational speeds and allowed to gel. After gelling, the thickness was investigated using Dektak profilometer.

4.1.3 Results & Discussion

Figure 4.2 shows the results obtained for this experiment. From the results it can be seen that very thin layers of agarose gels can be obtained using spin on technique. The thickness is seen to be inversely related to the concentration of agarose used. As the concentration of agarose was increased from 1 to 5%; the initial thickness at 200 rpm reduced by about 200nm. But this effect seems to decrease as higher rotational speeds are used. On an average, through this technique it was possible to make biomimetic layers with agarose for microdevices in the range of 13 um with a rotational speed of about 1200 rpm which is not possible by conventional manual protocols.

Although this method proved to be easy and simple for immobilization purposes, the main limitation with the use of this biomimetic layer was the reduction in sensitivity

as more noise was observed during sensing procedures. The 3D gel formed by agarose served to trap other non-specific molecules thus reducing the device sensitivity.

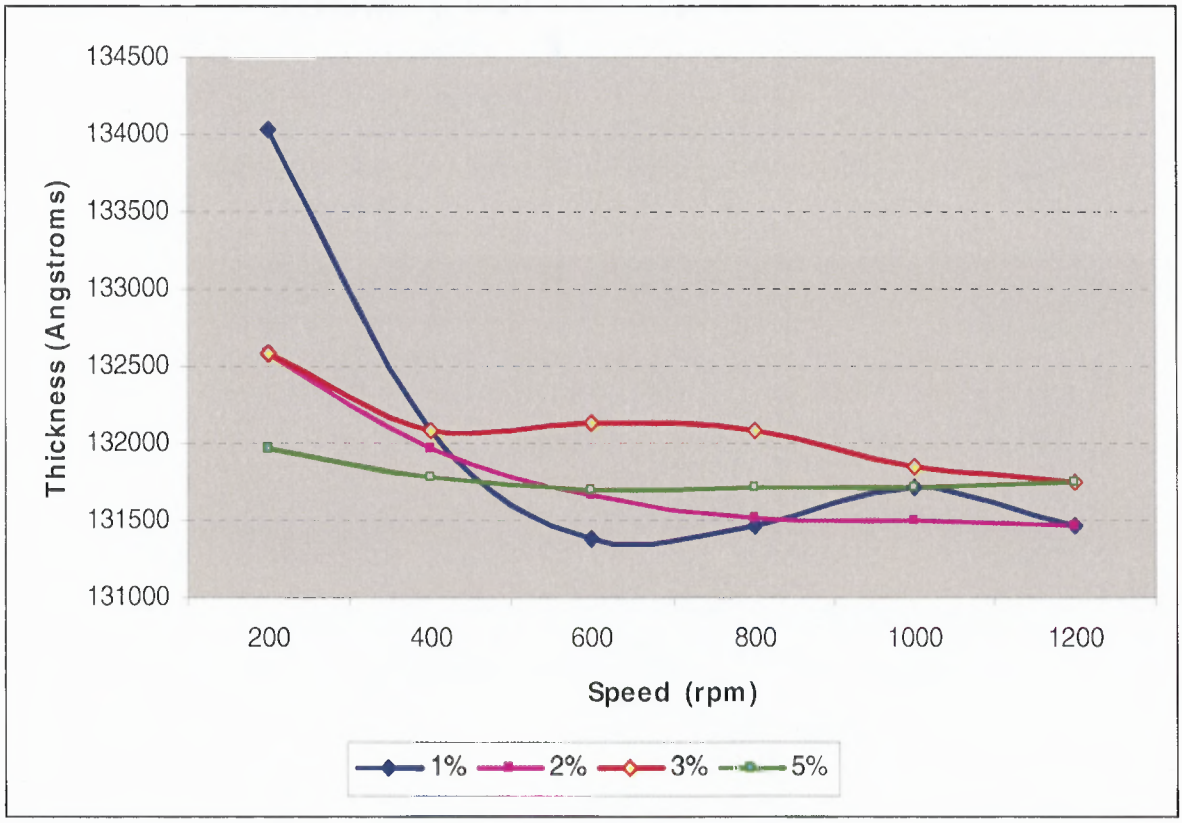

Figure 4.2 Variation of agarose thickness with speed and concentration.

4.2 Self Assembled Monolayers

Self assembly is one of the most common approaches used for surface modifications. Self assembled monolayers are surfaces that have a single layer of molecules attached onto them. The molecules that form these monolayers have a 'head group' that has a special affinity for the substrate. They can be prepared with different kinds of molecules on different substrates. The thickness of the SAM layer is mostly between 1-3nm[46]. Some common examples of monolayers include alkylsiloxane monolayers, fatty acids on oxidic

materials and alkanethiolate monolayers. In the development of this sensor, alkanethiol chemistry was chosen for immobilization. The self assembly is achieved by reacting thiol containing compounds with clean gold surfaces. The sulphydryl groups will covalently bind to the gold surface leaving the molecules to be arranged two dimensionally on the surface. The end group determines the surface properties of the SAM layer whereas the alkane group serves to act as a physical barrier and it alters the electronic conductivity and local optical properties of the material.

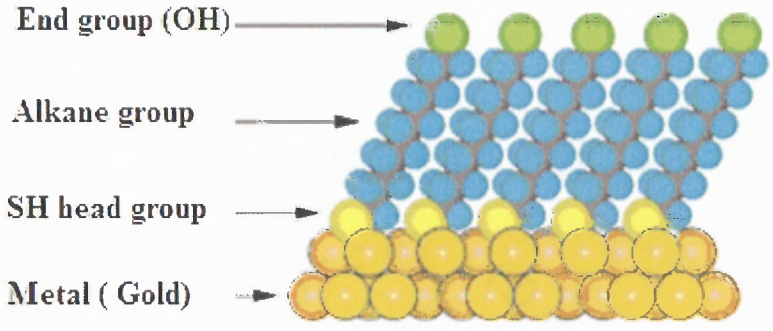

Figure 4.3 Schematic diagram of an ideal, single-crystalline SAM on a gold surface [46].

4.2.1 Theory

In thiolate linkage, the high affinity of sulphur atoms to gold/substrate is taken advantage of and the thiol molecules adsorb onto gold surface from the solution. An SH-modifier can be placed at either the 5'-end or 3'-end of the aptamer. These modifiers can be used to form reversible disulfide bonds (ligand-S-S-aptamer) or irreversible bonds with a variety of activated accepting groups. Aptamers with thiol end groups are chemically assembled onto the surface of gold. The long aptamer chains are thus tethered to the gold substrate at one end and the rest of the chain stays upright and fully extended, at an angle of approximately 30deg to the surface [47, 48]. The thiol – gold bond is a very strong

bond (semi-covalent bond) with binding energy of around 44kcal/mol [47]. The ordering of the aptamer chains parallel to each other occurs due to the hydrophobic, Van der Waals forces between the methylene carbons of adjacent chains. When the binding of aptamer chains onto the surface becomes dense enough, the chains begin to interact with each other and are lifted off the substrate with one end held on to the gold substrate by the thiol linkage. The crystal orientation of gold that gives excellent result for the formation of self assembled monolayers is found to be <111>, which can be obtained by thin layer deposition of gold by evaporation on silicon surfaces.

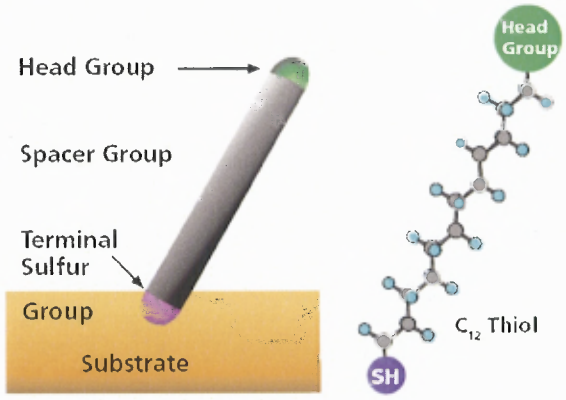

Figure 4.4 Schematic of an alkanethiol molecule attached to the gold substrate.

