APPLICATION OF BI-CELL SURFACE PLASMON RESONANCE FOR THE DETECTION OF APTAMER MEDIATED THROMBIN CAPTURE IN SERUM

By

RINOSH JOSHUA MANI

Bachelor of Veterinary Science and Animal Husbandry

Kerala Agricultural University

Kerala, India

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Thesis Approved:

Kenneth D. Clinkenbeard
Thesis Adviser

James H. Meinkoth

Rebecca J. Morton

A. Gordon Emslie
Dean of the Graduate College

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INTRODUCTION

Surface plasmon resonance (SPR) is a powerful technique for the detection and measurement of biomolecular interactions in real time. For the biology applications using SPR one of the interacting molecules is immobilized on the sensor surface as a capture probe while the other is passed over the surface in solution. An important advantage of this technique for biological applications is that it works in a label free environment. The most common biomolecule that is used as a capture probe for immobilization on the sensor surface is an antibody. In recent years aptamers have emerged as a promising substitute to antibodies. Aptamers are oligonucleotides that bind to target molecules in similar mechanisms to those of antibodies, with similar affinities and selectivities, but with added advantage of lower cost of production, ease of storage and also unlike antibodies aptamers can be produced against non-immunogenic targets.

Several SPR instruments are currently available commercially, each having their own advantages and disadvantages. The most common method of detection in SPR instruments uses either a linear diode array or charged coupled device. Recently a new detection system using a bi-cell photo detector has been introduced. This technique has

many advantages over the existing commercial instruments. Bi-cell SPR can cancel out the most important noise that current measurement methods encounter, which is the variation in the ambient light as well as the changes due to laser intensity fluctuations. Another advantage of this technique is that it is reputed to have a hundred times more resolution (sensitivity) than the other SPR instruments, and it is much cheaper to produce than the commercially available SPR instruments.

Our experimental objective was to assess a bi-cell SPR instrument as a model system for use of aptamers as capture probes for SPR in diagnostic testing. Our results demonstrate that an aptamer can be used as a capture probe in a bi-cell SPR instrument and thus validate the use of aptamers as an alternative to antibodies in SPR instruments. Because of the ease of use, better resolution and lower cost, this technique has a tremendous potential for use as a diagnostic tool for many clinical diseases.

A literature review of SPR, the role of SPR in biomolecular interaction analysis, sensitivity of the instrument, ligand immobilization techniques and specificity of the ligand-anlayte interaction is presented. Also included are reviews on thrombin aptamer and bi-cell SPR. The entire review is categorized under five different headings and the relative importance of each to my current research has been addressed.

CHAPTER II

REVIEW OF LITERATURE

Surface Plasmon Resonance

Principle and Background

The surface plasmon resonance (SPR) phenomenon was first discovered by R.W. Wood in 1902 (1). He observed the effect of surface plasmons on the intensity of light diffracted form metal gratings. The study of SPR phenomenon became popular in physics by the discovery that surface plasmons can be generated by attenuated total internal reflection of light, which was demonstrated by Kretshman and Otto (3). The interest in this physical phenomenon was further enhanced by the application of SPR phenomenon in biological science beginning in the late 1980s and early 1990s.

Surface plasmon resonance is a physical process, which occurs when a plane polarized light strikes a metal surface under total internal reflection (TIR) conditions. This TIR can be produced by passing a ray of light through a semi circular prism. When light strikes the prism it is diffracted and the light bends to the plane of the interface as it passes from the denser medium (prism) to the less dense medium (air) (Figure 1). As the angle of

incidence ( ) increases the angle of out coming light also changes. At a critical angle of incidence of light, incoming light is reflected within the prism and this is called total internal reflection (TIR). At the TIR even though no light is coming out of the prism, the electric field of photons extends about a quarter of wavelength of (incident light) beyond the reflecting surface. This wave, is called evanescent wave (Figure 1), has maximum intensity at the interface, and decays exponentially away from the interface.

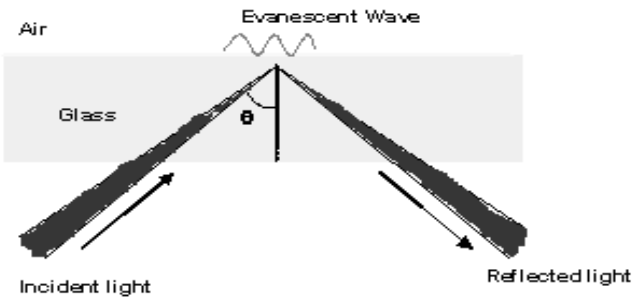

Figure 1. Under TIR the evanescent wave is formed at the interface of glass and air.

When the reflecting surface of the prism is coated with a thin film of a noble metal the photons will interact with the free electron constellations in the metal surface. The incidents photons are converted to surface plasmons (Figure 2). So surface plasmon resonance can be described as a quantum optical-electrical phenomenon arising from the interaction of light with a metal surface. In most cases the metal used is gold as it gives a SPR signal at convenient combinations of reflectance angle and wavelength. Also gold is comparatively chemically inert to solutions and solutes and can be easily applied in a variety of biochemical contexts (4). Under appropriate conditions, the plasmons in the conduction film resonantly couple with the light because their frequencies match. Since the energy is absorbed in this resonance, the reflected intensity I (Figure 2) decreases at

the angle at which SPR is occurring, and a black line or dip in the reflected light is observed (4).

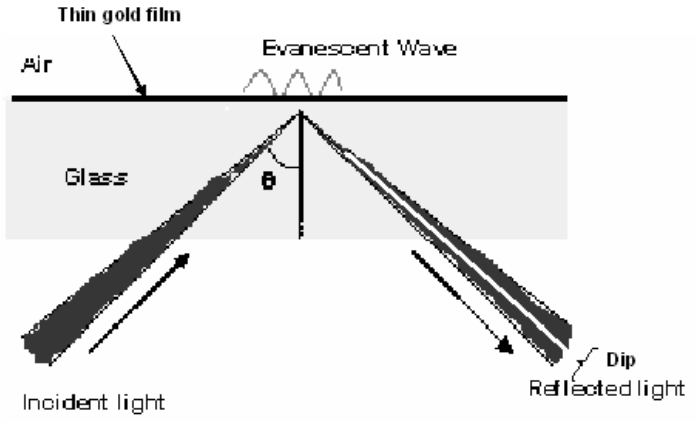

Figure 2. When a thin gold film is kept at the interface under TIR conditions, the some of the photons are taken up by the surface plasmons and a dip in the reflected light is produced.

As this resonance environment is extremely sensitive to refractive index of the medium adjacent to the metal film, any adsorption of molecules on the metal surface or any conformational changes in the adsorbed molecules on the surface of the metal can be accurately and sensitively detected. This can be explained by the principle of conservation of momentum. The velocities of the plasmons change as the medium changes and as a result the momentum of these particles change. Since there is a change in momentum, the angle of incident light at which resonance occurs also changes. This change in angle can be measured precisely. An SPR, which uses this principle, is called an angular SPR and is the most commonly used method for SPR instruments. In our present study the bi-cell SPR instrument we are using makes use of this principle. In contrast if a fixed angle of incident light is used, the wavelength is varied until the resonance occurs, this type of SPR is called a spectral SPR and is not commonly used (5).

The SPR phenomenon and the resonance angle is determined by three factors; the metal, the wavelength of the incident light, and the medium adjacent to the metal surface.

Metal

A variety of metallic elements satisfy this resonance condition. They include silver, gold, copper, aluminum, sodium, and indium. The metal must be free of oxides, sulphides and should not react to other molecules on exposure to atmosphere or liquid. Of these metals, gold is the most practical because it gives a strong and easy to measure SPR signal and is relatively chemically inert, but at the same time it can be made sufficiently reactive to accommodate coupling with wide variety of coating and functionalizing molecules including alkanethiol, dialkyl sulphide, and dialkyl disulphide derivatives. The best signal for the gold is obtained at a thickness of 50 nanometers (10). This thickness of the metal layer is of great importance because above an optimum, the dip in the reflected light becomes too shallow, and below the optimum, the dip becomes too broad for use in detection.

Wavelength

SPR is a physical process that can occur when light of a particular wavelength strikes a metal film under total internal reflection conditions. To obtain a sharp dip, the light source should be monochromatic and plane-polarized. All light, which is not plane-polarized, will contribute to the SPR and will increase the background intensity of the reflected light (10). The depth of the evanescent wave, which is useful for measurements, extends approximately 300nm from the sensor surface. In order to obtain a well-defined surface plasmon mode with the corresponding electro-magnetic field enhancement, an

excitation wavelength around 600nm is optimal for gold metal. This wavelength range falls in the visible and near-infrared parts of the spectrum and are particularly convenient because optical components and high performance detectors appropriate for this region are readily available. In our experiments a 5mW diode laser (λ = 670 nm, Hitachi), driven with a homemade laser controller, was collimated and then focused by a 14 mm local-length lens through the prism onto the gold film.

Medium

The movement of the surface plasmons produced by the resonance, like the movements of any electrically charged particle, generates an electric field. The plasmons electrical field extends about 100nm perpendicularly above and below the metal surface. The interaction between the plasmons electrical field and the material matter within the field determines the resonance wavelength. Any change in the composition of the matter within the range of the plasmon field causes a change in wavelength of light that resonates with the plasmon. The magnitude of the change in the resonance wavelength, the SPR shift, is directly proportional to the change in composition. In a sensor, the gold can be coated with capture probes like antibodies, receptors, or enzymes, which specifically interact with their target molecule. When the sensor is exposed to samples that contain the specific target molecule, the binding of the target to their cognate capture probe changes the composition of the medium at the surface and produces a SPR shift. The magnitude of the shift is proportional to the amount of binding. By comparing the observed shift to a known calibration curve we can quantify the concentration of the analyte in the sample.

Application

Any change in refractive index or any change in the mass at the surface can give an SPR shift. This means that the SPR phenomenon is non-specific with respect to the molecular composition of the target. This may appear to be a limiting factor but it has the powerful advantage in that a highly specific interaction like antigen-antibody, enzyme–substrate, or nucleic acid hybridization can be adapted to SPR measurement. One of the interacting pair is the capture probe and is attached to the gold surface, whereas the other is the analyte, which is usually exposed or passed over the probe in a flow cell. The shift in SPR angle is measured continuously to form a sensorgram as the target is flowed over and captured by the immobilized probe (Figure 3). SPR can be used as the basis for a sensor that is capable of sensitive and quantitative measurement of a broad spectrum of chemical and biological entities. It offers a number of important practical advantages over current analytical techniques. The time from sample application to reported result is limited only by the time the sample takes to reach the sensor surface, which can be in seconds. In most cases there is no need to pretreat the sample before its presentation to the sensor. Some of the potential areas of application include medical diagnostics, environmental monitoring, agriculture pesticide and antibiotic monitoring, food additive testing, military and civilian airborne biological and chemical agent testing, and real-time chemical and biological production process monitoring.

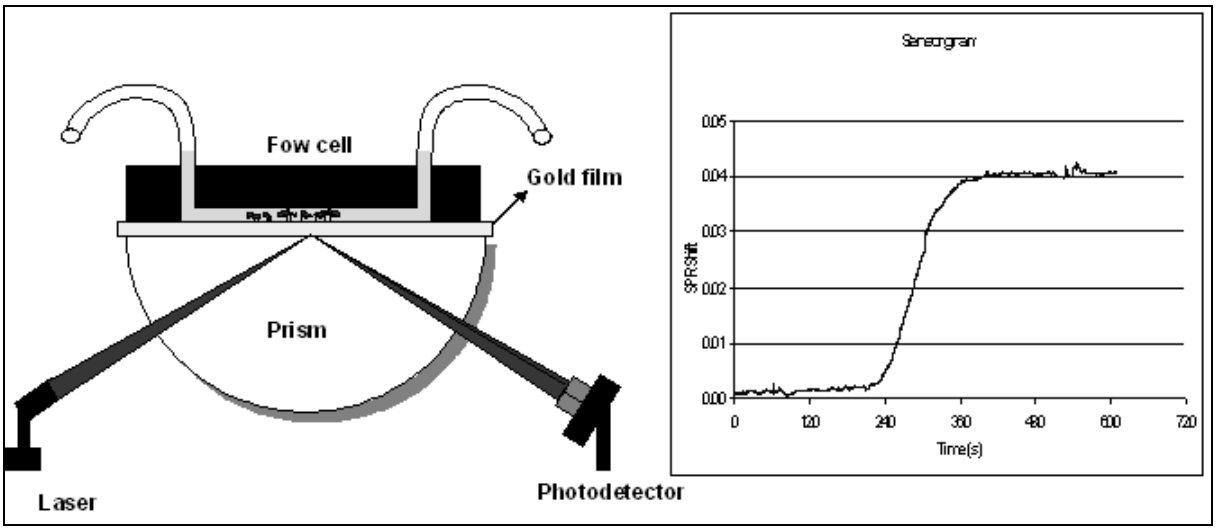

Figure 3. Typical SPR biosensor platform. TIR is produced by the prism. The capture probe immobilized on the gold film which is contact with a flow cell through which the analyte can be flowed at a constant rate. The real time capture of the target is detected and plotted in a sensogram.

