Fiber optic-based biosensor for detecting viral hepatitis

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Fiber Optic-Based Biosensor for Detecting Viral Hepatitis

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ABSTRACT

Hepatitis is an inflammatory disease of the liver that can present serious complications ranging from partial liver damage to liver cancer, and even death of the individual. Early diagnosis of this disease condition has been established as vital to prevent irreversible liver damage and to save life in patients. It has been well established that Hepatitis B virus infection causes cardinal serological changes in blood, which include the presence of Hepatitis B surface antigen (HBsAg), Hepatitis envelop antigen (HBeAg) and decrease in the serum protein content. It is therefore important to detect the levels of serological factors such as Hepatitis B surface antigen, Hepatitis B envelop antigen and serum proteins in the blood so that a more accurate and indicative measurement of the disease condition can be obtained when integrated together.

Current diagnostic methods for Hepatitis B include the Enzyme Linked Immunosorbent Assay (ELISA), liver biopsy and polymerase chain reaction. These methods are generally laboratory-based assays, tedious, time consuming and which require skilled personnel. Furthermore, hepatitis exhibits multiple clinical features and current diagnostic methods do not give a complete profile of the different serological variables.

This study therefore aims to research and introduce a novel single-step, rapid fiber optic-based biosensor system to detect different serological variables associated with the Hepatitis B virus. In this project experiments were conducted in two stages. In the first stage, a novel fiber optic-based biosensor for detecting total

protein content was introduced*. The protein sensor offers a single-step method for quantifying protein concentrations without destroying the sample. The highly sensitive Nano orange assay and the fiber optic spectroscopy were utilized in this study to develop the novel biosensor. The profiled fibers were used in order to enhance the fluorescent signal. The profiling of optical fibers were optimized. The sensitivity of the profiled fiber compared to the unprofiled fiber was found to give much better detection of fluorescence (about 35 times better) compared to the unprofiled one. A detection sensitivity level in the order of 10^{-6} g/ml was achieved by this protein sensor. The entire assay was completed in 2 minutes time. This is much lower than the conventional method for protein detection, which might take 30 minutes or more. This fiber optic-based sensor can be used to determine the level of total protein content in Hepatitis B patients to serve as an indicator of extent of liver damage.

In the second stage, a fiber optic-based immunosensor was developed to detect Hepatitis B surface antigen and envelop antigen. A sensitivity level down to 5 ng/ml of antigen was achieved with the sensor. This concentration of antigen was within the range that is normally present in Hepatitis B positive blood samples. A simple and fast process for coating antigen on the sensor surface was also introduced. Surface binding kinetics of the antibodies were examined and APTS-dextran based method was found to be promising for antibody immobilisation via silanisation. The entire assay experiment was completed within an hour's time and it is significantly lower than the conventional ELISA methods which typically take

* The author received the Far Eastern Economic Review's Young Inventor's Award (Finalist) 2003 for his submission "Fiber optic total protein sensor".

about 6 to 7 hours. One of the major advantages of this sensor is that it enables the simultaneous detection of Hepatitis B surface antigen and Hepatitis B envelop antigen which would allow a distinction amongst people who might benefit from antiviral treatment (HBsAg^+ and HBeAg^+), those less prone to respond to antiviral therapy (HBsAg^+ and HBeAg^-), and also those who are in need of vaccination (HBsAg^- HBeAg^-). A detection sensitivity level in the order of 10^{-9} g/ml was achieved with the sensor. A total protein sensor in conjunction with an immunosensor will provide a better profile of the disease state.

This thesis reports the investigations of a fiber optics-based technology for the rapid detection of total protein content and Hepatitis B surface antigen as well as envelop antigen. These have not been done before when a publication search was conducted. A novel multi-parametric biosensor for Hepatitis B virus is thereby introduced. The main advantage of this sensing strategy is its capability to detect multiple parameters, rapidly and sensitively.

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CHAPTER 1

INTRODUCTION

Hepatitis is an inflammatory disease of the liver, and the common cause is the infection with one of the five viruses called Hepatitis A, B, C, D and E. It is one of the serious diseases prevailing in the world today. Hepatitis caused by Hepatitis B virus is the most serious of all [1]. This is due to the risk of developing severe complications, such as scarring and hardening of the liver (cirrhosis), liver cancer and eventually death, if not diagnosed early. In Europe and North America, the incidences of known Hepatitis B virus carriers are about one in one thousand people. This ratio increases significantly in Asia, especially in countries like China and the Philippines. There is also a high incidence in Africa and the Middle East. World-wide, it is estimated that there are over 350 million Hepatitis B virus carriers which represent approximately 5% of the world's population. Moreover, recent studies have estimated that 10 to 30 million people become infected with the Hepatitis B virus each year [1, 2].

Because of the enormous impact of Hepatitis B virus infection, it is critical to be able to sense its manifestations as early as possible so that proper treatment can be given. Although conventional methods for detecting hepatitis exist, they are generally slow (1-2 days or more) and laboratory-based. These methods include the Enzyme Linked Immunosorbent Assay (ELISA) and Liver Function Test (LFT) [1]. Newer methods are gaining acceptance in hospitals such as Polymerase Chain Reaction (PCR)[3]. However disadvantages of the PCR-based method are

that it requires trained personnel in molecular biology and can give false positives because of cross contamination [1].

Hence, the author proposes the use of a combined fiber optic-based protein sensor and immunosensor as an integrated system to detect viral hepatitis caused by Hepatitis B. Hepatitis B exhibits multiple serological markers, and the current sensing method cannot perform the detection of multiple biochemical variables.

Figure 1.1 shows the schematic representation of the optical set up proposed in this study.

Figure 1.1 Schematic diagram of the optical set up used in the study.

The fiber optic sensing method is used in this research. The optical set up consists of an optic fiber coated with the biochemical sensing component, a detector and a

computer to translate the information obtained from the detector into a readable signal.

First, the fiber optic total protein sensor was developed as an initial part of the study by considering the fact that during chronic hepatitis condition, the amount of serum protein level drastically decreases [1]. Albumin is a major protein, which is formed by the liver, and chronic liver disease causes a decrease in the amount of albumin produced. Therefore, in liver disease, particularly more advanced liver disease, the level of the serum albumin is reduced which will lead to a reduction in the total protein content of the serum.