4.2.2 Experimental

4.2.2.1 Materials

1. Gold coated substrates (sensors)
2. Thiol compound- 6- Mercapto-1- hexanol solution HS- $(CH_2)_6$ OH
3. DNA aptamer solution 5'- GGT TGG TTT GGT TGG TTT - $(CH_2)_3$SH-3
4. Scrambled aptamer solution 5'-GTG TGT GTG TGT GTG TTT - $(CH_2)_3$SH-3'
5. Fresh 200 proof ethanol

4.2.2.2 Protocol. The concentration ratio of aptamer molecules to the MCH used in the immobilization procedure was 1:1000. 61 μM of aptamer solution and 61mM MCH solution was prepared using ethanol. The sensor surface was allowed to interact with MCH solution for a period of 15-20mins in an atmosphere of dry nitrogen with minimized exposure to air. This process was followed by the addition of 61μM aptamer solution. In the later experiments, a solution mix of both MCH and aptamer in the above concentrations were used for the SAM layer formation as both the procedures showed similar results.

4.2.2.3 Discussion. From this immobilization procedure, a dense coverage of the monolayer was obtained. According to calculation a surface coverage of about 3.6×10^{14} molecules/cm^2. The higher concentration of the molecules enabled this dense coverage of adsorbates; for millimolar solutions (milliseconds to minutes). Studies indicate the requirement of longer period of incubation for better reorganization process to maximize the density of molecules and minimize the defects in the SAM[46]. In the preparation of the sensor, ethanol was used as the solvent for preparing SAMs. Ethanol is usually used as the solvent in most of the cases due to the fact that it can solvate a number of alkanethiols with varying degrees of polar character and chain length; it is also inexpensive and available in high purity.

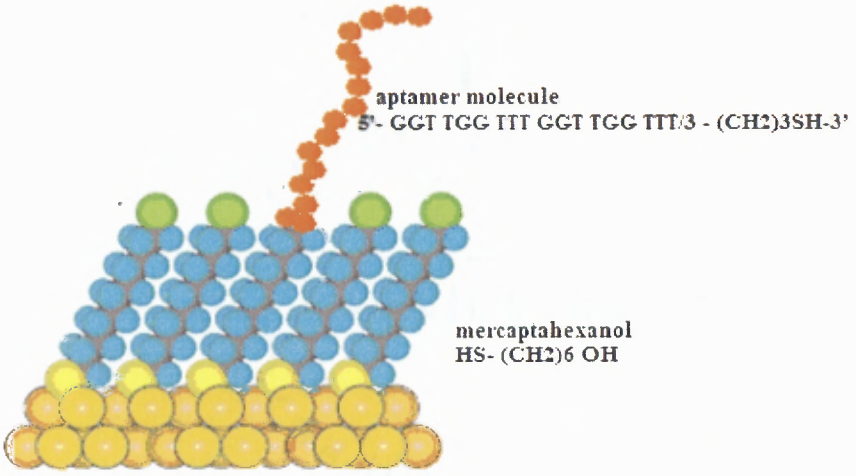

Figure 4.5 Schematic representation of an immobilized aptamer molecule in SAM layer.

The dynamic equilibrium is affected by the type of solvent used in the monolayer formation process. The choice of solvent is therefore very important as the interaction between the solvent and the substrate can at times hinder the rate of adsorption of thiols from the solution as the solvent molecules must be displaced from the surface prior to the adsorption of thiols, which are less prevalent in solution than the solvating molecules. The preparation of the monolayer for the sensor was done at a temperature of about 30°C as temperatures above 25°C have been reported to reduce the number of defects and improve the kinetics of the formation of the monolayers [49, 50].

CHAPTER 5

DEVELOPMENT OF PROTEIN SENSING MECHANISM

5.1 Introduction

The development of the label-free immunosensor was carried out in two separate stages. In the first stage, a piezoelectric platform was investigated for protein recognition. The following section deals with the information regarding this study. From the results of the study, it was found that a more sensitive system was required for real time, liquid phase detection of protein molecules. Hence, a novel nano cavity based electrochemical capacitive system was developed and was found to be more sensitive and more suitable for miniaturization purposes for applications such as point-of-care diagnostics. This sensor is presented in the second section of this chapter and is discussed in detail.

5.2 Piezoelectric Immunosensor

Piezoelectric effect is the basic principle behind the working of this sensor. This effect occurs in crystals without a center of symmetry [51]When pressure is applied to the crystal, the crystal lattice is deformed in such a manner that a dipole moment arises in the molecules of the crystal. Many types of crystals exhibit the piezoelectric effect, but the electrical, mechanical, and chemical properties of quartz make it the most common crystal type used in analytical applications[52].

Quartz crystal microbalance is a well established technique in non-biological applications. The device consists of a quartz crystal disc placed between two evaporated layers of metal electrodes. When an oscillating potential is applied to the disk through

the electrodes, a mechanical oscillation is produced in the disk due to its piezoelectric nature.

With this applied voltage, the physical orientation of the crystal lattice is distorted, resulting in a mechanical oscillation of a standing shear wave across the bulk of the quartz disk at a characteristic vibrational frequency (i.e. the crystal's natural resonant frequency)[52]. The direction of the oscillation depends on the orientation of crystal lattice in the electric field; in turn, the orientation of the crystal lattice depends on the exact geometry of the quartz disk with respect to the crystalline axes of the quartz from which the crystal is cut. Oscillation in the thickness shear mode (TSM) creates a displacement parallel to the surface of the quartz wafer. Only the region between the electrodes is piezoelectrically active; thus oscillation is maximum where the electrode pads overlap and diminishes rapidly from that point[52]

The frequency of vibration is sensitive to any loading effects on the crystal surface. Basic QCM operation is described by the Sauerbrey relation where a mass change (Δm) of the crystal results in a subsequent change in its resonance frequency (Δf). In air, the following formula applies.

$$\Delta F = \frac{-2f_0^2 \Delta m}{A\sqrt{\mu_q \rho_q}} = -C\Delta m$$

Where ΔF=measured frequency shift, in Hz; f_0^2=the fundamental resonant frequency (squared), in Hz; Δm=mass change, in g; A=piezoelectrically active area (area of electrode surface), in cm2; μ_q=shear modulus of quartz=2.947×1011 gcm−2; ρ_q=density of quartz=2.648 gcm−3; C=mass sensitivity constant (based on type of crystal used), in (sg)−1

In his work, Sauerbrey demonstrated that a thin film applied to the surface of a quartz crystal could be treated as an equivalent mass change of the crystal. This approach is based on the deposition of rigid layers that are infinitesimally thin and, thus, dampen the propagation of the bulk shear wave in a fashion identical to quartz itself [52]. Therefore, a small change in the mass of the crystal per unit area results in a change in resonant frequency. AT-cut crystals are used in quartz crystal microbalances. The AT-cut refers to quartz wafers cut at +35°15′ angle from the z-axis. AT-cut crystals have a temperature coefficient of nearly zero, indicating the resonant frequencies are stable over a wide range of temperatures[53].

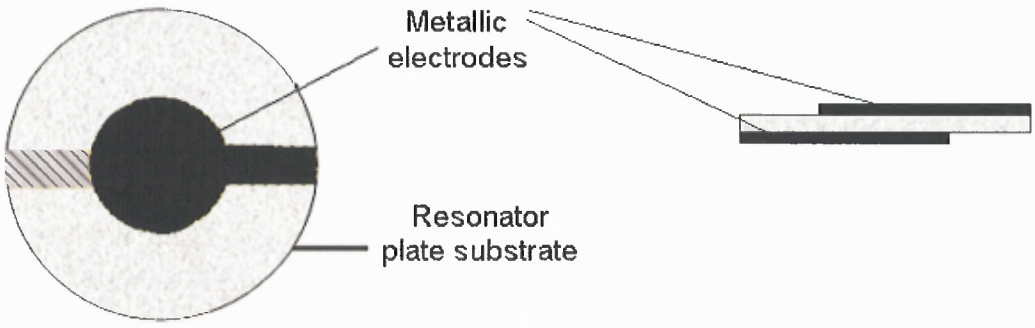

Figure 5.1 Schematic of a quartz crystal microbalance.

When biomolecular recognition occurs and biomolecular complexes are formed on the surface, additional mass is added to the crystal. This change in mass is identified by a corresponding change in the frequency of vibration of the crystal. The Sauerbrey relation is based on the overall mass sensitivity of the quartz crystal. Only the region under the electrode is piezoelectrically active; at the bare quartz, the mass sensitivity is negligible [54-56].

5.2.1 Experimental

The main part of the biosensor system is the quartz crystal with a diameter of 8mm and resonance frequency 6MHz. The crystal as described above was coated with gold layers. These gold layers serve as both electrical contacts and platforms for ligand support. The aptamers were immobilized on the gold surface as in the protocol in appendix. To monitor the binding and dissociation kinetics a network analyzer HP8713C was used. Samples of protein solutions were injected into the measuring cell using a syringe. The data was extracted and processed using the program LView Pro.