In recent years much research has been focused on different sensor platforms mainly targeting different applications areas and in providing solutions to specific applications requirements. The two main areas of current SPR biosensing applications is high throughput screening through multiple sensing channels and in the development of mobile SPR platforms for analysis of complex samples in field conditions.

One of the early interests for biochemists in SPR technology was in its ability to directly determine the separate association and dissociation rate constants of biochemical reactions. These parameters are of great importance in classifying different molecules, especially antibodies. With the increasing demand in therapeutic as well as research field for both polyclonal and monoclonal antibodies, high throughput analysis to determine antibodies with the greatest affinity for their target molecules is needed. The real time kinetic analysis with no sample pre-treatment offered by the SPR technique is the ideal

choice for such analysis. The antibody is usually immobilized on the sensor surface and the antigen is passed over it in a flow cell. This type of immuno SPR is the most popular and a number of experiments have been done using this kind of sensor platform for calculating the binding parameters of interactants and also to detect the presence of the analyte. In one such study the rate constants for the interaction between cancer testis antigen NY-ESO-1 and its monoclonal antibody ES121 were accurately measured using SPR (14). In another experiment a comparison was made between SPR and quartz crystal microbalance (QCM) which is another surface sensitive analytical device. In that study antibody to human IgE was immobilized on the sensor surface and IgE was the analyte. In this comparison, it was found that SPR is more than twice as sensitive as the QCM with better reproducibility and reliability (16). Several other comparison studies have been done between SPR and other common analytical techniques. A comparison between SPR and chemiluminescent immunoassay was done regarding the binding of human ferritin and its monoclonal antibody in which the coefficient of correlation between the two techniques was found to be 0.991(17). This model was also proposed to be developed as a biosensor for detecting human ferritin, which is known as a non-specific marker of the inflammatory processes and neoplasia.

In the area of SPR detection of a whole cell or an intact pathogen, very few studies have been done, as SPR technology is more suited for studying small molecular interactions. In one such study using SPR, antibodies against *Salmonella typhimurium* immobilized on sensor surface were able to detect the bacteria at levels of 10^2 to 10^9 CFU per ml (6). In another study by T.P Shevchnko *et al*, intact tobacco mosaic virus was detected using specific IgG immobilized on the sensor surface (18).

SPR applications using other types of interactions include protein-DNA and DNA-DNA hybridization interactions. A SPR bioassay useful for the detecting transcription factors were described by James P. Brody *et al*, in which a mutated promotor sequence would bind to a transcription factor leading to inhibition of the transcription (8). In another study a highly sensitive flow injection SPR was described which could detect DNA hybridization at femtomolar range (7). Other biological applications of SPR include environmental monitoring as of carbaryl detection in natural water samples (15) and drug interaction studies, and screening of novel drug targets. Bio-recognition events can be studied by mimicking the process as happening in biological system as described in the study in which a bio-recognition of G protein transducer and rhodopsin interaction occurring upon excitation by a particular wavelength of light (13).

Ligand Immobilization

Most biological molecules have a high recognition power to bind with their interacting partners. This binding, which can be in the form of antigen-antibody, ligand-receptor, enzyme-substrate, or hybridization reactions, is due to specific physical shape, electrostatic properties, and chemical binding that takes place between the two molecules. Such high affinity binding partners are usually employed in SPR analysis. However, most biological molecules also have a tendency to adsorb onto non-functionalized solid substrates which have not been derivatized with a specific capture probe (21). This type of interaction can produce high background noise and can interfere with the analysis. In

SPR instruments, one of the important factors which can hinder the sensitivity is this non-specific binding.

Self-assembled monolayers (SAMs) are described as ordered molecular assemblies formed by the adsorption of an active surfactant on a solid surface (25). SAMs of functionalized long-chain hydrocarbons are commonly used as building blocks in biosensor platforms and can diminish non-specific binding of the target to the sensor platform. SAMs also help to properly orient capture probes through a variety of coupling chemistries so that maximum contact with the analyte is possible. The most important discovery in this field was that alkanethiol SAMs on gold could be prepared by adsorption of di-n-alkyl disulphides from dilute solutions (27). Sulphur containing compounds like the alkanethiols have a strong affinity for the gold surface and the binding is thought to be to the oxidative addition of S-H bond and reductive elimination of molecular hydrogen, with the result that the thiol derivative is firmly bound to the gold crystal lattice. The capture probe is usually coupled to such SAM through a variety of binding chemistries including amine coupling, thiol coupling and non-covalent coupling methods (26).

Amine coupling of a capture probe to the alkane thiol SAM is done by coupling primary amines using N-hydroxysuccinimide/1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (NHS/EDC) coupling method. This coupling requires a free N-terminus or lysine residue on the capture probe. The thiolated alkanoicacid monolayer or the carboxy dextran surfaces (usually used in Biacore SPR) have free carboxylic groups and NHS/EDC activate these carboxylic groups making them more reactive to amino groups on the

capture probe. This kind of binding method is described in many SPR experiments. A tumor marker, IL-8, was detected using SPR by immobilizing anti IL-8 IgG on the sensor surface using such a method (23). In these types of reactions the excessive reactive carboxylic acid groups are usually blocked using ethanolamine. However, if the capture probe has a high concentration of lysine residues, the amino group on those residues may bind with reactive carboxylic acid group and this may diminish the proper orientation of the capture probe.

In other methods of immobilizing biomolecules on sensor surfaces, especially antibodies, protein A is widely used. Protein A is a cell surface protein and has high specificity for the Fc portion of the immunoglobulin. In the SPR system used for detecting tobacco mosaic virus, an antibody against the virus was first immobilized on the protein A (18). In other systems, a concanavalin A is used. Concanavalin A is a lectin with affinity for carbohydrate moieties, and proteins with free carbohydrate groups can be attached to the sensor platform via immobilized ConA. In SPR detection of rhodopsin, ConA was immobilized on carboxy dextran monolayer on the gold through amine coupling (13).

Other common immobilization methods include cystamine-gluteraldehyde method wherein the capture probe is coupled to the gluteraldehyde by amine coupling (17) and biotin-streptavidin method where the biotinylated capture probe can be immobilized on to the streptavidin layer (8). In a novel technique polyhistidine–tagged tumor antigens were bound to a nickel-nitriloacetic acid functionalized surface (14).

In all these different methods, initial binding to the gold is through a sulphide, disulphide, or thiol group, and the capture probe is immobilized on these monolayers. All the above mentioned binding chemistries have their advantages and limitations, some may show better reusability of sensor surface as in cysteine-gluteraldehyde method and some may orient the ligand in the best conformation possible thereby increasing sensitivity as in the case of the nickel-nitriloacetic acid immobilization method.

One commonly used method for immobilizing DNA on the gold surface is to immobilize biotinylated nucleic acid using streptavidin-biotin binding chemistry. Alternatively a direct immobilization can be used by modifying the DNA with a S-H group attached to the deoxyribose group on the 3 prime end of the nucleic acid and thus directly immobilizing it on to the gold. This method was utilized for the detection of oligonucleotide hybridization and gene analysis of *Arabidopsis thaliana* leaf extract (7). This study demonstrated particularly high sensitivity which may be due to the bi-cell SPR technology used as well as the relatively high molecular mass target used. For small length oligos, the proper orientation of the probe may be extremely important especially when the interaction depends on special chemistry as in the case of aptamer and its target. In our present study, the DNA aptamer that we used was a 15mer oligonucleotide modified with a thiol group at the 3' end. We used 6-mercapto hexanol, which is an alkanethiol, to form a SAM on the gold via a thiol linkage. This mercaptohexanol layer helps to block the remaining gold surface where the aptamer has not bound and also to elevate and properly orient the aptamer for analyte binding.

Aptamers

Aptamers are macromolecules composed of short, single strand nucleic acids, such as RNA or DNA molecules of a particular sequence which cause the oligo to assume a particular structure that complex with another target molecule, such as, a particular protein. The word aptamer was coined form the Latin 'aptus', to fit (29). Aptamer was initially used to refer to RNA molecules that bind to a specific molecular target. The term was later extended to DNA molecules (30). Aptamers are selected *in vitro* from random nucleotide libraries by multiple rounds of target screening and enrichment. This procedure was first developed by Craig Tuerk and Larry Gold and was named SELEX (**S**ystematic **E**volution of **L**igands by **EX**ponential enrichment) (28).

SELEX

SELEX is a protocol for isolating high affinity ligands to a target protein from a pool of variant nucleic acid sequences (28). Although the initial use of SELEX involved the identification of sequence information crucial to naturally occurring protein-RNA interactions, it became apparent that this procedure could be used in the design of useful reagents (32). Thus, RNA ligands could be developed for proteins with the intent of inhibiting or otherwise affecting the function of target molecules. If the target molecules already have natural nucleic acid ligands, one would expect that SELEX could provide

ligands of higher affinity because: (a) nature may select against optimal affinity, (b) natural evolution conducts an incomplete survey of all possible sequences, or (c) overlapping sequence constraints, such as those of protein-coding sequences, introduce competing priorities over genetic drift to higher affinity sequences (32).

Aptamer Chemistry

Aptamers range in size from approximately 6 to 40 kDa and some can assume complex three-dimensional structures produced by a combination of Watson–Crick and non-canonical intramolecular interactions (38). They bind to their targets with dissociation constant (K_D) typically in the low nanomolar range and can distinguish enantiomers of small molecules or minor sequence variants of macromolecules with frequency several orders of magnitude in K_D ratio. In contrast to proteins, nucleic acids are strikingly uniform in their hydrophilicity and low isoelectric point (p*I)*. In spite of these limitations, hydrogen bonds and stacking interactions of their component bases provide a diverse toolbox of structural motifs (38). High-affinity nucleic acid–protein interactions require specific complementary contacts between functional groups on both the nucleic acid and the protein. Because the specific three-dimensional arrangement of complementary contact sites that mediate the protein-aptamer interaction are unlikely to be recapitulated in other proteins, aptamers are generally specific for their targets (20). Aptamers are different from antibodies, yet they mimic properties of antibodies in a variety of diagnostic formats (37).

Advantages of aptamers over antibodies:

Antibodies have been the primary choice for capture ligands in bioassays. But there are some inherent limitations of using antibodies in assays, which owe to the physical and chemical properties of these molecules and also to their manufacturing process. The antibody identification process starts within an animal; therefore, antibody generation becomes difficult with molecules that are not well tolerated by animals, such as toxins (37). Furthermore, antibodies against molecules that are inherently less immunogenic are difficult to produce. By and large, the generation of hybridomas are restricted to rat and mouse, limiting the use of antibodies in therapeutic applications. Antibodies of non-human origin have implications in diagnostic applications as well. Heterophilic antibodies (human antibodies that recognize antibodies of non-human origin) that exist in humans could potentially link a capture antibody with a detector antibody of non-human origin in the absence of the specific analyte, leading to false positive results (70). Rheumatoid factors and auto antibodies also interfere in immunoassays.

Another disadvantage of monoclonal antibodies is that the identification and production technologies are laborious and could become very expensive in searches for rare antibodies that require screening of the death of cell lines. Typically, high yields of monoclonal antibodies are obtained by growing the hybridomas in the peritoneal cavities of animals and purifying the antibody from ascites fluid (37). Some hybridomas are difficult to grow *in vivo*, thus restricting this route of antibody production. The performance of the same antibody tends to vary from batch to batch, requiring

immunoassays to be reoptimized with each new batch of antibodies. Although the production of antibodies is subject to *in vivo* variations, the identification of antibodies is restricted by *in vivo* parameters (37). In other words, identification of antibodies that could recognize targets under conditions other than physiological conditions is not feasible. Antibodies are sensitive to temperature and undergo irreversible denaturation. They also have limited shelf life.