Second, the fiber optic immunosensor was developed to detect the Hepatitis B surface antigen (HBsAg) and core antigen (HBeAg). The detection of Hepatitis B surface antigen (HBsAg) and Hepatitis B envelop antigen (HBeAg) would allow a distinction to be made amongst persons who might benefit from antiviral therapy ($\text{HBsAg}^+ \text{HBeAg}^+$), those who are less prone to respond to antiviral therapy ($\text{HBsAg}^+ \text{HBeAg}^-$) and those who are in need of vaccination ($\text{HBsAg}^- \text{HBeAg}^-$). As the immunosensor alone is not enough to assess the extent of liver damage, a multiple probe approach consisting of both fiber optic total protein sensor and immunosensor is required to diagnose viral hepatitis more efficiently. With a more accurate diagnosis, physicians will be able to prescribe more effective treatment.

1.1 Objectives

Based on the above considerations, the objectives of this work are as follows:

- To research a new, portable, fiber optic-based biosensor for the single step, rapid, sensitive and accurate detection of Hepatitis B virus infection.
- To characterise the fiber optic-based biosensor for the detection of total protein content in the liquid sample. This will help in the detection of the non-immunological clinical variables of hepatitis such as total protein content.
- To test different protein samples at different concentrations, using the developed fiber optic-based protein sensor.
- To investigate the optical and material parameters in order to optimise the biochemical variables of the antigen-antibody reaction employed to create the optical biosensor.
- To translate the above characterisation and optimisation parameters to a novel fiber optic-based immunosensor for Hepatitis B virus detection.

1.2 Scope

Chapter one covers the general introduction and objectives of the proposed research work.

Chapter two is a detailed review of Hepatitis B virus infection, current diagnostic methods and treatments. The review next describes the physics and background of fiber optics, and finally a critical review is given to describe the essence of the work.

Chapter three deals with the methods, experiment, results and discussion in relation to studies of the fiber optic-based protein sensor.

Chapter four deals with the methods, experiment, results and discussion in relation to the investigations of the fiber optic-based immunosensor to detect Hepatitis B virus antigens.

Chapter five gives the conclusion drawn from the experiments conducted in this study.

Chapter six discusses the suggestions for future studies.

CHAPTER 2

REVIEW OF THE LITERATURE

There are 350 million people worldwide who are Hepatitis B virus (HBV) carriers. About 227 million Asians are HBV carriers. This represents 80% of the world Hepatitis B virus carriers [1]. Hepatitis also causes up to 80% of the world's liver cancer, and annually about 0.5 million people die from HBV related diseases such Hepatocellular carcinoma (HCC) [1]. Chronic Hepatitis B infection is a common disease in Singapore affecting 4% of the population. It may lead to further complications 20 to 30 years later in the form of chronic hepatitis, scarring and hardening of the liver (cirrhosis), as well as liver failure and liver cancer. Death from chronic liver disease occurs in 15-25% of chronically infected persons. Current estimates indicate that the average or mean incubation period from the time of infection to the development of chronic liver disease and death is approximately thirty five to forty years [1]. Further still, it is crucial to note that Asian countries have large numbers of HBV carriers and high prevalence of mortality due to HCC [1, 2]. In this chapter, the first part of the review covers the details of hepatitis infection and current diagnostic methods. The next part of the review describes the physics background for the research. Finally, a critical review is given to support the feasibility of this research.

2.1 Clinical features of Hepatitis B infection

The HBV infection usually shows a long dormant phase. This means that although the individual is infected with HBV, he may not experience illness or symptoms. Hepatitis can be classified into two groups based on the time intervals between first recognition of the illness and onset of hepatic encephalopathy; they are acute and chronic hepatitis. The commonest symptoms in acute hepatitis include fatigue, fever, poor appetite, nausea and abdominal discomfort. Sometimes there is the characteristic sign of jaundice, during which there will be a yellowish discoloration of the sclera of eyes. These symptoms can last from a few days to a few months and referred to as acute hepatitis. Itching skin and pale stools may also occur. In some cases infection may last for more than six months and this is considered to be chronic hepatitis and can be fatal, especially in the elderly people, where the reported mortality rate is as high as 10 - 15% [1]. People with chronic hepatitis infection are at risk of liver damage and around 20-30% of these progress to cirrhosis of the liver (chronic irreversible damage of liver) [1]. Serological testing is the most important test for the detection of HBV. These tests will demonstrate the presence of surface antigens and other serological markers [4]. Table 2.1 represents the percentage of population who are Hepatitis B virus carriers in major Asian countries. Taiwan shows the highest percentage of carriers (14.6%). Table 2.2 shows the clinical complications of Hepatitis B virus infection. Figure 2.1 shows the healthy liver and liver with cirrhosis.

Table 2.1 The Hepatitis B carrier rate in Asia [5].

Country	Percentage of population who are Hepatitis B surface antigen positive.
China	11.7
Hong Kong (SAR)	9.6
India	5
Indonesia	5.5
Japan	1.9
Korea	12.3
Philippines	9.2
Singapore	5
Thailand	10
Taiwan	14.6

Table 2.2 Clinical complications of Hepatitis B virus [1].

Complications of Hepatitis B
<ul style="list-style-type: none">• Chronic hepatitis (inflammation of the liver).• Hepatitis flare (sudden increase of the ALT (Alanine Amino Transferase) , > 5 times upper limit of normal).• Cirrhosis (hardening of the liver).• Ascites (fluid accumulation in the abdomen).• Bleeding of the esophageal or gastric varices (burst of vessels in gullet or stomach).• Liver cancer.• Liver failure (confusion and coma due to poor liver function).• Spontaneous Bacterial Peritonitis (SBP) - infection of ascites fluid by bacteria.• Hepato Renal Syndrome (HRS) - kidney failure secondary to liver failure.

Figure 2.1 Healthy liver and a liver with cirrhosis [1].

2.1.1 Viral detection methods

There are different varieties of laboratory diagnostic tests for diseases caused by viruses. These tests can be grouped into four main categories: morphological, serological, virus isolation, and nucleic acid technology. Each method has its own advantages and disadvantages [6].

- *Morphological*

In a morphological test, one directly examines the clinical specimen for evidence of the virus. A morphological test, although simple to perform, requires a high

concentration of virus components in the clinical specimen. The procedure commonly includes electron microscopic examination of the clinical sample. In this method samples are prepared on glass slides and are coated with electron conducting materials such as gold or platinum and are observed under electron microscope, typically it will take a day for completing this analysis [6]. After getting the morphological features of the virus, one has to compare it with the virus classification standards. The chance of making false conclusions is very high as it depends mainly on the morphological image and experience of the person doing the diagnosis [7].