5.2.1.1 Objective. Determination of selectivity of thrombin aptamer immobilized quartz resonator to various types of protein was investigated. The sensitivity of the crystal was also investigated in a second set of experiments.

5.2.1.2 Materials & Method.

1. Buffer solution 0.25xSSC,
2. Acetone
3. Isopropanol
4. Protein solutions (Albumin, Thrombin and Lysozyme)

Initially, the quartz resonator is cleaned using acetone, isopropanol and deionized water. Then, oligonucleotides are immobilized on the gold surface according to the immobilization procedure described in Subsection 4.2.2 and the resonance frequency was noted. 10μl of 50μM concentrations of protein solutions made up in buffer solutions were allowed to incubate on the surface of the crystal for short amount of time. The surface of

the resonator is washed off with deionized water to remove unbound protein and the resonant frequency of the crystal was noted after drying.

In a second set of experiments the sensitivity of the quartz crystal microbalance was determined using thrombin solution. The quartz resonator was first cleaned using deionized water and various concentrations of thrombin solutions made up using the prepared buffer solution. After noting the original resonant frequency of the quartz crystal, 10ul of the prepared protein solution is placed on the quartz resonator.10-12 mins is given for incubation and the crystal is washed with DI water and then allowed to dry and resultant resonant frequency is noted.

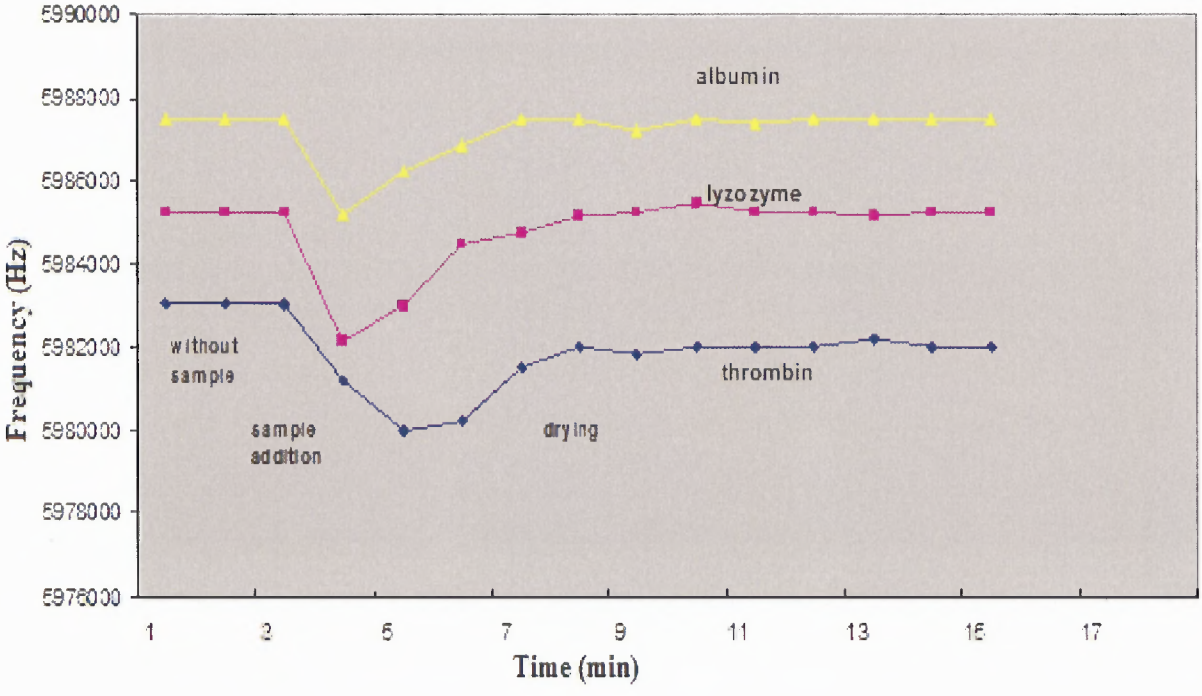

Figure 5.2 Variation of resonant frequency with time various protein solutions.

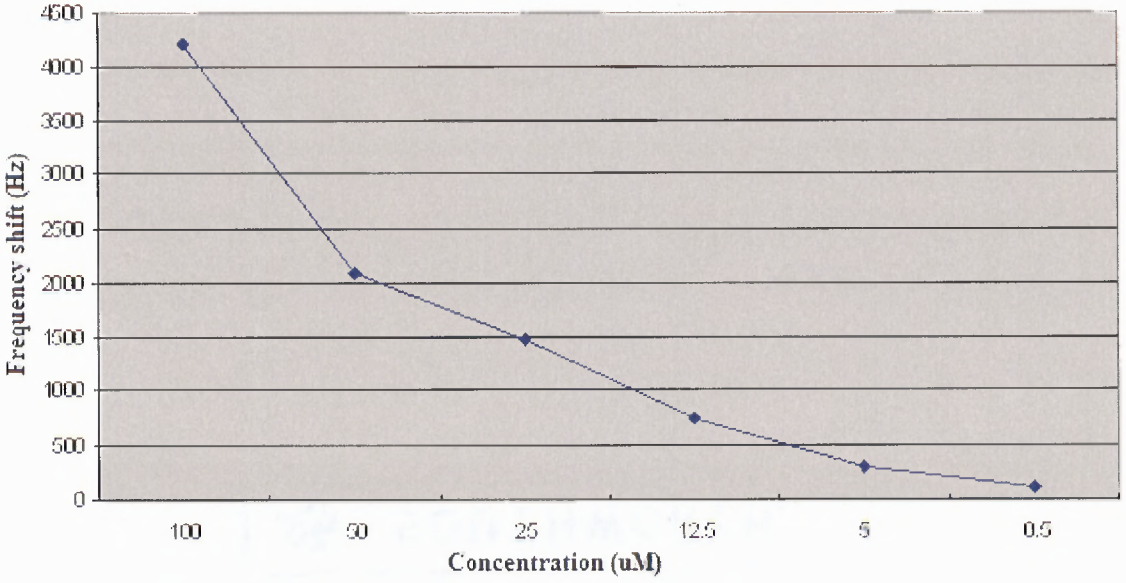

Figure 5.3 Variation of resonant frequency with protein concentration.

5.2.1.3 Results & Discussion. The mass change of the crystal resulting from the association or dissociation of a protein to the quartz sensor surface is shown as frequency decrease in the graph. In Figure 4.1, the first plot the control protein albumin was added to the functionalized sensor surface. After incubating for few minutes and unbound molecules were washed off. From the graph it can be seen that after drying, the frequency returns to the original point, indicating that the molecules did not bind to the surface. In the case of thrombin addition, the frequency shift was apparent, indicating an additional increase in crystal mass due to protein binding. As the third control experiment, lysozyme, a positively charged protein molecule similar to thrombin was also tested. From the graph, a small shift in frequency can be seen after about 15mins indicating the presence of some nonspecific binding. Figure 5.3 shows the response of the crystal to various concentration of protein. Almost 100 Hz frequency shift is observed at 0.5uM concentration, indicating the possibility of detecting much lower concentration. Although

this proves to be a good sensitive method for protein recognition, this method provides no real-time data. To date, no unifying equation has been developed to describe the quartz crystal response in a liquid medium since the response depends greatly on the exact interfacial structure formed between the coatings on the crystal surface and the solution environment. Therefore, development of a new type of biosensor was investigated.

5.3 Electrochemical Immunosensor

5.3.1 Dielectric Spectroscopy as Monitoring Technique

Biomolecular recognition events occurring at the interface of the sensor leads to the formation of biomolecular complexes; altering the state of the immobilized probe molecules, the fluid boundary layer in the proximity of the probe-target complex and the bulk fluid in the vicinity of the biomolecules. The alterations in these equilibrium states are reflected as alterations in the electrical properties (relative permittivity and conductivity) and such changes can be measured using dielectric spectroscopic techniques.

The dielectric constant, ε, of any polar liquid or solution (proteins or oligonucleotides) can be interpreted as being almost entirely a measure of the number of molecules oriented by an external electrical field of unit strength. These molecules are oriented by a torque depending on the field strength and the dipole moment ρ, a constant or each molecular species. The orientation of these biomolecules is hindered by the frictional forces in the solution depending on the frequency υ, and a constant τ, designated as the "relaxation time." Thus it can be seen that the number of molecules oriented at unit field strength will decrease in the frequency region where the hindering

frictional forces and the orienting torque become of the same order of magnitude. Figure 5.4 shows the dispersion curve for pure polar liquid. At lower frequencies the orienting torque is sufficient to overcome completely the resisting forces, and resulting in a high dielectric constant (ε_s). At very high frequencies the resisting forces completely overcome the orienting torque resulting in a low dielectric constant (ε_∞).