Aptamers are identified through an *in vitro* process that does not depend on animals, cells, or even *in vivo* conditions. As a result, the properties of aptamers have the potential to be changed on demand. Selection conditions can be manipulated to obtain aptamers with properties desirable for *in vitro* diagnostics. For example, aptamers that bind to a target in a non-physiological buffer and at non-physiological temperatures could be identified. Similarly, kinetic parameters, such as the kinetics of association – dissociation rates of aptamers, have the potential to be changed as needed. Because animals or cells are not involved in aptamer identification, toxins as well as molecules that do not elicit good immune responses can be used to generate high-affinity aptamers. Another advantage of aptamers is that they are produced by chemical synthesis with extreme accuracy and reproducibility. They are purified under denaturing conditions to a very high degree of purity. Therefore, little to no batch-to-batch variation is expected in aptamer production.

Reporter molecules such as fluorescein and binding molecules like biotin can be attached to aptamers at precise locations identified by the user. Functional groups that

allow subsequent derivatization of aptamers with other molecules can also be attached during the chemical synthesis of aptamers. Aptamers undergo denaturation, but the process is reversible. Once denatured, functional aptamers could be regenerated easily within minutes. They are stable to long-term storage and can be transported at ambient temperature (37).

Applications of aptamers

The concept of using nucleic acids to bind to and inhibit the activities of target proteins grew out of early HIV gene therapy studies that employed RNA ligands, termed decoys, to competitively inhibit the activities of essential HIV proteins and in the process block viral replication (35). However, the therapeutic potential of aptamers depends on many issues. First, aptamers must interact tightly and specifically with their targets. The large size and surface area of nucleic acids is a decided advantage, in that they can potentially form many more interactions with targets than can smaller molecules. Similarly, the large size of aptamers gives them multiple opportunities to discriminate between epitopes on related proteins, and aptamers have been shown to distinguish between even closely related targets, such as protein kinase C (PKC) isozymes that are 96% identical. Secondly, aptamers must specifically disrupt the function of their targets. Finally, aptamers must be able to not only disrupt the function of a particular target, but also inhibit or modify the metabolism associated with that target. For example, anti-thrombin aptamers have been shown to block blood clotting (33). Some of the disadvantages of using aptamers in therapeutics are that being an RNA or DNA

molecules they are relatively susceptible to degradation by nucleases and will be unstable in sera or within cells. The development of stable nucleic acid aptamers now allows researchers to proceed to the more difficult problems of delivery and bioavailability. Also, the specificity of aptamers for their targets may ward off the systemic side effects often associated with pharmaceuticals; this same specificity may encourage the evolution of metabolic or viral resistance (36).

Applications of aptamers in diagnosis assays and biosensors

Because of their reputed robustness and high specificity for its target molecule, aptamers have great potential to be used in a variety of diagnostic assays. For instance, radio-labeled aptamers bound to a protein target can be separated from unbound aptamers with nitrocellulose filters that generally bind protein but not RNA or DNA. However, in a clinical setting, it would be preferable to avoid radioactive labels and extensive enzymatic manipulations. One way to simplify the detection of aptamer-protein complexes is to link the aptamer to an enzyme that has an activity that is easily assayed. An aptamer based detection system for the detection of binding of *Francisella tulerensis* subspecies japonica bacterial antigen, using a set of 25 DNA cocktail sequences by aptamer-linked immobilized sorbent assay (ALISA) was described by Jeevalatha and co-workers (71). Here a biotin linked secondary aptamer was used in a sandwich manner and streptavidin conjugated horse radish peroxidase was used as the reporter enzyme. A detection limit of 1.7×10^3 bacteria per ml was possible using this method and the detection limit for an assay suing antibodies for the same antigen was 6.9×10^3 bacteria

per ml. Capillary electrophoresis/ laser induced fluorescence assay was developed to detect flourescently labeled aptamers that was bound to HIV-reverse transcriptase enzyme (72). In another work alkaline phosphatase linked aptamers were used to detect bile acids (73). Other novel methods of using labeled aptamers include aptamer beacons for detection of thrombin (74) and pyrroquinoline quinine glucose dehydrogenase labeled aptamer for detection of thrombin. In the latter assay two aptamers against thrombin, the one discovered by Bock *et al* and the other discovered by Tasset *et al* which binds to thrombin at two different regions were used in a sandwich type assay.

Aptamers have also been used in a label free system, as in the case of detection of IgE (76) and HIV Tat proteins (77) using quartz crystal microbalance. Also ultrasensitive biosensors can be developed by combining aptamer and PCR technology. In one such report, a few hundred molecules of thrombin were detected using the DNA aptamer linked with 7 additional bases which functioned as template for PCR (78).

Thrombin protein and thrombin aptamer

Thrombin is a serine protease responsible for the conversion of fibrinogen to fibrin, platelet activation, and the cleavage of coagulation factors V, VIII, XI, and XIII (33). The delicate balance between hemostasis and hemorrhage is maintained by a complex system of plasma, cellular, and endothelial factors. Coagulation, the normal process by which a fibrin clot is generated in response to a vascular injury, is to be distinguished from thrombosis, the pathological formation of clot in response to injury, stasis, or hypercoagulability. The latter is widespread in conditions such as acute

coronary syndrome, stroke, peripheral vascular disease, and deep vein thrombosis, and can also occur in response to iatrogenic vascular injury. The clinical demand for more and better antithrombotics, that inhibit the initial formation of the platelet plug, and anticoagulants, that inhibit the cascade of reactions leading to the crosslinking of fibrin, is testimony to the importance of this process. In addition, the variety of patients and scenarios in which such agents are utilized requires an array of inhibitors with different mechanisms, properties, and toxicity profiles (39). In addition to its role in blood coagulation, thrombin can act as a potent mitogen and can also exert a chemotactic effect on monocytes. Because of its pivotal role in both thrombosis and hemostasis, thrombin is a major target for anticoagulation and cardiovascular disease therapy (33).

Using a novel *in vitro* selection/amplification technique, a new class of thrombin inhibitors based on single-stranded DNA (ssDNA) oligonucleotides were identified by Bock *et al* (30). These thrombin inhibitors are the first example of the use of the SELEX technique to obtain ssDNA oligonucleotides that bind a target protein with no known specificity for nucleic acids. One oligonucleotide sequence, GGTTGGTGTGGTTGG (thrombin aptamer) was capable of nanomolar inhibition of fibrinogen cleavage at nanomolar concentrations *in vitro* and was shown to inhibit clot-bound thrombin and reduce arterial thrombus formation in an *ex vivo* whole artery angioplasty model (30). Recent *in vivo* studies in cynomolgus monkeys have shown the thrombin aptamer to be a potent anticoagulant with a rapid onset of action and a short half-life (33). Unlike hirudin and other active site thrombin inhibitors, the thrombin aptamer does not inhibit the cleavage of small chromogenic amide substrates indicating that the aptamer does not bind

directly to the active site of thrombin. However, distinct from the catalytic center of thrombin are two highly basic regions that form secondary binding sites on the surface of the molecule. These sites are important for thrombin specificity in interactions with several macromolecular substrates and receptors. One site, the anion-binding exosite, contributes to a tight, specific complex with fibrinogen. The second site, the putative heparin recognition site, contributes to the significant increase of thrombin inactivation by antithrombin III in the presence of heparin. Considering the polyanionic nature of the nucleic acid phosphodiester backbone, these basic sites on thrombin, shown to interact with acidic regions of other thrombin-binding molecules, are likely targets for thrombin aptamer binding.

Various studies have been done to determine the structure of the thrombin aptamer. The oligonucleotide folds into a unimolecular quadruplex in the DNA-thrombin complex containing two G-quartets linked by two TT loops at one end and a TGT loop at the other end in the X –ray structure (34). Solution NMR structures of the 15-mer have also been determined (31), where the quartet structure is essentially the same as that found in the complex. However, the TT loops span the narrow grooves while the TGT loop spans the wide groove in the NMR structure while the opposite occurs in the complex. The difference thus appears to be a reversal in strand polarity between the two. These two structures are referred to as the NMR and the X –ray structures (Figure 4).

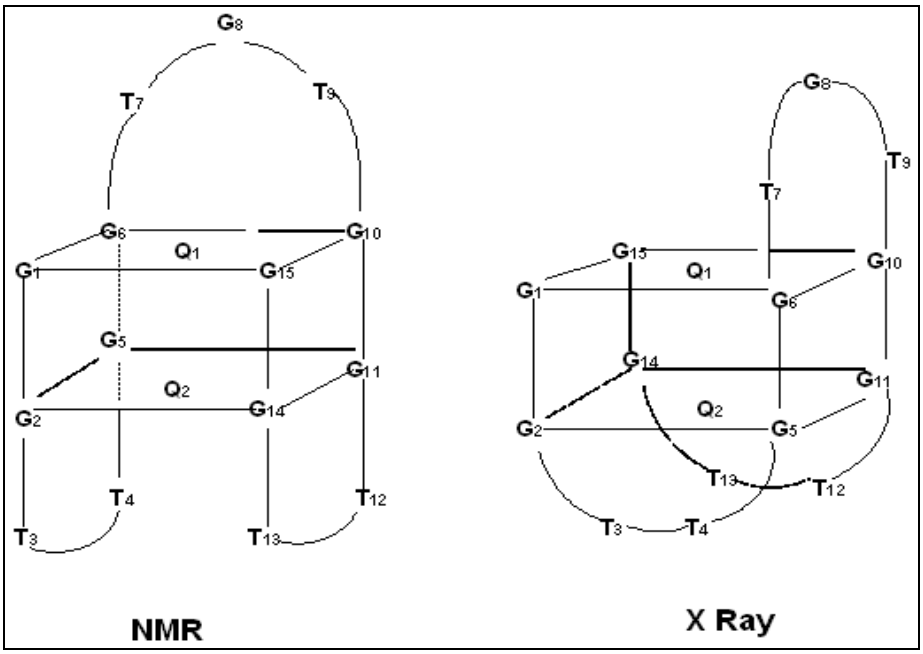

Figure 4. The double quartet structure of thrombin aptamer showing both NMR and X ray structures.

Sensitivity and Specificity of SPR

High sensitivity is an essential requirement of any bioassay instrument. In the case of SPR the detection of the analyte is directly proportional to the molecular mass of the analyte. As a result sensitivity decreases as the molecular mass of the analyte decreases. In general in SPR instruments the sensitivity is in the range of micromolar to nanomolar range for analytes, which are greater than 1000 Da, and in millimolar range for analytes less than 1000 Da (60). The sensitivity is also increased by the affinity between the interacting molecules. A greater affinity results in increased number of analytes being bound to capture probes which in turn increases the mass at the surface leading to enhanced sensitivity. Several strategies to increase the sensitivity of SPR

instruments have been tried, which include better orientation of capture probes or amplification of ligand binding events by sandwich type assays and using high affinity probes. In the SPR instrumentation itself, there are several areas, which could increase sensitivity. These include using different light detection systems, like charged coupled device, linear diode arrays or bi-cell photodetectors. Decreasing the noise in the instrument, optimizing the flow channel volume and maintaining optimum flow speed can also increase the sensitivity of SPR instruments.

Orientation and density of the probe

Forming a monolayer on the gold surface serves not only for a proper binding of the capture probe, but also to orient the probe in a way that the binding area of the probe is properly exposed to the analyte in the solution. Each of the immobilization techniques discussed earlier has some specific advantages, which is suited to the specific experimental purpose. The nickel-nitriloacetic chemistry for immobilizing his-tagged ligands helps in orienting the ligand in a homogenous fashion, which is very important in the binding of the ligand with the analyte. Also this kind of surface chemistry confers a hydrated environment through hydrogen bonding of water to the triethylene glycol molecules and thus helps in preventing protein denaturation and non-specific binding (14). In the cystamine-gluteraldehyde immobilization chemistry, the free amine groups of the ligand bind to the activated gluteraldehyde by forming a Schiff's base. The strong bonding by the disulphides in cystamine to the gold surface and the Schiff's base formation between the gluteraldehyde and the ligand provides a very strong

immobilization chemistry, and this greatly increased the reusability of sensor chip after repeated regeneration cycles. Ten cycles of measurements could be performed on the same sensor surface and the operating stability of the sensor was 15 days (17). In the SPR study of G protein receptor coupling, bovine rhodopsin was bound to the ConA lectin, which was first immobilized on the carboxymethyl dextran sensor chip. This method helped in uniformly orienting the rhodopsin molecules so that the cytoplasmic domain of the receptor had access to a coupling G protein (13).