- *Serological*

In a serological test, one examines the serum directly for viral antigen or antibody with procedures based on antigen-antibody reaction. The procedure commonly includes ELISA and latex agglutination. The enzyme linked assay method is one of the sensitive detection methods but it can, however, give rise to false positive results. For example in ELISA, microwell plates are coated by simple physical adsorption which often results in inappropriate coating of the wells. In ELISA the conjugate is an enzyme-bound molecule so that the ultimate colour reaction depends on the activity of the enzyme, substrate quality and incubation time rather than the concentration of the analyte. An ELISA test will take 6 to 7 hours to complete. The latex agglutination test will give only a qualitative result and it is also less sensitive compared to ELISA and time taken is 3 to 4 hours [6, 7].

- *Virus isolation*

In this procedure, one inoculates appropriate clinical samples into cell culture or laboratory animals and then observes for the evidence of virus replication. This technique is a sensitive procedure for detecting viruses in clinical materials. The cell cultures and laboratory animals must be susceptible to infection by the virus under study [8]. In general, virus isolation is a slow and expensive process and is not commonly used in clinical practice except in the diagnosis of certain specific species such as herpes simplex virus [6, 7].

- *Nucleic acid technology*

Virus genomes in the tissue or the secretion can be detected by the hybridisation technique. This technique is not a sensitive procedure and it generally requires 10^5 genome equivalents to give a positive test. The sensitivity of the test can be increased by PCR (Polymerase Chain Reaction) [3], in which specific DNA probe against the pathogen is used. This method is very expensive and consumes a lot of time. It gives little information about the disease activity. This method requires technical personnel who are trained in molecular biology. One of the main problems associated with nucleic acid technology is the false positive reaction as a result of contaminants, for e.g. DNA fragments from other cellular sources, which got similar reading frames as that of probes [6, 7]. The different viral detection methods and its limitations are summarised in Table 2.3

Table 2.3 Current viral detection methods.

Detection Method	Materials Used	Limitations
Morphological	Electron microscope examination of the specimen	Chances of getting false result very high and need an experienced microbiologist to do a morphology analysis.
Serological	Antigen-antibody reaction (ELISA), detection of the activity of serum enzymes.	Time consuming, require trained persons.
Virus isolation	Cell culture technique	Time consuming, need highly trained professional and very expensive.
Nucleic acid technology	Virus genome isolation using molecular biological methods.	Time consuming, chances of false positive reaction due to cross contamination and need trained people.

2.1.2 Diagnostic methods for Hepatitis B

Current diagnostic methods for Hepatitis B can be broadly classified in to two groups:

(a) Immunological methods and (b) non-immunological methods.

(a) Immunological methods.

In this method the affinity binding of a specific antigen with its antibody is taken into consideration. The first serologic marker that appears following an acute infection is Hepatitis B surface antigen (HBsAg), which can be detected as early

as 1 or 2 weeks and as late as 11 or 12 weeks after exposure to HBV. HBsAg is an antigenic protein produced by HBV. Since this is the earliest indicator of acute hepatitis, they are frequently used to identify infected people before symptoms appear. In some patients (particularly in children or patients with a weak immune system, such as those with AIDS), chronic hepatitis occurs. In chronic hepatitis, surface antigen will also be present along with other serological markers such as envelop antigen. Common methods for detecting Hepatitis B surface antigens and envelop antigens include Enzyme Linked Immunosorbent Assay (ELISA) and Radio Immuno Assay (RIA) [1].

(b) Non-immunological tests

This method includes liver function test, which measures various protein components present in the blood. This can be useful in determining the extent of liver damage. Albumin is a major protein formed by the liver. In chronic liver disease, a decrease in the amount of serum albumin results in the damage of the liver cells. Therefore, in advanced liver disease, serum level of albumin decreases (less than 3.5 mg/dl) compared to the normal level which is 3-5g/dl [1]. Apart from this, the determination of specific enzyme activity of the common liver enzymes such as alanine aminotransferase, aspartate amino transferase and gamma-glutamyltransferase are also used as targets to predict liver damage. In some cases, a liver biopsy has to be performed in order to confirm liver damage. Modern molecular biological methods such as PCR are also used to determine the amount of HBV-DNA present in the sample [1].

It is important to realise that the current sensing methods for Hepatitis B require a skilled technician to conduct the test. Conventional immunoassay methods including ELISA or radio immunoassay need sufficient time to be available for the reaction to reach equilibrium before performing the assay (6-7 hours) [9]. The sensitivity of these reactions largely depends on different parameters such as activity of the enzyme-conjugated antibody used for detection and substrate quality [9]. If the serum level of the antigen is very low, it will not be detected by the classical ELISA. Furthermore, it cannot be practised as a single step, bedside or chair-side procedure. Hepatitis displays multiple serological markers, which include both immunological and non-immunological molecules. It is not possible to detect multiple serological markers with a single sensing device using current diagnostic methods. Thus, new and more sensitive methods are needed that exceeds their performance.

2.1.3 Treatment for hepatitis

Current treatments for HBV include both injectable and tablet therapy [1].

- **Injectable therapy**

Interferon is an injection that is given three times per week, and a course of treatment is 4 to 6 months. A number of different forms of interferon are available: interferon alpha 2a (Intron A), interferon alpha 2b (Roferon), and consensus interferon. Interferon works by both an anti-viral effect as well as altering the body's immune system. A response to treatment is seen in about 30% of patients.

However, it has numerous side effects: mainly flu-like symptoms such as, fever, muscle aches, tiredness, loss of appetite, drop in blood counts, some hair loss, effects on thyroid and worsening of depression. A stronger form of the drug is now being tested in Hepatitis B patients called pegylated interferon, which can be given once a week.

Thymosin alpha-1 is another injectable drug given 2-3 times a week. But in contrast to interferon, it has virtually no side effects. Its effectiveness, however, is unclear with a response rate as low as 12% and as high as only 40%. The response of the drug takes place 6-12 months after therapy has been completed.

- Tablets

Lamivudine is the first oral tablet for Hepatitis B virus and suppresses the virus in almost all patients, but stopping therapy usually results in return of the virus that can result in a "flare" or severe inflammation of the liver. After 3 years of continuous treatment, about 30-40% of patients "respond" and can stop therapy without fear of a flare. Although the medication is very safe, it tends to lose its effectiveness as the virus becomes more resistant over time (14% in one year and 50% in 3 years). The development of lamivudine resistance usually indicates a need to change treatment, and adefovir dipovoxil is the only treatment which is universally effective in this situation [1].