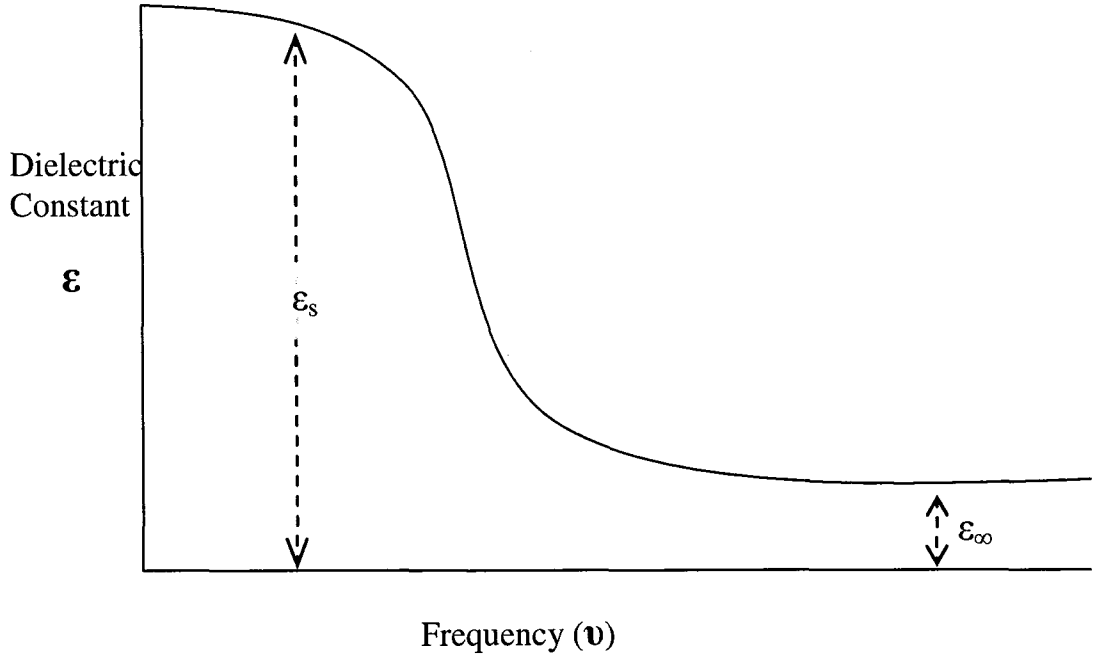

Figure 5.4 Dispersion curve for pure polar liquid.

As a result of this dielectric property of the biomolecules, application of an external electric field produces a different internal field within them. There are two main categories of this electrical interaction in biomolecules (i) charge displacement, which subsides on the removal of the field and (ii) charge drift. Charge displacement causes electrical polarization whereas charge drift leads to the formation of ionic currents. In an electric field of time varying in strength or direction, polarization and charge displacement do not occur instantaneously. If the measuring frequency is low enough for

all the charges to change their position, polarization is maximal. But as frequency increases, they do not get sufficient time and thus the polarization and permittivity will decrease. *Relaxation* is the term given to this characteristic property and it occurs in the time domain as the system is allowed to *relax* to the new equilibrium. Relaxation time is therefore the parameter that describes this property and it's defined as the time required for dipolar molecules to orient themselves

The frequency dependence of the relaxation process will depend on the contribution of polarizability of the material. For biomolecules Schwan [57] proposed 3 main different frequency ranges where these processes can be observed – α, β and γ regions. In the case of protein molecules α dispersion occurs in the lower frequency range (kHz) and β dispersion occurs at slightly higher frequencies due to the rotation of protein molecules The γ dispersion occurs at microwave frequencies (10^{10}Hz) indicative of the relaxation of the free water in the solution. In addition δ dispersion occurs in between β and γ regions and it is indicative of the internal conformational motion of protein and water bound to protein.

The dielectric spectra of biomolecules such as proteins, oligonucleotides etc. presents a very complex shape involving the above three or more different partially overlapping, contributions, each of them originated by different molecular level mechanism[58, 59]. These may be classified as due to polyion dipolar orientation relaxation, polarization of condensed counter ions, polarization of ionic atmospheres, long-range solvent ordering effects and at the higher end of frequencies, orientational polarization of the water molecules[60]. Proteins have complex three dimensional structures as well as complex charge distribution and thus may have a high linear charge

density. In the case of F actin filament the linear charge density is 1 electron charge per 2.5 Å[61, 62]. Same is the case with oligonucleotides; DNA in aqueous solution has a linear charge density of 1 electron charge per 1.7 Å. These high charge densities give rise to condensed structures and they are characterized by large dielectric constant in the lower frequency range (tens of KHz).

Since water is present in most aqueous solution of biomolecules, the main characteristics of the biomolecules do not differ much from the bulk water dispersion. The difference is confined to moderate variation in the dielectric permittivity (75.9) [63] which is contributed partly due to the large dipole of the water molecules (1.84 Debye) and also due to the high Kirkwood correlation factor [64]. In general, there is certainly a dependence of dielectric parameters on the biomolecule type and concentration. But this dependence relies on the very delicate interplay between the biomolecular chain conformation and its charge, which is indirectly dependent on the concentration, contour length and also its interaction with small ions.

Dielectric spectroscopic measurement is a well established method and the principle of measurement is as follows. The electrical impedance of the device containing the sample is measured as a function of the frequency of the applied electric field [60]. However, this method faces a number of limitations which hampered its application to biosensing. The main limitations is that biomolecular solutions usually display a very high ionic electrical conductivity which causes a giant frequency-dependent dielectric dispersion, falling in the low-frequency tail of the spectrum that generally masks the relaxations associated with the polymer component [60]. In addition, there is also a strong polarization in the vicinity of the electrodes that can dwarf the relaxation of

interest in the sample. Therefore experimentally, it is quite difficult to remove parasitic effects that screen the real information especially in the low frequency region.

5.3.2 The Electric Double Layer

The double layer at the electrodes of the sensor can be thought of as a molecular capacitor where one plate is represented by the surface charge of the metal and the other plate by the ions at a minimum distance from the solution .Since the distance between these 'capacitor plates' are in the order of nanometers, the capacitance values become enormous (proposed by Helmholtz in 1879).

At the sensor electrode/ electrolyte interface, the surface charge σ^M created by the application of potential forms an electrostatic field that affects the ions in the bulk of the solution. This field causes the bulk solution to create a counter charge σ^S to screen this surface electric charge σ^M. The properties of this layer depends on the surface charge on the electrode, the DC bias voltage, the concentration of ions in the solution and the charge of the individual ions This net electric screening charge σ^S is equal in magnitude to the surface charge and has opposite polarity. As a result, the complete structure is electrically neutral.

There are three theoretical treatments of the double layer on the interface, Helmholtz Plane Theory, Gouy-Chapman Theory and Gouy-Chapman Stern Models. Helmholtz model is only valid for high concentration electrolyte solution. In more dilute solution; the thickness of the double layer will increase as the transition is not so abrupt. The thickness is related to the distance from the metal surface at which the ions can escape to the bulk by thermal motion. In such a case, the counterion atmosphere is commonly referred to as diffuse layer. In the theory of Gouy and Chapman, the exchange

of counter ions between the double layer and the bulk solution due to thermal motion is taken into account. The theory of Stern takes into account the finite size of the counter ions and their binding properties at the surface. The diffuse layer is divided into an inner layer (Stern layer) and outer layer (Gouy layer). The Stern layer refers to the layer formed when the counter-ions specifically adsorb near the surface. Grahame made a further division of the Stern layer into the Inner Helmholtz layer and Outer Helmholtz layer. These layers are separated by the IHP at a distance from the surface corresponding to the radius of non-hydrated specifically adsorbed ions. It assumes a "plane of closest approach", which is based on the fact that ions have finite size, so they cannot approach the surface closer than a few nm. The IHP is defined by the plane of the center of the dehydrated ions and the excess ions are adsorbed to form the OHP.