The amount of immobilized ligand also leads to better sensitivity. This aspect was specifically addressed in the SPR detection system developed for the detection of small plant viruses (18). In this study the concentration of the antibody was important. It was found that for proper quantification of the virus, the concentration of epitopes on the virus should be greater than the concentration of the IgG used and the concentration of the IgG should be greater than the concentration of the virus itself. Proper ligand density is also important in immobilizing DNA on the sensor surface. Closely packed, high density DNA regions may lead to weak binding with the protein analytes or may lead to non-specific binding with proteins. Therefore proper spacing between these DNA ligands is essential in increasing the sensitivity and specificity. Using a streptavidin linker layer to immobilize biotinylated double stranded DNAs on a planar gold surface has been shown to produce adequate spacing for the binding proteins (47). In studying protein-DNA interactions by SPR, the streptavidin was bound to SAMs on the gold surface, which was composed of biotin and ethylene glycol terminated alkanethiols. The ethylene glycol terminated alkanethiols functioned as spacer and also prevented non-specific

adsorption (61). In our present study single stranded DNA aptamer was first immobilized on the gold surface via thiol linkage and then mercaptohexanol was adsorbed on the same surface. The mercaptohexanol also binds to the gold via a thiol group. It has been shown that the mercaptohexanol also displaces some of the non-specifically tethered DNA probes and also binds to vacant gold surface. The mercaptohexanol thus acts as a spacer and also helps in preventing the non-specific binding. It has also been shown that nearly 100% of the tethered DNA probes hybridize with complementary strand and the spacing helps in extending the probe by 1nm into the surrounding buffer solution (11).

Ligand enhancement

Ligand enhancement is important particularly for detecting low molecular mass analytes. The detection can be increased by a second molecule, which binds to the analyte in a sandwich assay. Biotin labeled oligonucleotide was shown to hybridize to DNA immobilized on gold and subsequent hybridization of streptavidin to the biotinylated oligo enhanced the detection limit (62). Two mouse monoclonal antibodies against IL-8 were used in a similar SPR sandwich assay, and the sensitivity limit was increased making possible the detection of 2.5 picomolar IL-8 (23). In another format, conjugating the analyte with a larger molecular mass substance can increase detection. In a study by Seves and Schasfoort, it was shown that latex particles coated with antigen was bound to antibody on the sensor surface, thus improving the sensitivity of SPR immunoassay for human chorionic gonadotropin (63). Inhibition assays here has also been shown to increase the sensitivity. For this strategy, analyte is immobilized on the sensor surface,

and the sample containing the analyte is mixed with known concentrations of the antibody and passed over the sensor surface. The SPR signal is inversely proportional to the concentration of the analyte. Using this format carbaryl concentration as low as 2.7 g per liter was detected in a study aimed at developing a SPR biosensor for pesticides in water (15). Also a similar format was used to detect warfarin in plasma filtrate with a detection limit of 2 g per liter (20).

Instrumentation

The flow cell volume, flow rate and the type of light detection play a role in the sensitivity of SPR instruments. The flow rate influences the response time of the sensor (12). A syringe pump attached to the flow cell can deliver a precise and constant flow with minimal fluctuation. An optimum flow rate is essential for proper binding and also to minimize the drift in SPR signal. In some of the new SPR instruments multiple flow cells are being used, which helps in high throughput analysis and also the flow cells can function as reference channels and thus can eliminate the bulk effect and non-specific adsorption.

Increasing the angular resolution of SPR instruments can also enhance sensitivity of SPR instruments. Angular resolution can be described in terms of the smallest detectable change in refractive index of the analyte. The resolution is usually 10^{-2} to 10^{-3} degrees for SPR instruments. This means that a change in 10^{-3} degrees in SPR angle could be detected. Two widely used detection systems for SPR are charged coupled device and linear diode array. The limitations of these methods include laser intensity fluctuations and thermal and mechanical drift (45). In 1999 N. J. Tao *et al* described a bi-

cell SPR instrument using a bi-cell photodetector SPR instrument, which increases the angular resolution to 10^{-5} degrees (42). In another study using the bi-cell SPR oligonucleotide hybridization at femtomolar level was detected (7). In our present study we used a bi-cell SPR instrument (Nomadics Inc, Stillwater, OK). This highly sensitive detection system has been shown to cancel out any light intensity changes in the laser or in the ambient environment.

Bi-cell SPR

Most of the SPR instruments that are currently in use have a typical angular resolution of 0.01° to 0.001°, which means that analytes causing 0.01° to 0.001° change in the angle of reflected light can be detected. For higher angular resolution, a large distance between the prism and the photodetector is required. Unfortunately, this change makes the instruments design too bulky and more susceptible to mechanical noise and thermal drift which also increases the response time of the instrument. Mechanical movements can be avoided by fixing the photodetector at an angle near resonance and measuring the intensity change in the reflection due to SPR angular shift. A major advantage of this approach is that the response time is only limited by the photodetector and the associated electronics, which can be as fast as nanoseconds. A drawback, however, is that the relationship between the intensity and the resonance angle is sensitively dependent on the angle at which the photodetector is fixed. Major limitations in the resolution of the method come from the intensity fluctuation in the laser and from thermal and mechanical drift in the setup. Another widely used attenuated total internal

reflection (ATR) -based method is to replace the collimated incident light in the above design with a convergent beam that covers a range of incident angles. The reflections from different incident angles are collected simultaneously with a linear diode array (LDA) or charge coupled device (CCD). This method involves no mechanical movements, but simultaneous detection of many channels (of the LDA) slows down the response time. The typical angular resolution obtained for this method is 0.01° to 0.001°. As in the method with a rotating prism, high angular resolution of this method requires a large distance between the prism and the photodetector. In 1999, N. J. Tao *and* collaborators described a new bi-cell SPR detection method that that was able to achieve an angular resolution of 0.00001° and time response of 1 μs (42). The method has several additional features, which include simplicity, good linearity, compactness, and immunity to ambient light.

Bi-cell SPR instrumentation

In bi-cell SPR instruments a plano-cylindrical lens is used as the prism to create the total internal reflection. On the prism the sensor surface which is usually 50nm gold coated coverslip is placed using a matching fluid with the same refractive index as that of the prism. The gold surface is usually decontaminated by Piranha solution or hydrogen flame. Collimated laser light is focused on the gold film through the prism. Light reflected from the prism is detected using a bi-cell photo detector which was mounted on a precision translation stage. The photocurrents from the two cells of the bi-cell detector (A and B) are converted to voltages with a homemade circuit. The circuit also calculates the differential, A-B, and the sum, A+B, signals, which is then sent to a PC computer equipped with a 16-bit data acquisition board. Before each measurement the prism is

rotated so that there was a dark line located at the center of the laser beam. The dark line is due to the absorption of the light by the surface plasmon, which occurs at the angle of resonance. The reflected light falling onto the two cells of the photodetector is then balanced by adjusting the photodetector position with the translation stage until A-B approached zero. Because of the high sensitivity of the method, drift in the A-B signal due to mechanical stress was clearly visible immediately after alignment but it settles down typically over a period of 15–30 min when all the screws were properly tightened. The ratio of the differential to sum signals, which is linearly proportional to the SPR angular shift, is obtained numerically by dividing A-B with A+B (42).

Sensitivity of bi-cell SPR

Errors in the ATR-based SPR methods come mainly from three sources; laser intensity fluctuation, mechanical vibration and thermal drift; and noise in the photodetector; and its electronics. The intensity fluctuation in a typical diode or HeNe laser is between 0.1% and 1% which is a serious source of errors in the methods based on detecting the ATR intensity. This problem is greatly reduced in the bi-cell SPR design because the common mode noise in the laser intensity is subtracted out in the differential signal. The measurement of the ratio of the differential to the sum signals further reduces errors due to the intensity fluctuation. The detection of the differential signal also makes the present model largely immune to noise due to ambient background light. These advantages are not shared by the methods using either a single cell or an array of photodetectors. Noise in the photodetector and its electronics is another source of errors in all the ATR-based methods. In the bi-cell SPR instrument, the noise in *A-B* due to the

photodetector and electronics has been calculated to be very low, so the resolution achieved is clearly not limited by the noise. Another source of errors comes from mechanical vibrations and thermal drift in the systems and depends on the design of each system. This is the dominant source of errors in the bi-cell SPR instrument. It can be minimized by placing the laser diode, prism, and bi-cell photodetector close together on an optical breadboard, which can be placed on a vibration-isolated plate used for a scanning tunneling microscope.

Taking advantage of a sensitive SPR instrument using a bi-cell photodetector, J. H. Fendler and coworkers readily observed pH-dependent differences in the reflectivity of a mercapto propionic acid surface assembled monolayer on a gold substrate and elucidated the rate of mercapto propionic acid self-assembly (43). Additionally, in this experiment time-resolved SPR measurements were performed using the 635 nm incident wavelength (using a 5 mW diode Power Technologies laser) and a bi-cell photodiode detector (Hamamatsu). The photocurrents, from the two photocells, were converted to voltages, and collected on a computer. It was found that the method provides an accurate, time-resolved detection with high angular resolution ~ 0.0001deg and fast time response ~1 μs. All the SPR experiments reported in the experiment were performed at room temperature (43).

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CHAPTER III

APTAMER MEDIATED THROMBIN CAPTURE IN SERUM

Abstract

A novel bi-cell surface plasmon resonance (SPR) spectrometer was demonstrated to detect protein capture by specific binding with an immobilized aptamer. SPR is increasingly being used to detect a variety of target-ligand interactions in real time with high sensitivity and no labeling or tagging required. Our SPR spectrometer consisted of a gold surface, to which the capture probe was attached, a diode laser as the light source, a prism to focus the light on the gold, and a bi-cell photodetector. The resonance angle, where maximum energy from the light waves is transferred to the gold as electromagnet waves called plasmons, changes with capture of a target molecule at the gold surface and is detected by the photodetector. Reported herein is the novel use of an aptamer as a capture probe. Aptamers are oligonucleotides whose secondary structure specifies its affinity to bind structurally and electrostatically to non-nucleic acid targets. In this case, a thrombin aptamer (5'-GGTTGGTGTGGTTGG-3') with a thiol linker was attached to the gold by a covalent thiolate bond, allowing it to be available for binding to thrombin, a serine protease, in aqueous samples. The liquid samples were passed through a low volume flow cell resting on the gold surface. Following the construction and optimization of the bi-cell SPR platform, two experiments were performed: (1) the thrombin aptamer was used as a hybridization probe with the complementary and non-

complementary oligonucleotides to demonstrate immobilization and functionality of the oligonucleotide capture probe, and (2) thrombin capture was performed using the thrombin aptamer as capture probe. A misaptamer, nucleotide content identical to the aptamer but with a different sequence: (5'-TGG GTT GGG TTG GTT TT-3') immobilized to the gold served as the negative control. Factor X, another serine protease, was used as a target control for these experiments. Our SPR spectrometer was able to detect the hybridization of 5 picomoles of complementary oligonucleotide target and the capture of 1 picomole of thrombin with specificity for the protein and low background without reaching its limit of detection. We also investigated the affinity of the aptamer for prothrombin, which was detected at 2 picomole of prothrombin with the thrombin aptamer. Using serum samples, the capture of 1 picomole of thrombin was detected. However use of serum samples was associated with significant background drift. Strategies to reduce this background drift was explored. We conclude that the bi-cell SPR platform with an aptamer as a capture probe can be used as a highly sensitive real-time, label-free biosensor model for the detection of biomolecules in diagnostically relevant samples.

Introduction

Surface Plasmon Resonance spectroscopy is being increasingly used as a real time label free system for the detection of biomolecular interactions. Initially the use of this technique was to study the chemical kinetics of various biomolecular interactions. However, in the past few years with better techniques of immobilizing biomolecules on

sensor surface, thus making the interaction free from any non-specific binding, the SPR instrument is used in detection of various biomolecules.

The advantages of this method of detection include real-time, label-free binding with specificity and sensitivity in the femtomoles range. In SPR instruments the sensor surface chemistry is of outmost importance. The most common biomolecules that is being used as capture ligands are monoclonal antibodies. However, limiting the sensor platform to just an antibody based immobilization chemistry has its limitations. Some of the biomolecular targets may not be immunogenic and an antibody cannot be produced against such targets. Also the stability of antibody and variation between different batches of the same monoclonal antibody can be problematic in a biosensor application. Aptamers, which are composed of oligonucleotides such as RNA or DNA have in recent years emerged as a substitute for antibodies. As these molecules are produced *in vitro* by a process called SELEX (Systematic Evolution of Ligands by Exponential Enrichment) there is very little variation between batches. Aptamers have high specificity for their target molecule and potentially can be produced against any molecule. Another advantage of aptamers are that they are chemically stable and can be boiled or frozen without loss of activity. Because they are synthetically made molecules they are amenable to a variety of chemical modification for specific applications.