Adefovir dipovoxil is the second orally available anti-Hepatitis B drug and is currently the only effective treatment for patients who develop lamivudine resistant mutants. It works in a similar manner to lamivudine by suppressing Hepatitis B

virus. However, the risk of developing mutants resistant to adefovir is extremely low (<3% after 3years). There are some concerns about kidney side effects although long-term studies have shown this to be <2% over 3 years. The cost of adefovir is about 50% more than lamvidine, hence the drug tends to be reserved more for those who fail lamivudine therapy. In patients who have HBeAg in their blood (HBeAg positive), a continued therapy for 6 months after this becomes negative is normally required. For those who started therapy when their HBeAg were already negative, there is no agreement on the length of therapy. Instead, this will be evaluated from time to time by their doctor. Some patients may need to be on indefinite therapy, particularly those who have cirrhosis or have had a liver transplant. Those who are taking medication because they are on chemotherapy or steroids should only need short term therapy, usually a few months (4-6 months) after stopping their chemotherapy or steroids. Treatment will usually need to be individualized with the doctor who monitors progress and provides advice and prescription accordingly.

2.2 Biosensors

A biosensor combines a biochemical molecular recognition scheme with a suitable transducer to provide a signal that can be related to the presence or concentration of the desired analyte [10, 11]. A biosensor can be defined as an analytical device that incorporates a biological or biomimetic material (e.g. tissue, micro-organisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc.) that is intimately associated with or integrated within a physicochemical transducer or transducing microsystem. This transduction system may be optical,

electrochemical, thermometric, piezoelectric or magnetic [12, 13]. The research in this area has attracted extraordinary attention in recent years [14]. This is because of the key role that biosensors can play towards the development of highly sensitive and selective biochemical analysis[4, 15-21]. These devices promise an interesting alternative to the conventional techniques in a broad range of areas (e.g. medical, pharmaceutical and environmental applications) [22-28]. Biosensors are gauged based on their sensitivity, selectivity, versatility, rapidness, and capability for simultaneous multianalyte monitoring. Figure 2.2 shows the schematic representation of a generalised biosensor.

Figure 2.2 Schematic layout of a generalised biosensor.

2.2.1 Biosensor classifications

Biosensors can be classified based on the recognition molecule and on the transducer element. In general, depending on the recognition properties of the components, the following biosensor categories are recognised [11, 12, 29-31] .

- *Catalyst based biosensors.*

These are also known as metabolism sensors and are kinetic devices based on the achievement of a steady-state concentration of a transducer-detectable species. The progress of the biocatalyzed reaction is related to the concentration of the analyte, which can be measured by monitoring the rate of formation of a product, the disappearance of a reactant, or the inhibition of the reaction. The biocatalyst can be an isolated enzyme, a microorganism, a subcellular organelle, or a tissue slice.

- *Affinity based biosensors.*

In these the receptor molecule binds with the analyte irreversibly and non-catalytically. The binding event between the target molecule and the bioreceptor, for instance an antibody, a nucleic acid, or a hormone receptor, is the origin of a physicochemical change that will be measured by the transducer.

• *Biochemical sensors*

In this type of sensor, a chemical recognition species is immobilized on the transducer surface. In response to the biochemical reaction, the chemical recognition species elicit certain optical changes such as absorbance or fluorescence, which can be used to detect the target analyte.

Based on the mode of operation of the transducers, biosensors can further be classified into four groups [32]: (1) optical (2) electrochemical (3) mass sensitive, and (4) thermometric.

Table 2.4 provides information on some of the above mentioned transducers and biological recognition elements. The first column represents the common biological recognition element and the second column represents the signal transducers employed for recognising the biological elements. Since this project primarily focus is on the fiber optic-based biosensors, a more detailed review has been carried out in this direction.

Table 2.4 Classification of the biological recognition elements and signal transducers employed in biosensor development [12, 20, 29, 32].

BIOLOGICAL RECOGNITION ELEMENT	SIGNAL TRANSDUCERS
<p>Catalyst based biosensor</p> <p>Enzymes (purified or crude mixtures , single or multiple enzymes) Catalytic transformation of analyte into a sensor detectable product or which induce detectable signal. The analyte inhibits the enzyme activity. A detectable characteristic of enzyme is changed up on interaction with analyte.</p> <p>Cellular structure and whole cells Pollutant dependent increase in the microorganism respiration. Pollutant dependent inhibition of microorganism respiration.</p> <p>Affinity based biosensor</p> <p>Antibodies or antigen Based on the specific and high affinity antigen antibody binding reaction. A tracer (fluorescent molecule, enzymes) is used to generate the signal.</p> <p>Nucleic acid detection Detection of DNA ligand interaction Detection of specific sequence by hybridisation</p> <p>Biomimetic receptors Genetically Engineered molecule Artificial membranes Molecularly imprinted polymers</p>	<p>Electrochemical Potentiometric Amperometric Conductance Capacitance</p> <p>Optical Absorbance Reflectance Luminescence Refractive index Light scattering</p> <p>Mass Sensitive Piezoelectric Surface acoustic</p> <p>Thermometric Thermistor</p>

2.2.2 Immunosensors

An immunoassay is a quantitative technique that depends on the reaction between the molecule of interest, the antigen, and a complementary molecule, which is the antibody. An antigen is a molecule capable of eliciting an immune response when injected in an animal that treats it as foreign species, for example, bacteria and viruses. An antibody has two roles to play: first, to bind antigen; and second, to interact with the host tissue and effector system in order to ensure the removal of the antigen. The basic antibody structure consists of two-glycosylated protein consisting of two heavy and two light, polypeptide chains. There are two regions in the antibody Fab (Fragment antigen binding) and Fc (Fragment which can be heavily crystallized by enzymes like papain) [33]. The region, which binds to the antigen, is known as Fab. The other region is known as Fc and is responsible for evoking the effector system, which helps in the removal of the antigen [9]. Figure 2.3 shows the molecular structure of immunoglobulin G (IgG).

Figure 2.3 Immunoglobulin G structure adapted from [33].

Immunosensors are a combination of an immobilized immunological receptor used for detecting a target analyte by structural complementarity, and a transducer, which converts a biological interaction into a measurable signal [34-36]. The most frequently used transducers in immunosensor development are based on electrochemistry, fluorimetry, interferometry, resonance and reflectometry [35, 37]. The term immunoassay is used for tests based on immunoreactions, while the term immunosensor is specifically employed to describe whole instruments, i.e. immunoreaction-based biosensors.