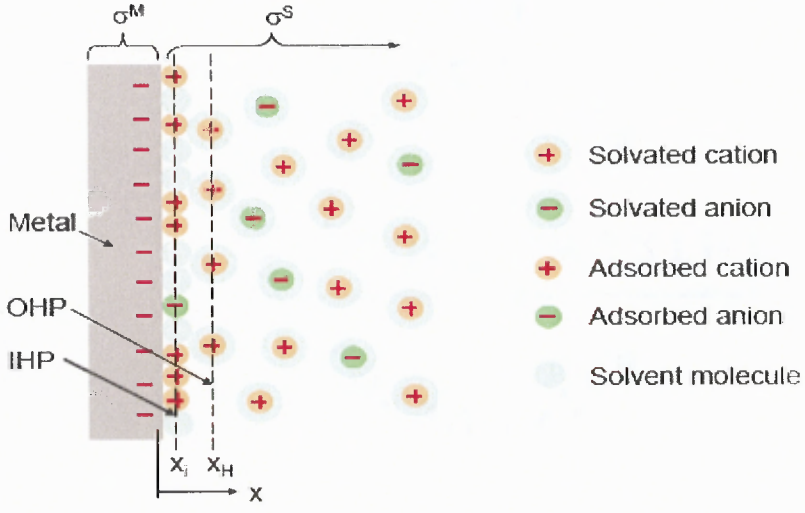

Figure 5.5 Schematic of electrical double layer.

This accumulation of ions on the electrode surface causes huge parasitic impedance that masks the small changes in relative permittivity produced by biomolecular interactions. This is because a potential gradient exists in the area of the

electrical double layer. As the measurement frequency is decreased this effect becomes more pronounced thus making low frequency measurement impossible. Several methods have been proposed for eliminating this double layer effect. In this sensing system, the effect of the electric double layer has been eliminated by reducing the electrode distance to the same order as the double layer length. The characteristic thickness of the DL is the Debye length which is the reciprocal of the Debye-Huckel parameter κ. This value is reciprocally proportional to the square root of the ion concentration C. Therefore the thickness of the double layer decreases with increasing valence and concentration of the buffer electrolyte. In aqueous solution it is on scale of a few nanometers and the thickness decreases with the concentration of the electrolyte. Experimentally, the double layer thickness is generally found to be somewhat larger than calculated

5.3.3 Experimental

5.3.3.1 Sensor Fabrication. In the first process step 500nm thick Silicon Nitride is deposited on the single side polished <100> Si wafer followed by the patterning of 1μm thick photo resist spacers to act as the sacrificial layer for the formation of the first set of Au electrodes. Gold electrodes are deposited using E-beam evaporation under ultra high vacuum conditions. The selective removal of the photo resist sacrificial layer defines the first set of Au electrodes. In the next step a very thin and uniform layer of SiO_2 is deposited using Plasma Enhanced Chemical Vapor Deposition (PECVD), to form the nanometer spacers between the electrodes. A second layer of 1μm gold metallization is applied using E-beam evaporation and SiO_2 is etched off to form the nano cavities.

Figure 5.6 Microscopic Image of the fabricated sensor.

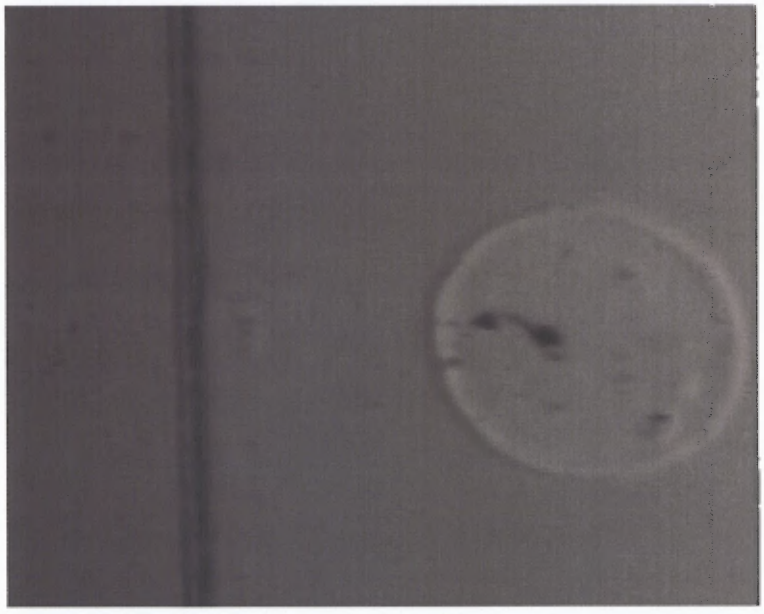

Figure 5.7 Microscopic image of the nanoscale sensing area in comparison to a cell

5.3.3.2 Materials. Alpha-thrombin (α-thrombin) aptamers modified with thiol linkers (5'- GGT TGG TTT GGT TGG TTT - $(CH_2)_3$SH-3') were used for the specific binding

experiments. A scrambled version of the specific aptamer, (5'-GTG TGT GTG TGT GTG TTT - $(CH_2)_3SH$-3') also modified with the thiol linker was used for control experiments. The DNA aptamers used for the experiments were purchased from IDT (Integrated DNA Technologies). α-thrombin samples were purchased from Haematologic Technologies, Essex Junction, VT. Lysozyme and the buffer solution 20xSSC (3.0M Sodium Chloride + 0.3M Sodium Citrate) were purchased from Sigma-Aldrich.

Aptamer Sequences:

Alpha thrombin aptamer (apt1) – 5'-GGT TGG TTT GGT TGG TTT/3 ThioMC3-D/-3'

Scrambled aptamer (apt2) – 5'-GTG TGT GTG TGT GTG TTT/3 Thio MC3-D/-3'

5.3.3.3 Aptamer Probe Immobilization. Prior to the immobilization procedure the structure was cleansed using acetone, isopropanol and deionized water. Single stranded probe DNA aptamer sequences pre-modified by the thiol linker (5'- GGT TGG TTT GGT TGG TTT/3 - $(CH_2)_3SH$-3') were immobilized on the gold electrodes using a concentration of 10μM in 0.5xSSC buffer (75mM Sodium Chloride + 7.5mM Sodium Citrate). By taking advantage of the high affinity of sulphur atoms to gold substrate, the DNA molecules with thiol end groups are chemically assembled onto the gold surface from the solution [65]. The Van der Waals forces between adjacent chains helps to order the oligomers parallel to each other. After immobilization of the DNA aptamers, mercaptohexanol (HS- $(CH_2)_6$ OH) self assembled monolayers (SAMs) were immobilized in between the DNA strands in order to passivate the vacant spaces. The <111> crystal orientation of gold which is obtained by thin film deposition provides an optimum substrate for the formation of such monolayers.

5.3.3.4 Measurement Procedure. The dielectric properties were investigated over a frequency range of 10Hz to 100 kHz, with 0V DC bias and 20mV AC signals using an SR 785, 2 channel dynamic signal analyzer. A Lab View program was used to collect and record data through a GPIB interface. The electrical contacts and the functioning of the entire system including the capacitive element were verified by measuring the dielectric spectrum with air and deionized (DI) water between the electrodes.

In order to monitor the formation of the bio-recognition layer, measurements were taken before and after aptamer immobilization. After immobilization, α-thrombin solution varying from 0.1μM to 1μM was added on the sensor surface. After a short incubation time, the variation of the dielectric spectrum was recorded. To monitor the exact response of the sensor towards the bound protein, the measurement was taken after the removal of unbound proteins by washing. As a first control experiment, the above experimental procedure was repeated with lysozyme, a positively charged protein which does not bind to the α-thrombin aptamer. The interaction of α-thrombin molecules to a scrambled version of the specific aptamer is performed as a second control measurement. Each step of the aptamer immobilization and the target binding was characterized by dielectric spectroscopic measurements conducted on the nano-cavity capacitive electrodes.

5.3.4 Results and Discussion

Dielectric spectra of the binding experiments were obtained by conducting impedance spectroscopy analysis on the nanogap capacitive structure. The relative permittivity of air was measured and found to be close to 1 and the relative permittivity of DI water was used for calibration. The result is shown in Figure 5.8. From the result it can be seen that

the relative permittivity of water is seen to be around 78. It is slightly lower than the reported value of 80 due to the presence of unfilled air gaps in the nano-space cavities.