In our study we have used the thrombin aptamer as our capture probe on the biosensor surface. The SELEX procedure has been used to create both a RNA (19) and a DNA (11) aptamer against human alpha thrombin. Thrombin is a multifunctional serine

protease with both coagulant and anticoagulant properties. The 15mer DNA thrombin aptamer is the most studied aptamer. Griffin et al (1993) have studied the pharmacological activities of this aptamer in cynomolgus monkeys and found that the aptamer increased the plasma prothrombin time and in ex vivo model it inhibited platelet aggregation (18). Thrombin aptamer have been shown to have high affinity for its target molecule which is the serine protease thrombin (18). We used the 15mer DNA thrombin aptamer, which was chemically modified with a thiol group attached to the 3' end of the oligonucleotide. The thio group allowed coupling of the aptamer to the gold surface by a thiol linkage.

One of the most common problems associated with the biosensor application is the non-specific binding of non-target biomolecules to the sensor surface. This non-specific binding can be explained as the tendency of biomolecules to physically adsorb on to a solid substrate without specific receptor recognition interaction (16). Self Assembled Monolayers (SAM) of sulfur containing compounds like alkanethiols, dialkyl sulphides and dialkyl disulphides on noble metal surfaces have been extensively studied and have been shown to prevent the non-specific interactions. However, most of the surface chemistry studies have been based on gold surface due to its inert character. The structure of these SAMs has been well established and detailed (17). SAMs of alkanethiols on gold formed by adsorption of a long chained alkanethiol (XCH2-SH, n=11-18) have been shown to prevent non-specific binding. In our experiment, to prevent any non-specific adsorption on to the gold surface, a 6-mercapto-1-hexanol SAM was adsorbed on to the gold. This six carbon alkanethiol monolayer not only prevents the

non-specific adsorption, but it also purported to lift the aptamer from the gold surface which in turn will increase the efficiency of binding with the target molecule (7).

SPR instruments use the total internal reflection of light off of a metal surface, usually gold, to detect the capture of a target molecule. At total internal reflection, the light waves are reflected, but there is a small amount of energy transferred from the waves to the metal surface, resulting in electromagnetic waves within the metal called plasmons. Additional binding to the surface increases the mass of the surface, changing the refractive index and ultimately the resonance angle. The resonance angle is the angle at which the greatest amount of energy is being transferred to the plasmons, and appears as a dark line (dip) in the reflected light. There are many different instruments being used with SPR, including flow injector cells, to deliver the target, and bi-cell photodetectors that measure the shift in the resonance angle by dividing the difference in light waves detected by the two cells, by the sum of the two cells (A-B)/(A+B). Compared to instruments using single-cell detectors that can detect binding around the nano-molar range, bi-cell detectors have been shown to detect hybridization in the femto-molar range with specificity (2). This novel method of detection by using the ratio between the differential to the total sum of light not only make the bi-cell SPR more sensitive but also cancels out the noise which arise due ambient light variation and laser intensity fluctuations.

In this study we combined this bi-cell SPR and aptamer technology to create a biosensor that detects the capture of thrombin by its aptamer, in low levels with

specificity for the protein. Nomadics, Inc. (Stillwater, OK) has developed a SPR instrument with a bi-cell photodetector equipped with a teflon flow cell. We used this novel bi-cell SPR instrument for our experiment (Figure1). The 15mer DNA aptamer was modified with a thio group on its 3' end, and it was tethered to the gold surface by its S-H modified 3' end. After the oligo monolayer was formed on the gold, a SAM monolayer of mercaptohexanol was accomplished by binding the mercaptohexanol to the surface of the gold by thiolate bonds. The Tris EDTA buffer was spiked with the thrombin or with factor X as a negative control. The targets in the solution were introduced to the flow cell sequentially and capture was be detected. The ability of the thrombin aptamer to detect prothrombin was also investigated. Five hundred femtomoles of thrombin and two picomoles of prothrombin were detected in Tris EDTA buffer. Experiments to study the inter-day and intra-day variations were conducted. After optimizing the experimental protocols, capture of thrombin in diagnostically relevant samples was assessed using the thrombin aptamer. Using this model, one picomole of thrombin was detected in fetal bovine serum, but we were not able get rid of the background drift which was seen on flowing the serum through the flow cell. This work demonstrates that an aptamer can be used as a capture probe in bi-cell SPR with high specificity and sensitivity of detection and this model could be used as real time label free detection system which can easily be used in a clinical setting more and affordable compared to the commercially available SPR instruments.

Materials and Methods

Materials

The thio linked DNA thrombin aptamer, its complimentary sequence and a misaptamer with thio group were purchased from Integrated DNA Technology (Coralville, IA) (Table 1). Human -thrombin and prothrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Optical fluid (LS-5252) from Lightspan (Wareham, MA), Cleland's Reductacryl Reagent from Calbiochem, Microspin G-25 columns from Amersham Biosciences (Pittsburg, PA), Microcon YM-3 centrifuge filter devices from Millipore (Burlington, MA), 97% 6-Mercapto-1-hexanol from Aldrich (St Louise, MO), distilled deionized water from Cellgro. Defined fetal bovine was bought from HyClone (South Logan,UT). All other materials for buffers, bovine serum albumin and Tween 20 were purchased from Sigma Aldrich.

Thrombin aptamer with the linker	5'GGT TGT TGT GGT TGG AA/ 3ThioMC3-D/3'
Misaptamer with the linker	5'TGG GTT GGG TTG GTT TT/ 3ThioMC3-D/3'
Complementary strand of thrombin aptamer	5'TTC CAA CCA CAC CAA CC 3'
Non-complementary strand	5'AAA CCC AAC CCA ACC CA 3'

Table.1. The thrombin aptamer and the misaptamer was modified with a thio group using the 3' thio linker. Two adenine and two thiamine bases were attached to the 3' end of the aptamer and the misaptamer respectively, which acted as a spacer.

Coating gold on the cover slips

Clean Fisherbrand 18'x18' BK-7 glass cover slips (Fisher Scientific) were washed in soap solution, isopropanol and acetone, each for 10 minutes and then air dried. Coverslips were first coated with chromium at 2nm thickness using a bench top sputter coater from Denton Vacuum (Moorestown, NJ). The 2nm chromium coating helps in proper attachment of the gold film on to the glass cover slips. Then gold was coated at 50nm thickness. Proper thickness of the gold coating is very important because thickness of more than 50nm or less than 50nm will cause the SPR resonance dip to be narrow or broader which in turn affect detection.

Coating the aptamer on the gold

First the gold coated slips were cleaned in freshly prepared Piranha solution for 10 minutes on a rocker to remove any impurities. The Piranha solution is made of concentrated sulphuric acid and 30% hydrogen peroxide in the ratio 7:3. The cover slips are then rinsed with double deionized water and ethanol, two times each and air dried.

The oligos, the aptamer and the misaptamer was supplied as disulfides. To prepare these for use, the disulfide bonds were reduced with a dithiothrietol (DTT) coated beads (Reductacryl, Calbiochem Inc.). The beads were first resuspended with the oligos in Tris-EDTA, pH 7.5. A ratio of 1 mg oligo with 50 mg resin was used to ensure complete reduction and were stirred or agitated at room temperature for 15 minutes. Reductacryl beads were removed using a G-25 Micro Spin Column and centrifuged for 3 minutes at 735 rcf. The beads stayed in the column and the oligo (aptamer) were taken in a tube. In

order to debuffer the solution, the oligos were added to Microcon YM-3 filter and centrifuged at 14,000 rcf for 30 minutes. The filtrate was discarded and KH_2PO_4 (1M, pH 7) was added to the other side of the filter, and again centrifuged at 1000 rcf for 4 minutes by keeping the filter tube inverted and the solution was collected and made up to 50µL by adding KH_2PO_4. The 50µL of purified oligo is coated on the Piranha cleaned gold slip and incubated for 4 hours at room temperature. The aptamers used also had two extra bases at the 3' end, which functioned as a spacer arm. The functionalized surface was then cleaned with double deionized water and air-dried. For the control experiments a misaptamer, which is a 15 mer with identical nucleotide content to the thrombin aptamer but in a scrambled sequence, was used by attaching to the gold surface as described above for the thrombin aptamer.

Coating mercaptohexanol on the gold

Following coating the oligo on the gold, the gold surface was further modified by a mercaptohexanol monolayer (SH-$(CH_2)_6$-OH) SAM. For this a 20mM mercaptohexanol was coated on to the gold for two hours at room temperature. In SPR experiments its important that the immobilized probe has a high target recognition ability, the tethering of the substrate must be engineered so that it does not interfere with their ability to hybridize or attach to the target molecule. The non-specific adsorption of the probe DNA at site other than the thiol group is minimized by a co-adsorbing mercaptohexenol, a short alkane thiol molecule, that also attaches to gold through a thiol group. The mercaptohexanol displaces the oligos which are not bound to gold via a thiol linkage and also occupies the vacant spaces on the gold which are not filled by the oligo.

It is been also proved that mercaptohexenol treatments lifts the probe backbone from the gold surface, leaving them anchored solely through the thiol-derivatized end (7).

SPR Instrumentation

A prototype of the bi-cell SPR was made by Nomadics Inc. (Stillwater, OK). The specifications of the prism, laser, photodetector and the electronics are as follows. A picture of the prototype is shown in Fig 1.

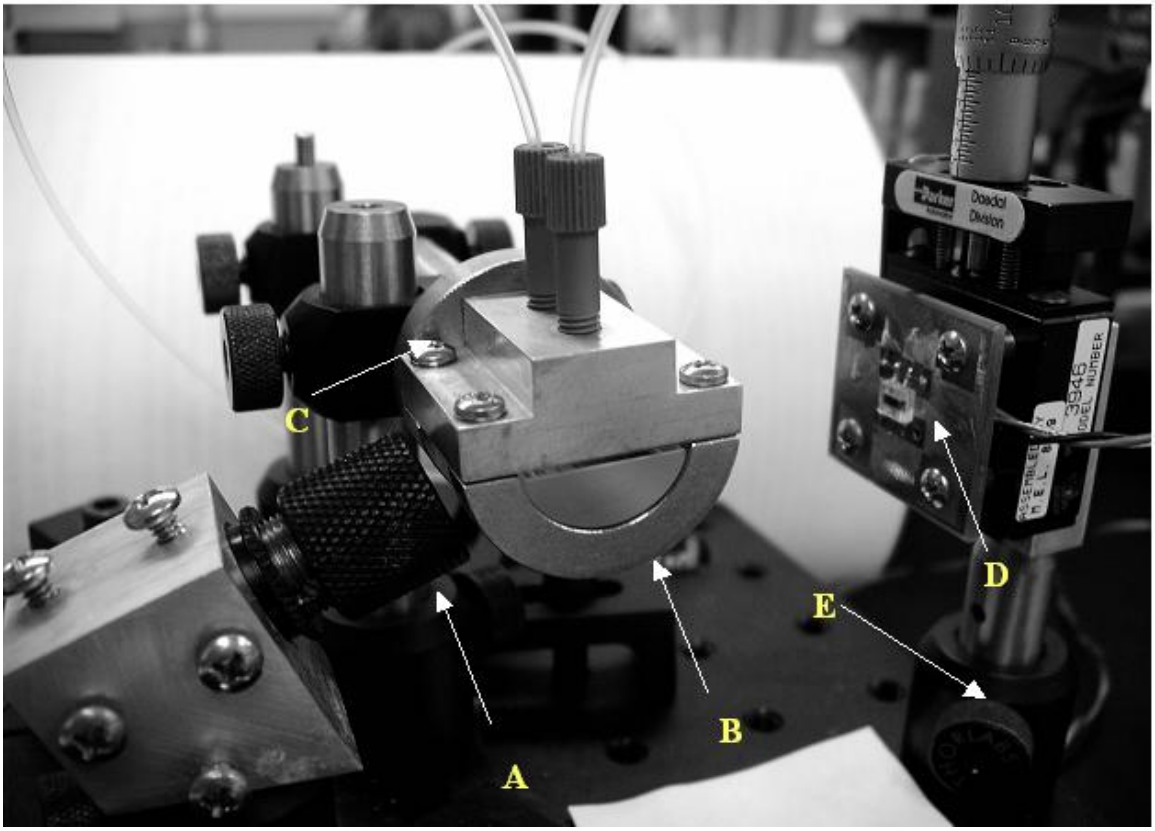

Figure 1. Home made bi-cell SPR instrument (Nomadics Inc, Stillwater, OK). A. Laser source. B. Prism holder with the prism. C. Flow cell. D. Bi-cell photodetector. E. Transition stage for adjusting the photodetector.