The measurement of the binding reaction can be performed by monitoring changes in the different physical phenomena associated with the biomolecules, the labels used and the configuration of the assay. Using indirect approaches, one of the

immuno-compounds is conjugated with an indicator molecule, e.g. an enzyme catalysing a redox reaction or a fluorophore emitting fluorescence and it can be achieved by applying the principle of sandwich solid-phase immunoassay format as shown in Figure 2.4.

In the sandwich assay format, the antigens are incubated with an excess of a primary antibody and the resulting antigen-antibody complex is incubated with a second labelled antibody, which binds to a second antigenic site. The amount of labelled antibody bound is related to the analyte concentration. The fact that two antibodies recognise different epitopes on the analyte molecule decreases the chance of interference by other similar species, but limits the application of this technique to sufficiently large antigens that fulfil this requirement [9].

The use of antibodies for sensor development has some limitations: (1) strong dependence of the antibody-binding capacity on the assay conditions, for instance pH and temperature, and (2) the irreversible nature of the antibody-antigen interaction. But chaotropic reagents alone or in combination with acidic buffers, or even ultrasonic radiation [38], have been used as effective agents to disrupt antibody-analyte association [39].

Figure 2.4 Sandwich immuno assay format.

Optical detection has a clear advantage over electrochemical methods in the development of immunosensors in that it can be used to monitor binding reactions directly (in situ) in real-time. Optical techniques such as evanescent wave (EW) spectroscopy have been widely applied in recent years, providing a way for direct evaluation of the antigen-antibody interactions occurring at the surface-solution interface. Some of the latest achievements in the area of optical immunosensing and their application towards clinical and food analysis are described below and listed in Table 2.5.

Table 2.5 Applications of fiber-optic evanescent wave immunosensors in clinical analysis.

Analyte (Antigen)	Application(s)	Assay format	Label(s) (Antibody)	Ref.
Cocaine	Clinical analysis	Competitive	Cy5-labeled antibodies	[36]
Coca alkaloids	Clinical analysis	Competitive	Fluorescein- labelled antibodies	[40, 41]
Benzopyrene	Clinical analysis	Direct	Flourescein labelled antibodies	[42, 43]
Protein C	Clinical analysis	Sandwich	Cy5-labeled antibodies	[44]
D-dimer	Clinical analysis	Competitive	Fluorescein- labelled antibodies	[45]
<i>Salmonella</i> <i>spp.</i>	Clinical and food analysis	Sandwich	Cy5-labeled antibodies	[46]
<i>Staphylococc</i> <i>us aureus</i>	Clinical and food analysis	Sandwich	FITC-labelled antibodies	[47]
Enterotoxin B	Clinical and food analysis	Sandwich	Cy5-labeled antibodies	[48]
<i>E.coli</i> <i>O157:H7</i>	Food analysis	Sandwich	Cy5-labeled antibodies	[49]
Fumonisin B ₁	Food analysis	Competitive	FITC-labelled antibodies	[50]

Figure 2.5 Different types of ELISA format described in the table 2.4, adapted from [35].

Experimental protocol the different ELISA format is summarised below.

Direct ELISA

Indirect ELISA

Competitive ELISA Sandwich

The symbols used are:

I = Microtiter plate well.

Ag = Antigen.

Ab= Antibody.

AB =Antibody produced in a different species to Ab.

Anti-Ab*E =Secondary antibody conjugated with Enzyme.

Ab*E= Antibody conjugated with Enzyme.

S= Substrate.

R= Read in spectrophotometer.

W= Washing step using Phosphate buffered saline.

Figure 2.5 shows the schematic representation of different assay formats mentioned in table 2.4. The main advantage of evanescent wave sensors is their suitability for measurement of molecules located on or near the outer surface of the optical fiber, even in the presence of turbid or absorbing solutions. In this type of device the selective receptor molecules are immobilised on the sensor surface and, in the presence of the analyte of interest, a biochemical reaction occurs on the

surface of the waveguide. It induces a change in its optical properties, which is detected by the evanescent wave. Most of the immunosensors based on this principle (using fiber-optics or planar optical waveguides) make use of fluorescent-labelled molecules that can re-emit the absorbed evanescent photons at a longer wavelength by total internal reflection fluorescence (TIRF).

Figure 2.6
Fiber optic immunosensor

ensor for intracellular measurements in mammary carcinoma cells [43].

Figure 2.6 shows the fiber optic immunosensor for intracellular measurements in mammary carcinoma cells. Since their appearance, immunosensors have become widespread in the field of clinical analysis [36]. A four-channel fiber-optic instrument using a competitive fluorescence immunoassay has been developed for analysis of cocaine and its metabolites (COC) in human urine [40].

A real-time fiber-optic-based biosensor has been described for the detection of protein C (PC), one of the human body's key anticoagulants, deficiency of which can lead to massive thrombotic complications [43, 44]. An average of six consecutive assays was achieved per fiber. The device has a linear working range of 0.2-2.0 μ g/ml, which includes the PC range of heterozygous deficient patients.

Fiber optic immunosensor analysis, moreover, requires significantly less time and skill than the standard ELISA method. In another example fluorescein-labelled D-dimer antibodies were immobilised on the tip of an optical fiber by means of a sol-gel technique [45]. The sol-gel process is discussed in Chapter 3 of this thesis.

Another area in which fiber optic immunosensors have found numerous applications in the last few years is the analysis of food toxins [50]. An increasing number of bacteria have been identified as important food- and water-borne pathogens. Infectious diseases caused world-wide by these micro-organisms account for nearly 40% of the total 50 million annual estimated deaths [46, 51]. Salmonellosis is an infectious disease caused by *Salmonella spp*, dangerous food-borne pathogenic bacteria. A portable sensor system, equipped with fiber optics and semiconductor laser excitation, was developed and it can be operated in ambient light. The biosensor test chamber serves as an ultrasonic standing-wave cell that allows microspheres or cells to be concentrated in parallel layers or in a column along the axis of the cell. The fluorescent labeled-antibodies themselves do not respond to the ultrasound, but, when attached to salmonella cells, the *Salmonella*-antibody complexes was moved acoustically to the axis of the cell,

increasing the fluorescent signal [52]. The sensor is rugged, cheap, stable, and can be used to detect *Salmonella* at concentrations of as low as 10^4 CFU (Colony Forming Unit)/ml. Figure 2.7 shows the schematic drawing of the *Salmonella* biosensor.

Figure 2.7 Schematic drawing of the acoustic test chamber for *salmonella* detection. The sensing fiber is located along the axis of the cell.