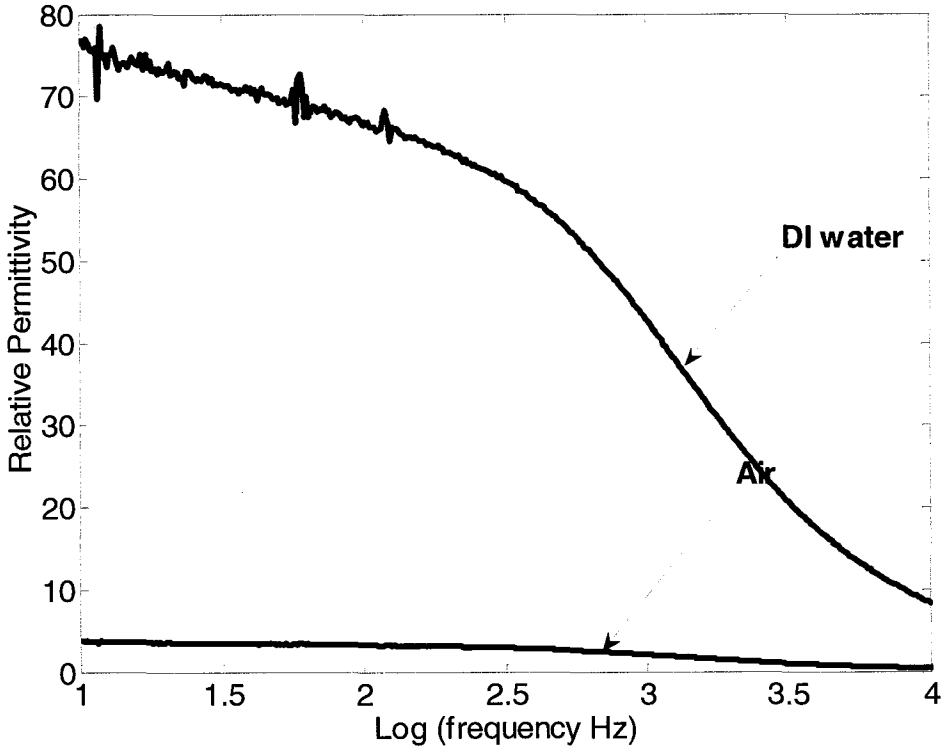

Figure 5.8 Dielectric spectra of deionized water and air.

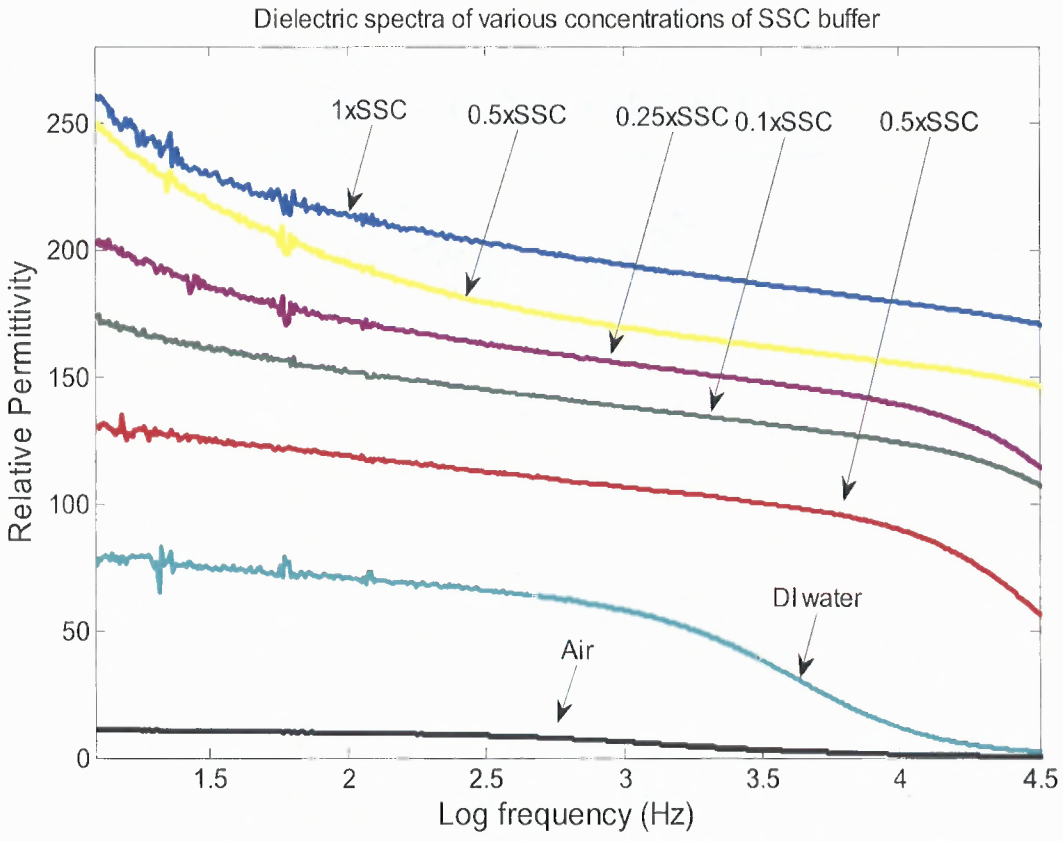

Figure 5.8 Dielectric spectra of various buffer concentrations.

Figure 5.8 shows the dielectric spectra of various concentration of buffer solution was analyzed and plotted. From the results it can be seen that the dielectric permittivity of the solutions increases with increase in buffer concentration. This can be explained by the fact that as the number of ions increase in the sample, the Debye length reduces thus increasing the double layer capacitance and hence the measured relative permittivity.

5.3.4.1 Dielectric Spectra for Alpha Thrombin- Aptamer Binding. In order to monitor the formation of the bio-recognition layer, measurements were taken before and after aptamer immobilization in 0.5xSSC. After immobilization, α-Thrombin solution concentration varying from 0.1μM to 1μM was added on the sensor surface. After a short

incubation time and rinsing process, the variation of the dielectric spectrum was recorded. The addition of positively charged alpha thrombin showed a decrease in the value of relative permittivity.

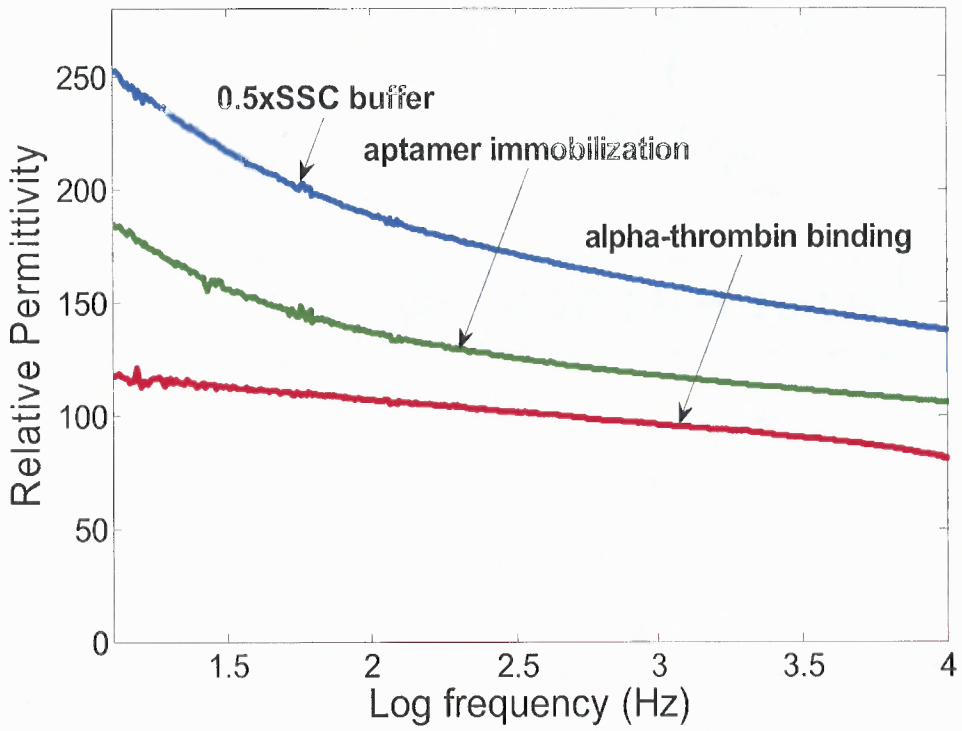

Figure 5.9 Dielectric spectra for alpha thrombin- aptamer binding.

Figure 5.9 shows the dielectric spectrum after aptamer immobilization and Alpha-thrombin binding. A reduction in the dielectric spectra was observed after immobilization and alpha thrombin binding. This decrease in overall sensor permittivity could be due replacement of water molecules (ε= 80) by oligonucleotide sequences during immobilization process. The binding of protein molecules further enhances this effect.

5.3.4.2 Dielectric Spectra for Lysozyme α-Thrombin Aptamer Interaction. In this control experiment, the immobilized α-Thrombin aptamer was allowed to interact with Lysozyme, a positively charged protein similar to α-Thrombin. The concentration used was 0.05 μM.