670 nm, 4 mW variable focus diode laser module (Coherent)

BK7 Plano-semi-cylindrical prism (Melles Griot, 01LCP004)

4 l Teflon sample flow cell with 5mm^2 exposure. (Nomadics, Inc)

Bi-cell photosensitive detector (Hamamatsu, S2721-02) mounted on a precision linear translation stage (Parker Hannifin, model 3946)

Circuit to convert the photocurrents to voltages (Nomadics, Inc.)

A 12-bit National Instruments AD/DA board (PCI 6071) was use to collect the SPR signal.

Control and data collection software is programmed with LabView for Windows 6.0

Bi-cell SPR

The aptamer immobilized gold coated cover slips were placed on to the BK-7 planocylindrical prism. A drop of optical fluid with the same refractive index as that of the prism and glass cover slip was placed between the prism and the cover slip so that the cover slip would not slide off. With the cover slip thus attached to the prism, the prism with cover slip on top was placed on a rotating holder. An injection flow cell was then placed on top of the prism and adjustment screws tightened. The flow cell has two ports, one in port through, which samples can be introduced, and one out port, which is connected to a volume rate controlled syringe pump. By adjusting the knob on the syringe pump a constant flow with desired flow rate can be maintained through the flow cell. At the start of the experiment, the Tris EDTA buffer was flown through the flow cell at a rate of 10 µl per min. The laser is then focused on to the prism, and then the prism was rotated till a dark line or dip was seen in the reflected light. Once the dip was clearly focused, all the screws on the prism holder were tightened. The bi-cell photodetector

which was mounted on a transition stage and the detector was placed in position in such away that the dip falls in between the two photocells. This procedure can be easily done by looking on the Labview screen on the monitor (Figure 2). The two photocells which were named A and B and as the dip falls in the exact middle of the two cells the light falling on the two cells will be same and on the monitor A-B will be zero. After focusing the dip in between the two photocells the screws on the photocell transition stage were also tightened.

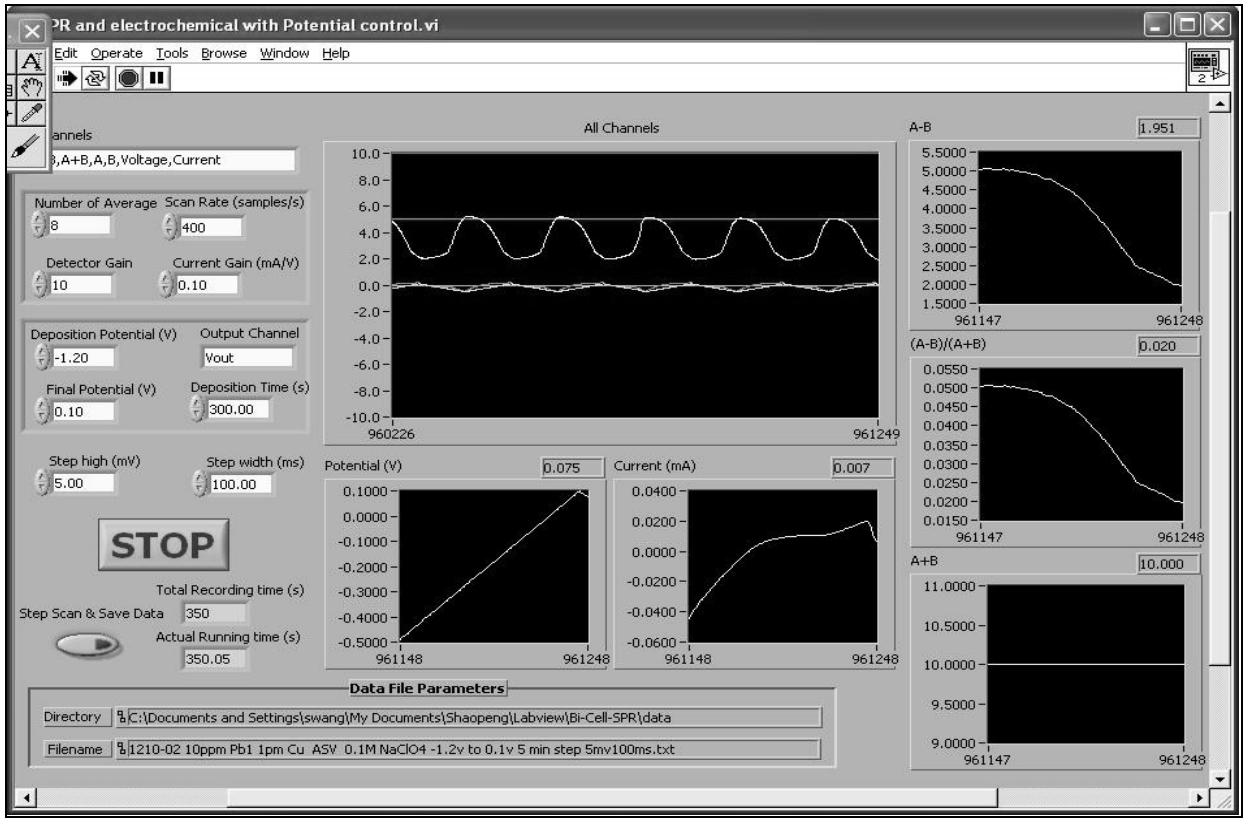

Figure 2. The computer window showing the Labview software screen (National Instruments). The smaller windows showing the different channels including total light falling on the photocells(A+B), differential sum of the lights (A-B) and the SPR shift (A-B)/(A+B).

Capture of Aptamer Compliment Using Bi-cell SPR

After the bi-cell SPR was set up, Tris-EDTA buffer (10mM Tris, 1mM EDTA, 5mM $MgCl_2$, pH 7.6) was flowed through the flow cell at 10µl per minute. The flow was maintained up to 10 to 20 minutes for the drift to settle and a stable base-line was reached on the Labview monitor. Then 5 picomoles of aptamer complement in Tris EDTA buffer was passed through the flow cell at the same flow rate. Change in SPR angle shift was calculated using the formula, SPR Shift = A-B/A+B which is the ratio of differential sum of the light falling on the two photo cells to the total light falling on the cells. This change was detected by the photocells and converted to electrical signals, amplified and the data collected was calculated using the Labview software. The data consists of the angle shift at each time frame. For each experiment the data was recorded for period of 10 minutes. The resulting data was plotted on an excel graph with time in seconds on the X-axis and SPR angle shift on the Y-axis. A 5 picomole non-complementary DNA sequence was used as the negative control. The sequences for the aptamer, aptamer complement and the non-complement is given in Table 1.

Capture of Human α-Thrombin using Bi-cell SPR

To prepare the analyte, α-thrombin, it was thawed at room temperature for 1 to 2 hours. After the bi-cell SPR was set up Tris-EDTA buffer (Tris 10mM, EDTA 1mM. pH 8.0) was introduced through the flow cell at a rate of 10µl per minute. This flow rate was experimentally determined, and was found to give a high level of binding with minimum detection time lag. The flow was maintained for 10 to 20 minutes until a

stable base-line was reached on the Labview monitor. Then 1 picomole of thrombin was passed through the flow cell at the same flow rate. Change in SPR angle shift was calculated using the formula, SPR Shift = A-B/A+B. The data consists of the angle shift at each time frame. For each experiment the data was recorded for period of 10 minutes. The resulting data was plotted on an excel graph with time in seconds on the X-axis and SPR angle shift on the Y-axis. One picomole factor X, which is a serine protease with molecular weight of 58800 Da, was used as the negative control. The same experiment was repeated by using a gold sensor which did not have the aptamer immobilized on it and contained only the mercaptohexanol monolayer. As another negative control the immobilized misaptamer gold sensor was used to capture both the factor X and thrombin and the SPR shift was calculated.

SPR Using Human Prothrombin

To prepare the analyte, prothrombin, it was thawed at room temperature for 1 to 2 hours. After the bi-cell SPR was set up Tris-EDTA buffer (Tris 10mM, EDTA 1mM. pH 8.0) was flown through the flow cell at 10µl per minute. The flow was maintained up to 10 to 20 minutes for the drift to settle and a stable base-line was reached on the Labview monitor. Then 2 picomole of prothrombin (MW 72000 kDa) was passed through the flow cell at the same flow rate. Change in SPR angle shift was calculated using the formula, SPR Shift = A-B/A+B and data handled and assessed as described above.

Intra-day and Inter-day Variations in the Thrombin Capture

In order to calculate the intra-day and inter-day variations in thrombin capture, three concentrations of thrombin, 0.5, 1 and 2 picomoles were used. To calculate the inter day variation, all the three concentrations were passed over the same gold sensor surface and the data was collected. Three different experiments with different gold sensor surfaces were done on the same day and the data from the three independent experiments were plotted on a graph and coefficient of regression and the coefficient of variations between were calculated. For calculating the intra-day variations three independent experiments were done on three separate days and the data was similarly plotted and r^2 value and CV was calculated.

Capture of Human α-Thrombin in serum

In order to assess whether a more complex biological media could be used in the bi-cell SPR, we extended the experiment by using the analytes spiked in serum. Defined fetal bovine serum (FBS) was thawed at room temperature and 10% FBS was made in Tris EDTA. We spiked the different concentrations of the analyte in the 10% FBS. For the SPR run we first introduce the 10% FBS through the flow cell at 10µl per minute for 45 minutes and then introduced the spiked samples. The change in SPR shift was measured and plotted on the sensorgram. In order to resolve the problems encountered with base-line drift, while using the serum samples, the sensor surface was first blocked with 1%

BSA. In addition different dilutions of the serum in Tris EDTA buffer were tried to see whether it had any affect on the base-line drift. The addition of 0.05% Tween 20 to the sample was also assessed for reduction, of any non-specific adsorption of serum components to the sensor surface. Finally to assess whether the flow channel had any effect on the base-line drift, the flow cell was replaced by a well of 0.5ml volume with an effective exposed sensor surface area of 2mm^2. The samples were introduced in the well by gently dropping the analytes into the well. As the analyte settles on the surface the SPR shift was measured.

Results

To optimize the binding environment and to minimize the time required to reach a stable base-line, several flow rates were tested. It was found that at a flow rate of 10 l per minute, the maximum SPR shift on binding experiments was observed. This rate was reasonably fast, and analytes could be initially detected in three minutes. Three minutes is the time required by the sample to reach the sensor surface from the point of sample injection. A base-line with minimum drift was obtained in about 10 to 15 minutes at this flow rate.

Hybridization of the Oligonucleotide Target

In the hybridization reaction, 5 picomole of complementary DNA could detected using the thrombin aptamer as a hybridization probe (Figure 3.). As a control the SPR signal shift was also monitored by passing a non-complimentary sequence over the

same sensor surface. In another experiment the sensor surface, which did not have the aptamer immobilized on it and which had only the mercaptohexanol monolayer on it was used and the signal shift for both the complementary and the non-complementary sequence was measured. In both these control experiments any deviation from the base-line was not observed. The absence of any shift with the controls confirmed the presence of the immobilized aptamer on the gold coated cover slips.

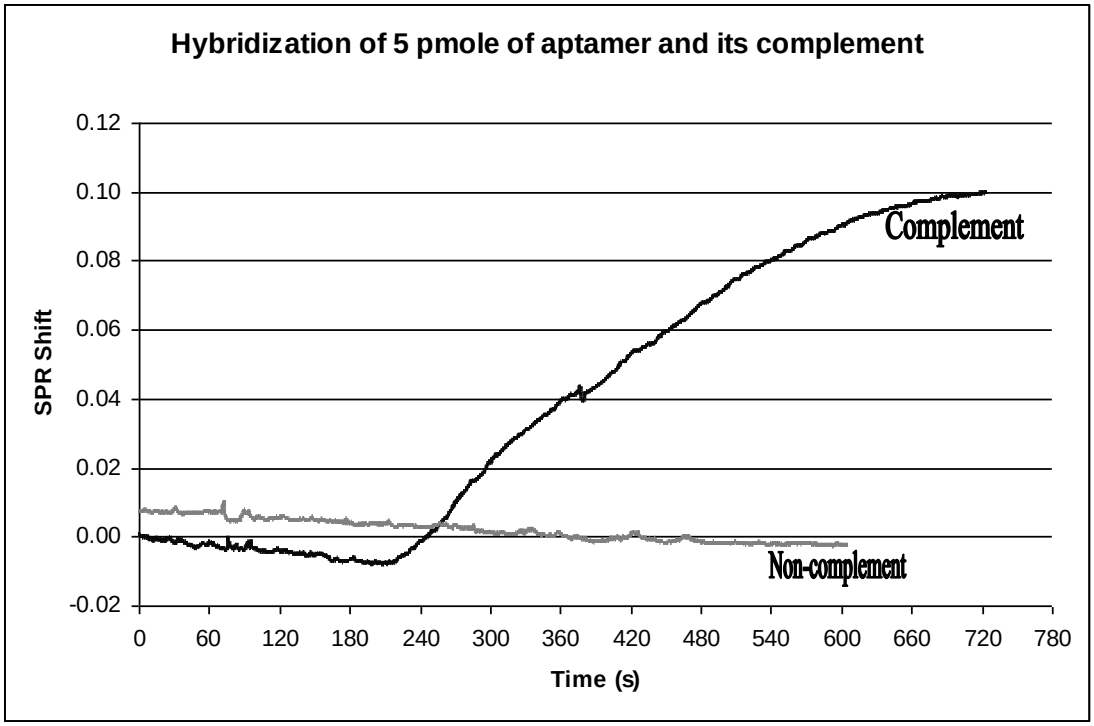

Fig.3. Hybridization of 5 picomole of aptamer complement detected around 3 minutes. No signal could be measured for the non-complementary sequence.