Staphylococcus aureus is another bacteria causing food-borne diseases. It can produce several types of endotoxin that cause gastro-enteritis. The presence of the bacterium in processed food is, therefore, a health hazard that must be controlled. An optrode was proposed for detection of protein A, a product secreted by *S. aureus* only [53]. Figure 2.8 shows the schematic of the staphylococcus endotoxin

B immunosensor. The detection limit of the immunosensor was 1 ng/ml protein A. The official US Food and Drug Administration methods for the analysis of the bacterium normally take from 5 to 7 days; the assay time using this biosensor can be reduced to 24 hours.

Figure 2.8 Schematic of staphylococcus endotoxin B immunosensor [48].

Thompson *et al.* [50], have developed a fiber optic evanescent wave immunosensor by using a direct competitive assay for measurement of fumonisin B₁ (FB₁), a mycotoxin produced by common fungal contaminants of corn. Figure 2.9 shows the schematic representation of the sensor. The sensor has a working range from 10 to 1000 ng/ml and a limit of detection of 10 ng/ml FB₁. The fiber optic biosensor was highly selective toward fumonisins and did not cross-react with other structurally related compounds. It has been successfully used for analysis of FB₁ in spiked and naturally contaminated corn, either by dilution or after affinity column clean up. The results obtained with the immunosensor agreed well with those from an established HPLC method for the analysis of these compounds..

Figure 2.9 Experimental set up of the fiber optic sensor for fumonisin [50].

2.3 Background optics related to the study

An optical fiber is a long cylindrical structure with two coaxial regions. The inner region is the light guiding core and is surrounded by another outer layer of coaxial region known as the cladding. The fiber with a core of constant refractive index and surrounded by a cladding of slightly lower refractive index is known as a step-index fiber. The fiber with a core of gradually varying refractive index is known as a graded-index fiber. Multimode fiber, the first to be manufactured and commercialized, refers to the fact that numerous modes or light rays are carried simultaneously through the waveguide. This fiber type has a much larger core diameter, compared to single-mode fiber, allowing for the larger number of modes, and multimode fiber is easier to couple than single-mode optical fiber [54].

Single mode fiber is basically designed for the transmission of a single ray or mode of light as a carrier and is used for long-distance signal transmission. Zhao et al. developed a fiber optic single mode pressure sensor [55]. In this study a self-referencing double interferometer was used for interrogation of the signal. The sensing interferometer is based on a white light Michelson interferometer. The sensor was evaluated through measurement of high pressures applied to sand samples. The sensitivity of the system was 0.03 MPa. Karen et al. conducted a preliminary investigation using a single mode fiber in conjunction with an infrared spectrometer to obtain spectra of a high-performance epoxy resin system [56]. Spectra were obtained using single mode fibers that contained Bragg gratings; however, the peaks of interest were barely discernible above the noise. According

to Keran et al. attempts to use this single mode fiber for spectroscopic analysis are problematic, as its small core diameter result in low signal intensity.

Bhatia et al. described a novel class of highly sensitive sensors based on long-period fiber gratings on single mode fibers [57]. Temperature, strain, and refractive-index resolutions of 0.65°C , $65.75\text{ }\mu\epsilon$ and 7.69×10^5 , respectively, were demonstrated. However, Bragg-grating based sensors suffer from limited temperature and strain induced spectral displacements, and it is often required that one should use complex interferometric techniques to detect these shifts. Moreover, these sensors can be difficult to manufacture because of stringent stability requirements. The use of expensive phase masks during fabrication and the need for isolators to prevent back reflection induced by source oscillations will be added to the overall cost of the system.

Elizabeth et al. developed a single mode fiber optic evanescent wave biosensor using an exposed core silica single mode fiber embedded in a silica block [58]. The device was able to monitor the concentration of a blue dye, Procion Blue MX-G, in overlayers of various refractive indices. The practicality of such a biosensor has been demonstrated with a colorimetric enzyme assay system. Penicillin G in the 0–0.4 mM concentration range was monitored at 633 nm by the decolouration of the starch-iodine reagent when *Bacillus cereus* penicillinase was immobilized over the exposed core of the monomode.

A fiber optic microsensor for surface plasmon resonance (SPR) was developed by Kazuyoshi et al. on the basis of the fabrication technology of optical fiber probes in

near-field scanning optical microscopy (NSOM) [59]. The microsensors were prepared by coating a gold-metallic film on the chemically etched single mode fiber. The microsensors were used to the real-time monitoring of the refractive index (RI) of transparent liquids flowing in the microfluidic device. When SPR microsensors were chemically modified with a self-assembled monolayer presenting a hydroxyl group, they responded to the refractive index from 1.33 to 1.40 RI with a sensitivity of 0.008 RI units.

One of the primary disadvantages of single mode fiber is that it is difficult to efficiently couple light into the small core diameter. The rationale for using multimode over single mode fiber in this study is the fact that, we are interested in taking advantage of their greater light collection diameter from diffused sources. We took advantage of its larger core diameter and NA (numerical aperture) as ours is a fluorescent based sensor[60]. The smaller core diameter makes coupling light into the core more difficult. Similarly, multi-mode connectors cost less and are easy to couple than single-mode connectors due to the more stringent alignment requirements of single-mode fiber. The tolerances for single-mode connectors and splices are also much more demanding. A powerful light source is required in order to excite fluorescent conjugate, which could be expensive. As we use antibodies as the primary conjugate greater core area at the sensing region could avoid over crowding of antibodies which could result in cross linking [54].

Optical fiber work as transmission lines to conduct electromagnetic energy. Here, the light source is the generator of electromagnetic energy. The ray propagation in a fiber has two components: meridional and skew rays (Figure 2.10). The former

passes through the optical z-axis and does not have a component in the direction of the curvature. Total internal reflection is experienced when their incidence angle exceeds that of the critical angle. The latter has a propagation component in the β direction and that exhibits a helical path down to the core, never crossing the fiber z-axis. Skew rays tend to propagate only in the annular region near the outer surface of the core. Most of radiation power coupled in the fiber will propagate as skewed rays. This, if measured, will increase the light gathering capacity of the fiber [61]. Indeed, skewed rays are accepted at larger axial angles than meridional rays [61].

Figure 2.10 Schematics of the geometrical representation of an arbitrary ray entering in cylindrical optic fiber.

The re-conversion of optical intensity into electrical power takes place at the photodetector. The aim of a photodetector is to achieve high sensitivity and low distortion in signal detection. The spectral response, the speed of response and the linearity of the photodetectors determine its performance [61].