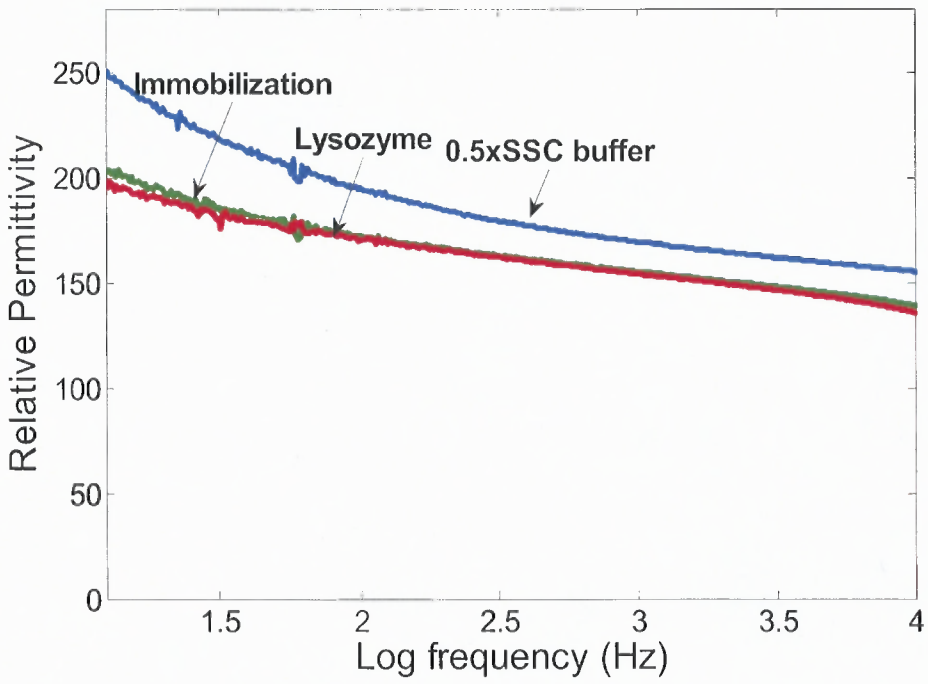

Figure 5.10 Dielectric spectra for Lysozyme α-Thrombin aptamer interaction.

An initial decrease of the relative permittivity was observed upon the addition of the positively charged molecules, but the spectra returned to the original value after rinsing off the unbound molecules. The Figure shows negligible change in dielectric property upon exposure to these non-specific target molecules. This supports the relationship between capacitance (relative permittivity) change and specific biomolecular interaction.

5.3.4.3 Dielectric spectra of Alpha Thrombin –Scrambled Aptamer Interaction.

In order to verify that the change in the value of rel. permittivity observed in the above experiment was due to the specific protein – aptamer interaction, a scrambled version of the aptamer was immobilized and allowed to interact with alpha thrombin. Even though an initial decrease in the value of relative permittivity from the SAM layer was observed due to the positively charged protein, rinsing off the non-specific bound molecules resulted in a spectrum close to the original one.

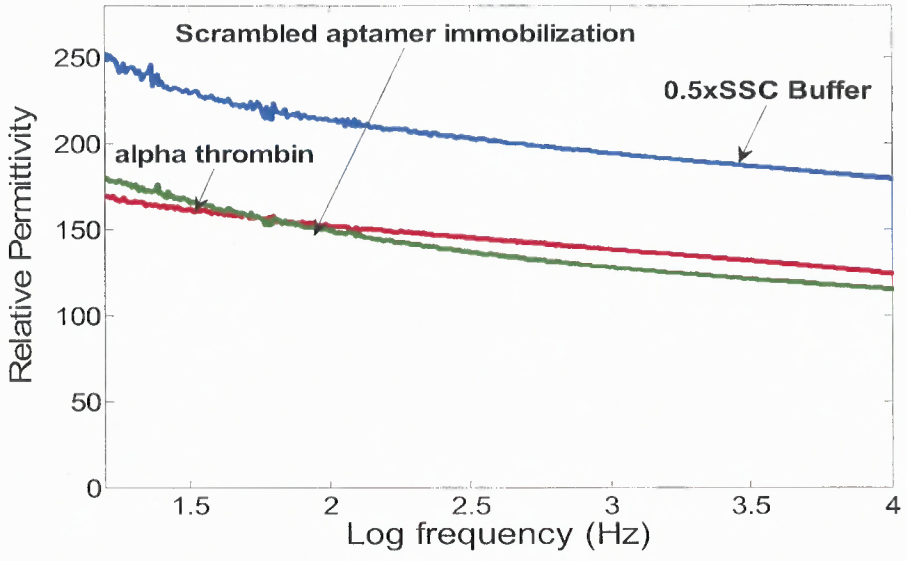

Figure 5.11 Dielectric spectra of alpha thrombin –scrambled aptamer interaction.

Dielectric spectra of the second control experiment; interaction of the α-Thrombin molecules with a layer of immobilized scrambled aptamer is shown in Figure 5.11. The Figure shows negligible change in dielectric property upon exposure to these non-specific target molecules. This proves the specific interaction between the α-Thrombin molecules and their aptamer sequences. The slight shift in the relative permittivity in the case of

and their aptamer sequences. The slight shift in the relative permittivity in the case of both the control experiments can be accounted by the electrostatic interaction between the positively charged protein molecules (α-Thrombin and Lysozyme) and the negatively charged aptamer molecules.

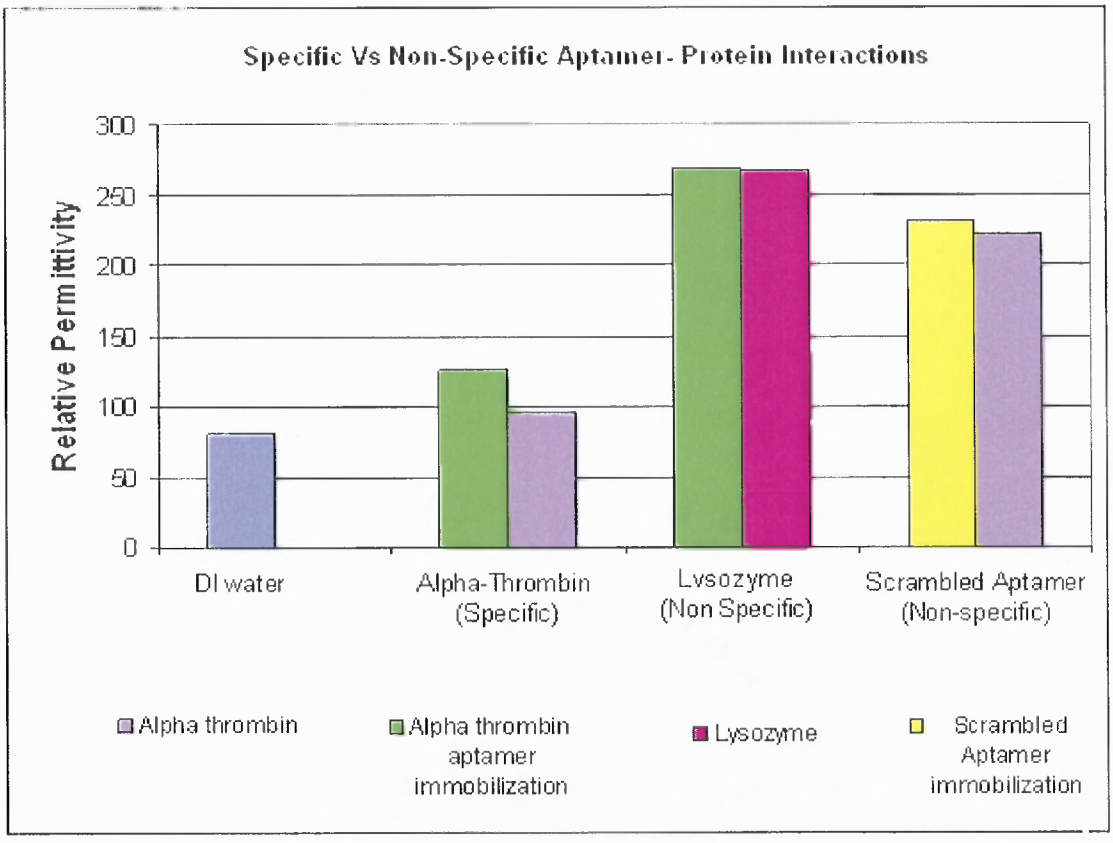

Figure 5.12 Relative permittivity change due to specific and non- specific interactions.