Thrombin Capture using Bi-cell SPR

Using the bi-cell SPR instrument, a detection range in femtomoles amounts of thrombin was observed. The lowest level of detection was 250 femtomoles of thrombin capture in a Tris EDTA buffer. However detection level was consistent in a range from 500 femtomoles to 2 picomoles range. For both the 500 femtomoles and 1

picomole thrombin capture, a consistent level of binding was observed. (Figure 4 and Figure 5). As controls, the factor X protein, which is another serine protease like the thrombin, with a little higher molecular weight than the thrombin molecule. No binding was observed with 500 femtomoles, 1 picomole or the 2 picomole of Factor X with the thrombin aptamer. This demonstrates that the binding between the thrombin aptamer and its target the thrombin molecule is highly specific. In another SPR run, the thrombin was flowed over a gold sensor with just the mercaptohexanol monolayer on it and also the thrombin was also passed over the sensor surface with another 15mer oligonucleotide strand immobilized on it. This oligonucleotide had the same guanine and thiamine bases similar to that of the thrombin aptamer but arranged in a scrambled sequence. In both the control experiments any change in the SPR angle shift was not observed.

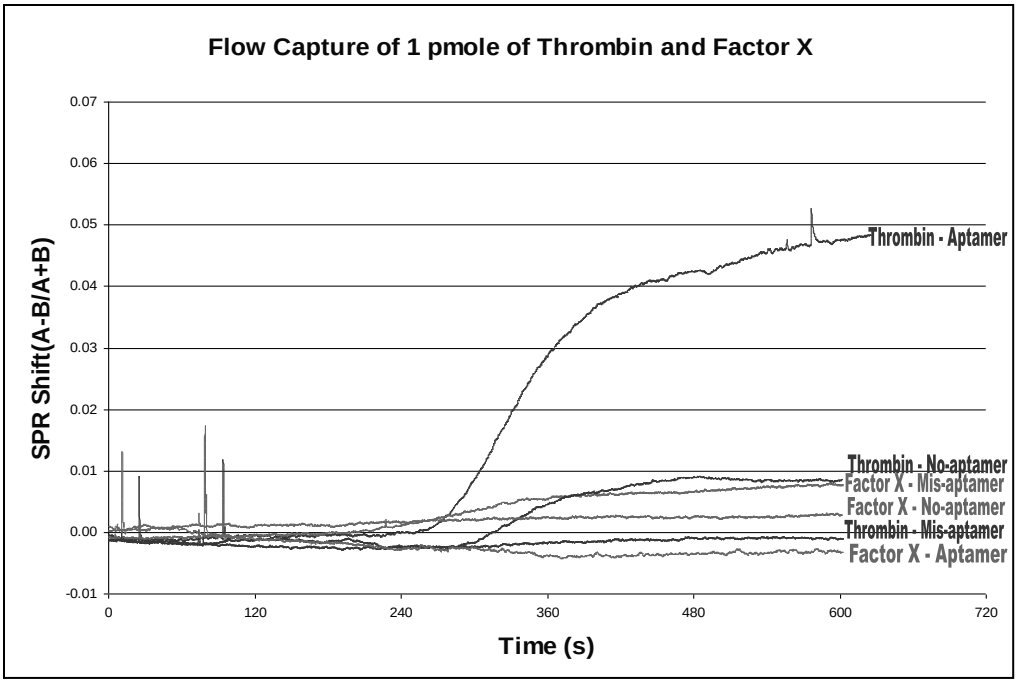

Figure 4. Capture of 1 picomole of thrombin using the thrombin aptamer detected just around 4 minutes. There was no significant SPR shift with the controls, which included factor X, capture using mercaptohexanol layer and misaptamer.

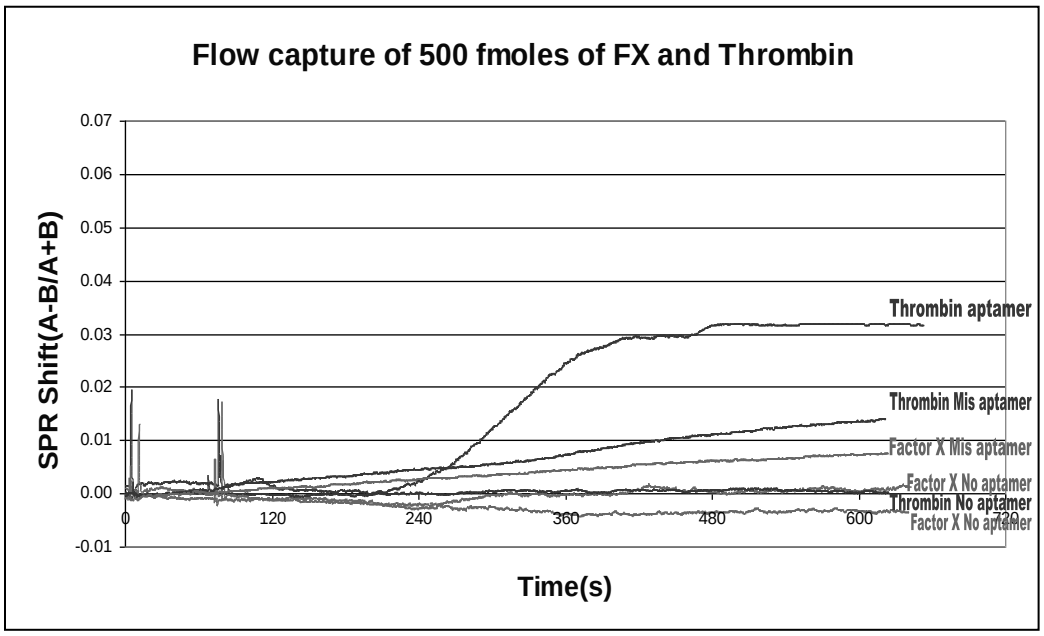

Figure 5. Capture of 500 femtomoles of thrombin using the thrombin aptamer detected just around 4 minutes. There was no significant SPR shift with the controls, which included factor X, capture using mercaptohexanol layer and misaptamer.

Prothrombin Capture using Bi-cell SPR

Two picomoles of prothrombin was detected with the thrombin aptamer as a capture probe using the bi-cell SPR instrument (Figure 6). A comparison was done between the levels of binding of both thrombin and prothrombin, and it was observed that the binding of prothrombin was slightly less than half the binding value of thrombin. However, a consistent level of binding with less than 2 picomoles of prothrombin was not obtained. It was also seen that with the prothrombin the sensor surface was easily saturated and multiple runs could not be made on the same sensor surface. As with other experiments any binding with the negative controls was not detected.

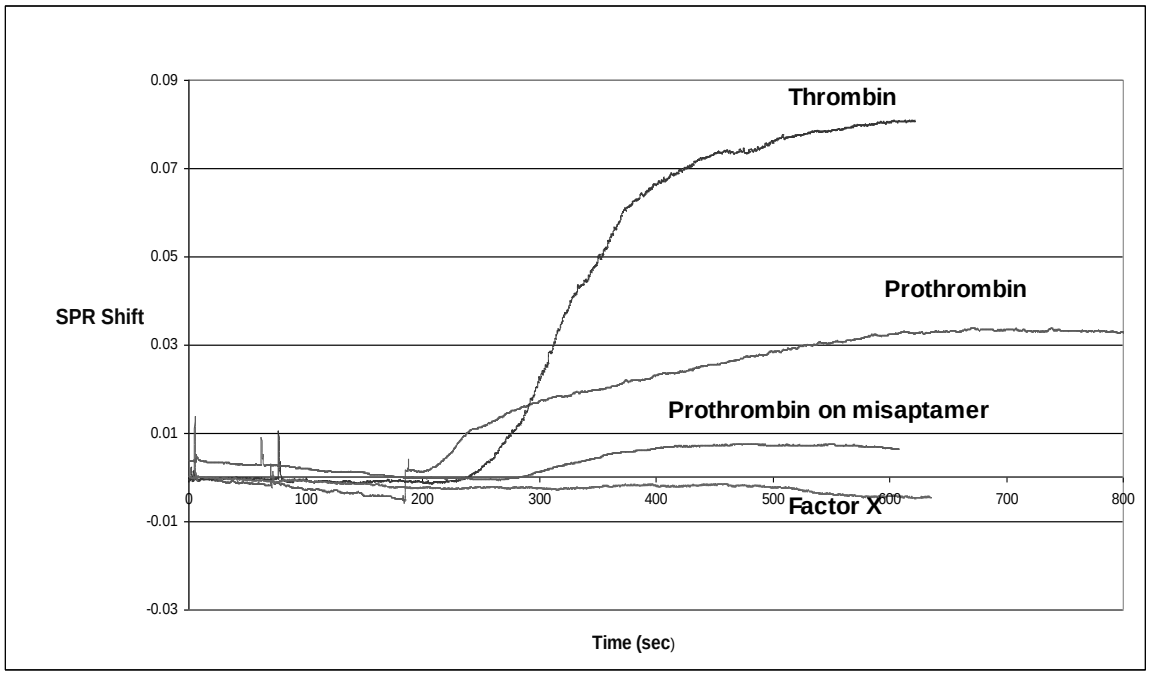

Figure 6. Capture of 2 picomole of prothrombin compared to the same amount of thrombin. No signals were detected for the 2 picomole factor X and for the control using misaptamer.

Intra-day and Inter-day Variations

In order to calculate the variations in thrombin capture, both inter-day as well as the intra-day experiments with three thrombin concentrations namely, 500 femtomoles, 1 picomole and 2 picomole was done. For the intra-day calculations three experiments were done on the same day with three different sensor surfaces, and the data was calculated. The mean value for each of the three concentrations was plotted on a graph, and r^2 was calculated and had a value of 0.9992 (Figure 7). The CV for the intra-day variation of 500 femtomoles thrombin capture was found to be 12.21% (Table 2).

For the inter-day calculation, three independent experiments were done on separate sensor surfaces on three different days. The data was taken and the mean value of the

three experiments was plotted on a graph and the r^2 value was found to be 0.9898. (Figure 8). The CV for the inter-day variations was of 500 femtomoles thrombin capture was found to be 12.43% (Table 3). For both these experiments a concentration of greater than 2000 femtomoles were found to be not linear.

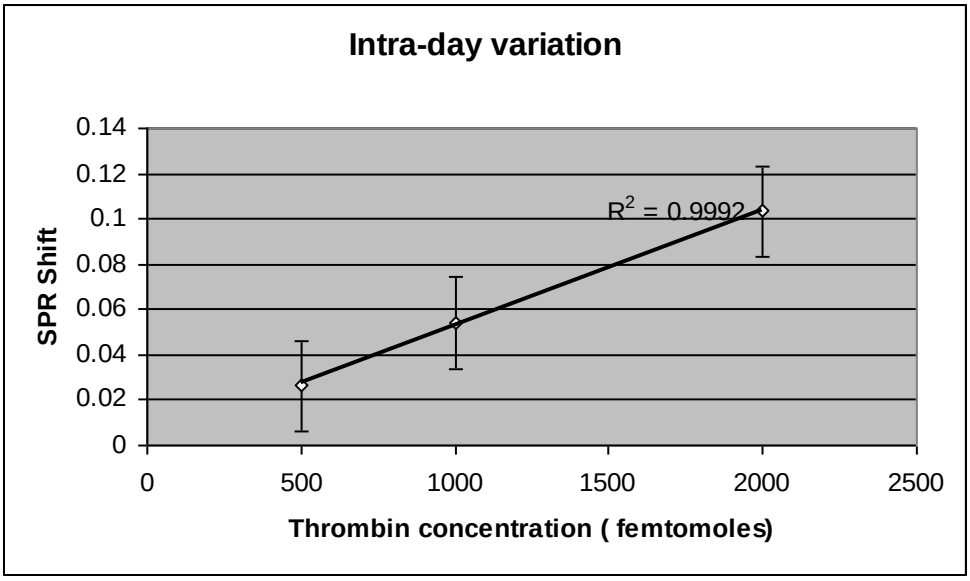

Figure 7. Intraday variation calculated by doing SPR runs on three different sensor surface on the same day. r^2 value =0.9992. . The error bars showing one standard deviation from the mean value of 3 measurements.