2.3.1 Refraction and total internal reflection

Refraction occurs when light passes from one medium to another and the light ray will be bent at the interface between the two mediums. Refraction is described in Snell's law.

Where :

(1)

$$n_0 \sin \phi_0 = n_1 \sin \phi_1$$

n_0 = Refractive index of first medium

n_1 = Refractive index of second medium

ϕ_0 = Angle between the incident ray and the normal to the interface

ϕ_1 = Angle between the refracted ray and the normal to the interface

Figure 2.11 Refracted ray and total internal reflection [62].

Figure 2.11 shows light passing from a high index n_0 to low index medium n_1 . As the angle between the incident ray and the normal increases beyond the point where $\phi_0 = \phi_c$, no refraction will occur (ϕ_c is the critical angle). For angles greater or equal to ϕ_c , total internal reflection at the interface occurs and no refraction takes place.

Numerical aperture (N.A.) is the light acceptance capability of the optical fiber. It is determined by the difference between the core and the cladding. Large N.A. allows more light to couple into the fiber at larger angles. Figure 2.12 shows the propagation of light through an optic fiber.

$$NA = (n_{\text{core}} - n_{\text{cladding}})^{1/2} \tag{2}$$

Figure 2.12 Light ray passing along an optical fiber [62].

2.3.2 Evanescent wave

The imperfect total internal reflection in a fiber causes some electromagnetic radiation to penetrate the cladding covering it. This radiation field intensity which extends beyond the boundaries of the waveguide is called the *evanescent wave* [63]. Its intensity decreases exponentially with the perpendicular distance from the interface between the core and cladding. This phenomenon can be described by calculating the depth of penetration of the wave [64] as follows:

$$d_p = \frac{\lambda}{2\pi(n_{co}^2 \sin^2 \theta - n_{cl}^2)^{1/2}} \quad (3)$$

Where θ is the internal incident ray angle with the normal to the n_1/n_2 interface, d_p is the penetration depth and λ is the fluorescent wavelength.

Evanescent waves can be used to detect variations in optical properties of chemical and biological samples placed around the surface of the exposed fiber. The sample is usually mixed in a fluorescent solution. On applying a light source to the sample, fluorescence will occur due to the excitation caused by the evanescent wave. The generated fluorescent signal is transmitted to the detector through the clad section of the fiber. Kronick and Little were the first ones to exploit the evanescent wave-generated fluorescence to detect antibody-antigen binding [65]. A few years after that, this revolutionary concept was further improved and applied to fiber optics by Hirschfeld [66] and Andrade [67].

2.3.3 *V-number mismatch*

By stripping off a few centimeters of cladding length from the distal end of a fiber, the sensing region of the optical fiber can then be formed. This simple method, however, was found to disrupt the signal acquisition of the fiber [68]. Part of the fluorescence signal collected at the sensing region was unable to return to the clad fiber due to V-number mismatch [69]. V-number or waveguide parameter is a parameter that determines the number of modes an optical waveguide can support. It can be calculated by,

$$V = \frac{2\pi r_{cl}}{\lambda} \sqrt{n_{co}^2 - n_{cl}^2} \quad (4)$$

where r = radius of core

λ = propagating wavelength of fluorescence light

The actual number of modes can be approximated to $V^2/2$ [70]. The sensing region is usually immersed in aqueous solution, n_{aq} such as phosphate buffered saline solution (PBS), which has a lower refractive index than the cladding. As a result, the number of modes at the sensing region is much higher than the clad fiber region. This will cause some of the fluorescence signal to be lost upon entering the clad fiber as it cannot support the same amount of modes.

Theoretically, to avoid the loss of fluorescence signal, the V-number of the clad and unclad regions must be matched. This can be done by calculating the matching radius, r_{match} using Eq. 3. There will be loss of fluorescence signal if the radius of the immersed fiber optic core is larger than r_{match} .

$$r_{match} = r_{cl} \sqrt{\frac{n_{co}^2 - n_{cl}^2}{n_{co}^2 - n_{aq}^2}} \quad (5)$$

2.3.4 Methods used to reduce V-number mismatch

In the earlier studies, the decladded core is step-etched to reduce its radius. Indeed, the sensitivity for the step-etched fiber was improved, by over 20 times [69].

However, it was also shown that the intensity of the signal still decreases along the probe length. Besides that, an excessive amount of light is lost to the bulk solution at the position of the probe where there is a sudden change in the core radius. This is undesirable as it reduces the ability of the fiber to differentiate between free and bound fluorophore (fluorescent dye molecules).

Continuous tapering is another method used for improving the signal acquisition of optical fibers which was suggested by Thompson and Villarruel [71]. They observed that a tapered probe converts the fluorescent signal from higher order modes in the immersed region into lower order modes. As a result, the fluorescent signal is able to propagate in the clad fiber. Golden *et al.* discovered that tapering increases the power of excitation light towards the distal end compared to the step-etched probes [72]. Signal losses at the front portion of the taper occur as it is above the V-number matching radius. To minimize this problem, they suggested the combination taper probe.

Apart from changing the diameter of the fiber probe, the V-number mismatch can also be reduced by using index matching liquids or oils [73]. The index matching oil helps to minimize the change in the refractive index between fiber and the surrounding media for into which the sensor is exposed. This method, however, may not be suitable as the liquids or oils used may react with the biological sample that is to be tested.

Compared to distal end sensors, evanescent wave sensors provide relatively small amounts of power for generating fluorescence signal [73]. Thus, it is very important to perform optical sensing in an environment, such as a dark room, where background noise is minimal in order for the fluorescence signal to be detected.

2.3.5 Ray tracing model

Figure 2.13 Diagram of ray tracing through a profiled fiber probe [72].

A ray tracing model (Figure 2.13) was developed by Golden *et al.* [72] to describe the evanescent wave penetration depth in a continuously tapered fiber probe. The model assumes that the internal core surfaces are totally reflective with the condition that the ray angles with the normal to the surface are larger than the critical angle. Applying Snell's law and taking $\alpha(0)$ as the launch angle of the light into the fiber,

$$r_0 \sin(\alpha_0) = r(z) \sin(\alpha(z)) \quad (6)$$

where $r(z)$ is given by

$$r(z) = \frac{(r_L - r_0)}{L} z + r_0 \quad (7)$$

where r_0 and r_L are the beginning and end radii of the probe respectively and L is the tapering length.