The measured values of Relative Permittivity at 210Hz for DI water, specific binding experiment. (alpha thrombin- aptamer 1), and control experiments are plotted. Due to the specific binding between alpha thrombin and aptamer 1, a significant change in relative permittivity is observed from the value of immobilized aptamer layer (114.4 to 84.22). The shift in relative permittivity observed due to the first control experiment

(alpha thrombin- aptamer 2) was considerably low as compared to the specific binding (234.1 to 224.4). The addition of lysozyme to the immobilized alpha thrombin aptamer layer produced slight change in the value of relative permittivity.

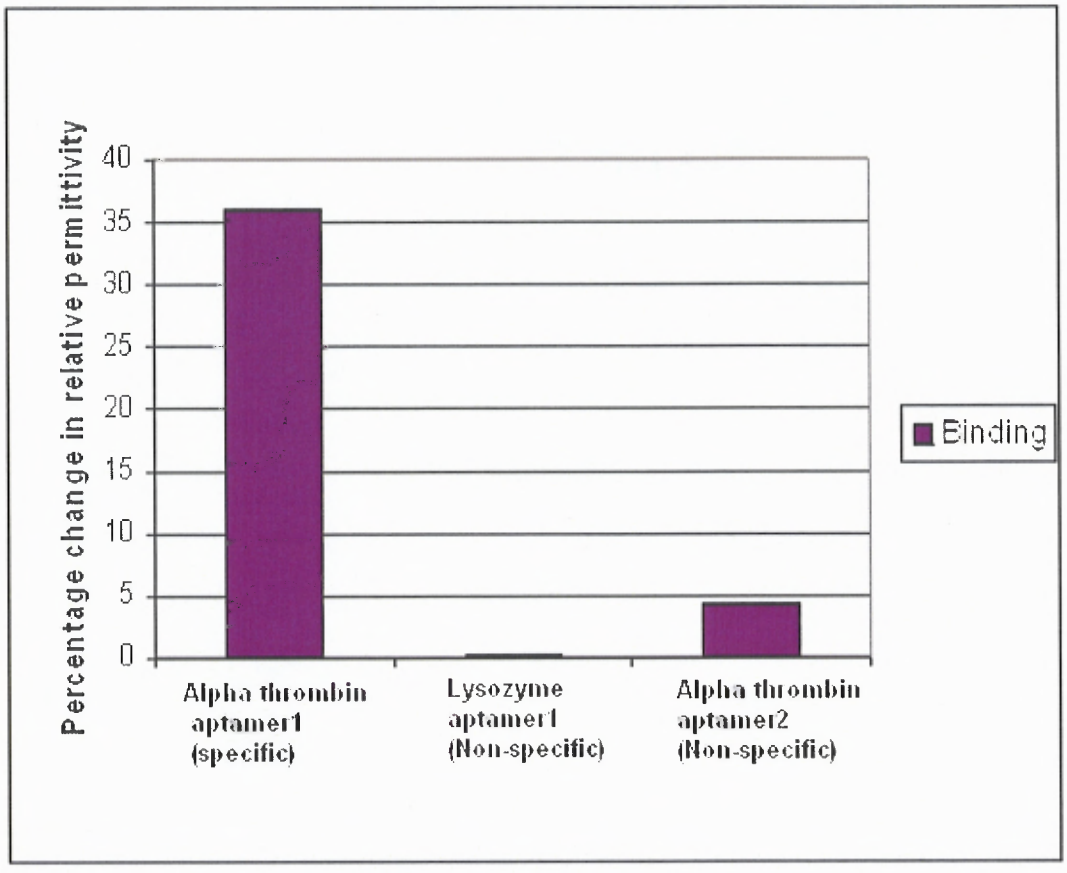

Figure 5.13 Percentage change in relative permittivity.

The percentage changes in relative permittivity for the three binding experiments were calculated with respect to the corresponding SAM layers. Due to the specific binding of alpha thrombin to its aptamer (aptamer1), a 35.83 % decrease in the value of relative permittivity is observed from the value of immobilized aptamer. This shows the improved sensitivity of the nanoscale dielectric sensing mechanism due to the minimization of electrode polarization effect through the reduced space confinement. The

interaction of alpha thrombin with aptamer 2 (scrambled aptamer) gave a variation of 4.32% in relative permittivity and about 0.23% change was observed for the interaction between lysozyme and aptamer 1 (non-specific interaction). This proves the specificity of the aptamer molecules to the specific proteins as well as demonstrates the selectivity of the entire sensing mechanism.

CHAPTER 6

CONCLUDING REMARKS AND FUTURE OUTLOOK

The aim of this thesis was to develop an aptamer based immunosensor that allows label-free detection of protein molecules. Improved sensitivity of electrochemical capacitive sensors towards biomolecular interactions due to reduced electrode spacing was demonstrated in this work. This sensing mechanism offer many advantages including small sample volumes, easy sensor array fabrication and portability over the macro-scale setups. Further this detection mechanism is label-free as it does not require samples to be labeled with fluorescent, radioactive or redox tags for sensing.

Development of two key components of the protein sensor was discussed in detail; the aptamer based recognition element and the electrochemical signal transducing element. Aptamer molecules were used in this sensor as recognition elements mainly due to their better shelf life and stability. These molecules were linked to the gold surface using the well known thiol-chemisty.

In the development of sensing mechanism, both piezoelectric and capacitive methods were investigated. Although good sensitivity was achieved by piezoelectric method, the inability of the system to measure in liquid phase posed to be a major problem. Development of capacitive sensing mechanism therefore proved to be better suited as real time monitoring of biomolecular recognition process in liquid medium was made possible.

Towards the development phase of this immunosensor; Lab-on-a-chip devices capable of identifying protein molecules in real time is envisioned. Integrating this microfabricated immunosensor along with well designed microfluidic processors and

MEMS components can enable the development of such miniaturized devices that are capable of purifying, isolating and characterizing samples, thus putting the entire assaying operation on a single chip. Figure 6.1 shows a graphical version of some of the process stages to be incorporated for the development of such a hand held device.

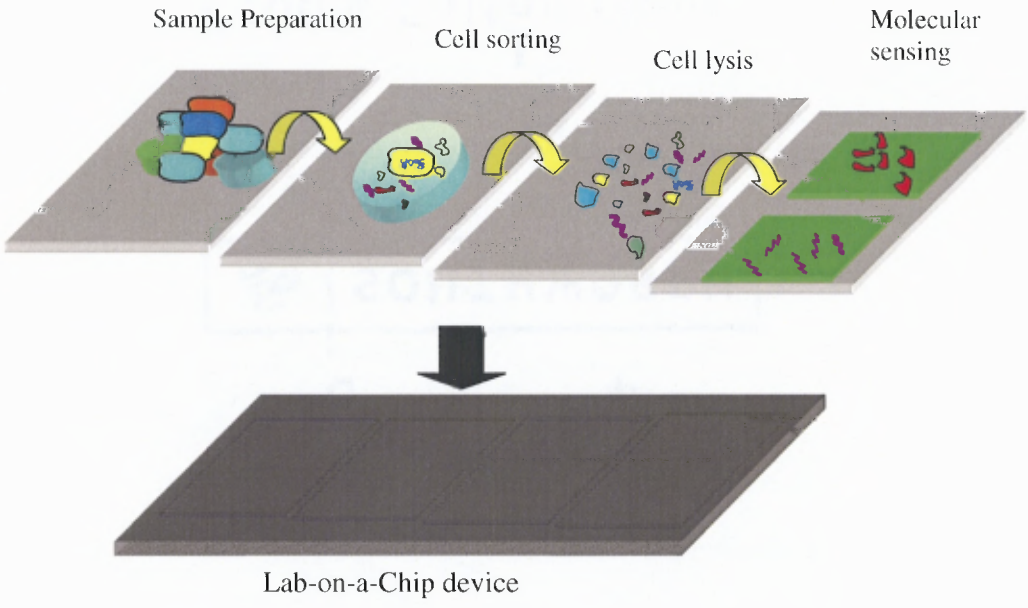

Figure 6.1 Future development towards a lab-on-a-chip device.

In addition, the design of the microfabricated immunosensor is such that a number of multiple sensing elements can be incorporated together and can be multiplexed electronically to detect many targets simultaneously. Due to the above mentioned advantages, this sensing mechanism can be used for the development of lab-on-a-chip and point-of care devices. The potential for such devices are many including detection of biological weapons through protein analysis, blood analysis and drug screening systems.

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