Intraday variation				
Concentration of Thrombin (femtomoles)	1	2	3	CV (%)
500	0.024	0.025	0.03	12.2104
1000	0.047	0.055	0.06	12.1426
2000	0.08	0.12	0.11	20.1394

Table.2. The table showing SPR shift and CV of 500,1000 and 2000 femtomoles of thrombin intra-day variation experiments.

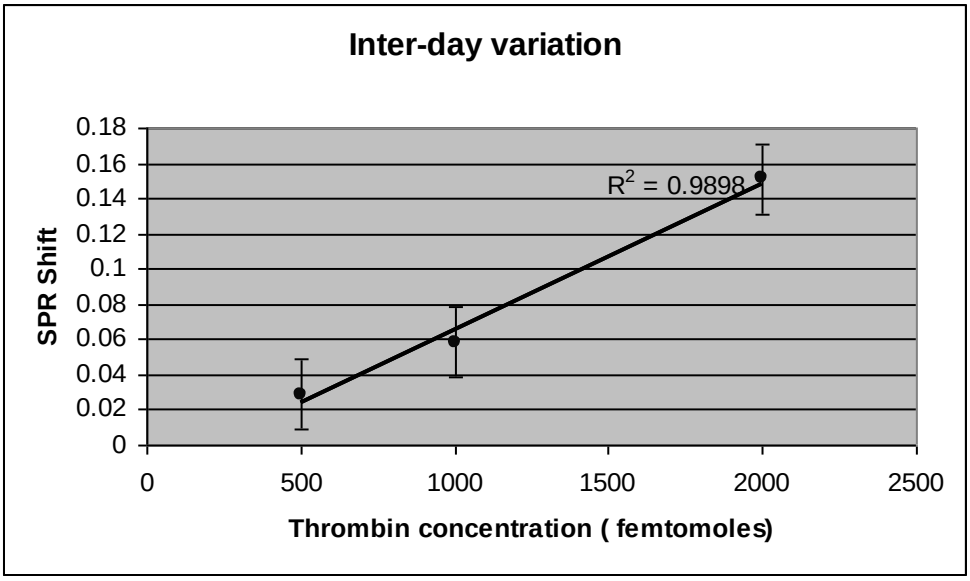

Figure 8. Inter-day variation calculated by doing SPR runs on three different sensor surface on the same day. r^2 value =0.9898. The error bars showing one standard deviation from the mean value of 3 measurements.

Inter-day variation				
Concentration of Thrombin (femtomoles)	Day 1	Day 2	Day 3	CV (%)
500	0.028	0.026	0.033	12.43
1000	0.065	0.06	0.05	13.0945
2000	0.183	0.14	0.13	18.6093

Table.3. The table showing SPR shift and CV of 500, 1000 and 2000 femtomoles of thrombin inter-day variation experiments

Thrombin Capture using Bi-cell SPR

The capture of 1 picomole of thrombin spiked in 10% FBS was detected using the same bi-cell SPR model (Figure 9). A constant base-line drift was observed during SPR experiments using 10% FBS as the running buffer. The drift would almost settle after 45 minutes of run time, but we could not completely get rid of the drift. The experiments with 0.05% tween 20 being added to the serum or blocking of the sensor surface with 1% BSA did not reduce the drift. It was also found that the drift was not due to the flow rate or the flow cell as experiments with a SPR using a well instead of a flow cell also produced similar drifts.

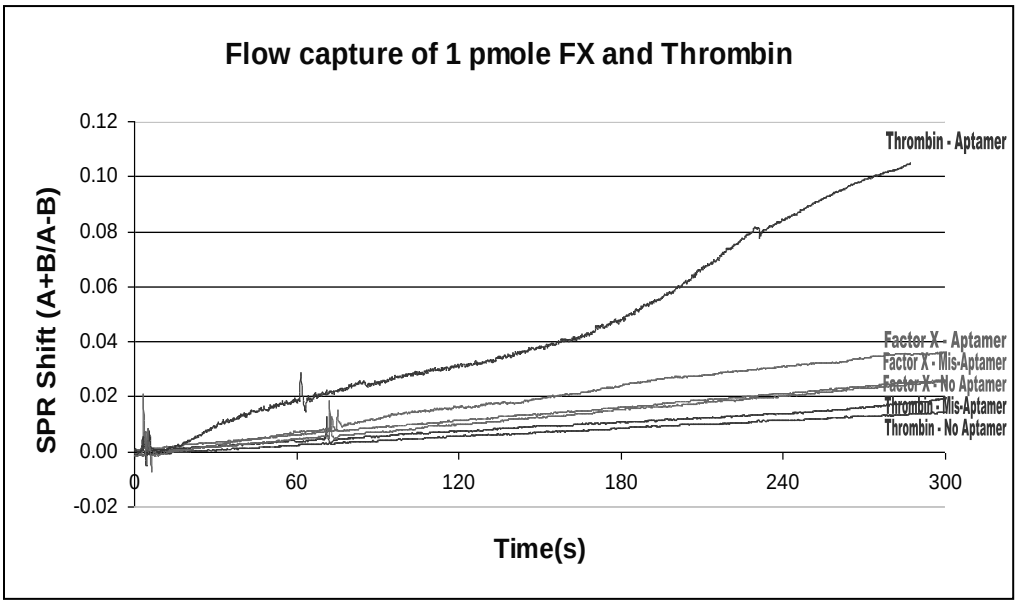

Figure 9. Capture of 1 picomole of thrombin using the thrombin aptamer, detected just around 4 minutes. The SPR run was calculated for 5 minutes. There was no significant SPR shift with the controls, which included factor X, capture using mercaptohexanol layer and misaptamer.

Discussion

The capture of 500 femtomoles of thrombin in Tris EDTA buffer and 1 picomole of thrombin in 10% FBS using the thrombin aptamer as capture ligand was possible using the bi-cell SPR instrument. The first experiment was to determine whether the 15 mer DNA aptamer was properly immobilized on the gold coated cover slips. In order to prove this, a hybridization reaction was done by introducing a complementary strand of DNA aptamer through the flow cell. Five picomoles of complementary strand could be detected and no SPR signal was observed for 5 picomoles of a non-complementary strand. Any binding was not observed when the complementary strand was passed over a gold surface with just the mercaptohexanol on it. This experiment demonstrated that the aptamer was properly immobilized on the gold surface and also that the complementary DNA strand was not binding non-specifically to the mercaptohexanol or the gold surface.

For capture of thrombin in buffer, Tris EDTA buffer with a pH of 8 was used. A couple of different buffers were tested, but the Tris EDTA buffer was found to show the best sensitivity with minimum amount of non-specific binding. It has been shown previously that the ions in the buffer would influence the interactions (22). The limit of detection was 250 femtomoles of thrombin using this buffer. The linearity in binding was observed for a concentration ranging from 500 to 2000 femtomoles. The specificity of the

thrombin aptamer for its target molecule, the thrombin protein was demonstrated in the control experiments. No SPR shift was observed for the same amounts of factor X using the aptamer capture probe. Factor X is a serine protease similar to thrombin with a molecular weight of 58000 Da, which is slightly higher than the thrombin (37000 Da). Also the results from the mercaptohexanol functionalized and the misaptamer immobilized sensor surface indicated that the thrombin protein was not binding to the sensor surface non-specifically.

Minimum variations in the detection levels between experiments and high reproducibility of the results is an important factor in biosensor applications. In the experiments with the variability in detection, linearity in binding for the concentrations ranging from 500 to 2000 femtomoles was obtained. The r^2 value for intra day and inter-day binding was 0.9922 and 0.9898 respectively. The coefficient of variation for 500 femtomoles was 12.21% and 12.43% for intraday and inter-day calculation. However, for higher concentrations the coefficient of variation was high. This may be due to the fact that the lowest concentration was first flowed and then introduced the higher thrombin concentrations over the same surface sequentially and for each run the available receptors for binding may become lesser and lesser, so we may not be getting the true value for subsequent binding. A better value could be obtained by using different sensor surface for each run or by using a regeneration solution by which all the previously bound molecules could be displaced. But for the current study the main objective was to find whether an aptamer could be used as a capture ligand in a bi-cell SPR and we were able to prove that part.

To explore whether the thrombin aptamer could detect prothrombin in a bi-cell SPR instrument, an SPR run with prothrombin in Tris EDTA buffer was used as the analyte. In the coagulation cascade prothrombin (72000Da) is cleaved into thrombin (37000Da) and two other smaller fragments by activated factor X, factor V and phospholipids (21). The bi-cell SPR could detect the binding of 2 picomoles of prothrombin using the thrombin aptamer as the capture probe. A comparison between the levels of binding was done and it was observed that prothrombin showed less than half the SPR shift when compared to the same concentration of thrombin. It has been shown previously that prothrombin has less affinity to the thrombin aptamer with a K_D value of 50nM while the K_D value for thrombin is 2nM (20). The level of binding that was observed for prothrombin in this experiment may be due to a combination of less affinity for the aptamer and also high molecular weight of prothrombin.

Finally the study was extended to see whether media could be used in the bi-cell SPR model. SPR phenomenon should not be affected by the complex media as it measures the refractive index changes of the solvent that takes place at the surface like a binding of molecules to the surface (19). A number of SPR studies have been done using complex solvents including saliva, cerebrospinal fluid and serum with varying results. A constant base-line drift in sensorgram was observed while introducing the serum through the flow cell. This drift would almost settle in 45 minutes, but a stable base-line was never reached. This was the case with different dilutions of serum. Several methods were tested to prevent the non-specific adsorption, including using Tween 20, which is a nonionic surfactant and spreading agent and is commonly used to prevent noon-specific

protein interactions. Bovine serum albumin was also used to block the gold surface. Neither of the above methods could reduce the non-specific adsorption. In order to find whether the flow cell or the flow rate was causing the drift we did a SPR with a well functionalized on the sensor surface, wherein the analyte is added into a miniature well having a volume of 0.5 ml and as the analyte settles on the sensor surface the angle shift could be calculated. In the well-SPR also the same constant drift was observed. This problem could be overcome by comparing the angle shift of the sample in serum to the angle shift of the serum in a reference channel simultaneously. But this was beyond the scope of the current objective.

In conclusion, it was able to prove that an aptamer could be used as a capture probe in a bi-cell SPR instrument with high sensitivity and specificity of detection. This real-time label free biosensor model can be easily adapted to a clinical setting and is much less costly than the commercially available instruments. This model has a great potential to be used for the detection of various biomolecules in a clinical setting.

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VITA

Rinosh Joshua Mani

Candidate for the Degree of

Master of Science

Thesis: APPLICATION OF BI-CELL SURFACE PLASMON RESONANCE FOR THE DETECTION OF APTAMER MEDIATED THROMBIN CAPTURE IN SERUM

Major Field: Veterinary Biomedical Sciences

Biographical:

Personal Data: Born in Kottayam, Kerala, India on 10th December 1975, son of T. J. Mani and Rajam Mani.

Education: Graduated from Girideepam Bethany High School, Kottayam, Kerala, India in March 1992; Graduated with Pre-Degree from CMS College, Kottayam, Kerala, India in June 1994; Received Bachelor of Veterinary Science and Animal Husbandry degree from Kerala Agricultural University, Mannuthy, Kerala, India August 2001. Completed the requirements for the Master of Science degree in Veterinary Biomedical Sciences at Oklahoma State University in July 2006.

Experience: Veterinary Surgeon and Extension Officer, MILMA, Pathanamthitta, Kerala, India. January 2002 to July 2003; Veterinary Surgeon, Veterinary Hospital, Mukkulam Idukki, Kerala, India. August 2003 to July 2004; Teaching Assistant, Veterinary Parasitology, Department of Pathobiology, College of Veterinary Health Sciences, Oklahoma State University. August 2004 until present.

Professional Memberships: Indian Veterinary Association (Kerala Chapter). American Chemical Society.