Combining equation (6 and (7),

$$\alpha(z) = \sin^{-1} \left(\frac{\sin(\alpha(0))}{\left[\frac{r_L}{r_0} - 1 \right] \frac{z}{L} + 1} \right) \quad (8)$$

which gives the expression of the ray angle makes with the z -axis along the profile.

For a profiled probe immersed in an aqueous solution of refractive index n_{aq} , n_{cl} in equation (3) becomes

$$d_p(z) = \frac{\lambda}{2\pi \left(n_{co}^2 \sin^2(\theta(z)) - n_{aq}^2 \right)^{1/2}} \quad (9)$$

where

$$\theta(z) = 90^\circ - \alpha(z) - \beta \quad (10)$$

and the profile angle, β is given by

$$\beta = \tan^{-1} \left(\frac{r_0 - r_L}{L} \right) \quad (11)$$

Applying Snell's law and combining equation (8) and (10),

$$\frac{\sin \alpha(0)}{\left(\frac{r_L}{r_0} - 1\right) \frac{z}{L} + 1} = n_{co} \sin(90^\circ - \theta(z) - \beta) \quad (12)$$

Which gives the relationship between $\theta(z)$ with z , the position along the z -axis at a given launch angle, $\alpha(0)$. According to Golden *et al.* [72], $\alpha(0)$ can be obtained by measuring the numerical aperture of the excitation light exiting the clad fiber.

Energy is carried in both the core and the cladding of the fiber during the electromagnetic wave propagation. A small portion of light penetrates the reflecting medium by a fraction of a wavelength, far enough for recognition of the different refractive index. This electromagnetic field, called the evanescent wave, has an energy distribution that is the maximum at the core-cladding interface and the energy in the cladding falls off exponentially as the distance is increased away from the optical fiber core [62].

In Figure 2.14, the incident light and the reflected light interact and the result is a standing wave close to the core-cladding interface. The standing wave decays exponentially away from the core-cladding interface into the low refractive index medium n_2 .

The evanescent wave exponential equation is given by:

$$\mathbf{E}_T = \mathbf{E}_2 e^{\alpha(-z)} e^{\beta(y)} \quad (13)$$

$\mathbf{E}_T$ is the electric field portion of a non-uniform wave propagating in the +y direction. The evanescent wave component is in the -z direction. $\mathbf{E}_2$ is the attenuated electric field in the lower refractive index medium n_2 . The attenuation constant α determines the spatial rate of decay of the field in lower refractive index medium n_2 . The phase propagation constant is β [54].

Figure 2. 14 Evanescent wave principle [62] d_p = penetration depth.

Most fiber optic sensors take advantage of these evanescent waves. As the number of reflections increases for a given length of fiber, the distance between reflection points is shortened. This has the practical effect of overlapping evanescent waves, and the overlap provides approximately a continuous evanescent wave region extending along the external surface of the wave-guide. This region circumscribed by linear extension along the boundary and depth of penetration creates an interaction space that is ideally suited to the dimensions of typical immobilised chemical layers. Evanescent waves are important because they provide additional options for excitatory interaction between light and matter. In some excitation configurations for the fiber cladding is stripped from a length of the fiber core, immobilised reagents may be layered directly on to the longitudinal portion of the

core surface, thus promoting the interaction with the evanescent wave. Interaction between the light and the immobilised layer can be monitored in several different ways, depending on the information sought.

The evanescent field is used in excitation of fluorescence. When a fluorescent molecule comes within the evanescent field region, and the wavelength of the radiation happens to be the same as that of the absorption spectrum of the fluorophore then the molecule will be elevated to an excited state. After a short period of time, the excited molecule returns to its ground state and emits photons. This emission may appear as a ray of light propagating away from the core-cladding interface to the lower refractive index medium n_2 . The emission may be channelled into the optic fiber and then guided into the detector.

The advantages a fiber optic-based sensor system include: (1) geometric convenience and flexibility, (2) low cost of production, (3) chemically inert and non-hazardous, (4) electrical isolation and free from electromagnetic interferences, (5) being dielectric, they are protected from atmospheric disturbances, (6) the miniature size economises the reagent used, and enables the portability of the system, and accessibility of the sensor to otherwise difficult areas, (7) they enable solid phase characterisation of analyte, and (8) optical fibers have a multiplexing capability in that they can guide light of different wavelengths at the same time and in different directions, this allows one to perform multiple parametric monitoring via a central unit without any cross-talk [61].

Some drawbacks of the fiber optic evanescent wave sensor can limit their applicability, these include the following [74] :

- Interference of ambient light, although this can be avoided by the use of suitable light isolation or modulated light sources.
- Possible photobleaching or indicator washout, when indicator phases are employed and limited stability of the immobilised biological component.
- Long response times if mass transfer to the reagent phase is needed.
- Limited availability of optimized commercial accessories for use with optical fibers.
- Fouling of the fiber surface could cause the degradation of the biomolecules as it could evoke proteolytic damage. This should be prevented by using chemical agents such as sodium azide.

2.3.6 Optical methods for biochemical sensing

Conventionally optical characteristics such as: (a) absorption, (b) Raman effect, (c) surface plasmon resonance (SPR), and (d) fluorescent techniques have been used for detection and monitoring of different biochemical processes. These aspects have been reviewed and discussed in the following sections.

- **Absorption**

The reagent is immobilised in a matrix and the sensor allows the analyte to diffuse through the matrix surrounding the reagent. The reagent is a dye that changes its absorption with changes in the concentration. The absorbed light generally increases as a function of analyte concentration.

In most absorption experiments, the Lambert-Beer law is used to measure the intensity of absorption. If I_0 is the intensity of a parallel beam of radiation incident normally on a layer of thickness b cm, and the concentration of the absorbing material is c , then the intensity of the emergent beam I is :

$$\log_{10} \left(\frac{I_0}{I} \right) = \epsilon cb \quad (14)$$

Where $\epsilon cb = A$

A - is the absorbance of the sample in the beam

ϵ - is the molar absorptivity

The expression shows that there is a linear relationship between the absorbance, A and concentration, c , of a given solution if the optical path length and the wavelength of the light signal are kept constant [61]. An example for absorption based sensing is protein detection using Lowry's method. In this method Folin-

Ciocalteu reagent changes its absorption maximum when it binds with protein. The sensitivity level up to 100 µg/ml is achieved using this method [75].

- **Raman effect**

Raman scattering (Figure 2.15) involves a momentary elastic distortion of the electrons distributed around a bond in a molecule, followed by re-emission of radiation in all directions as the bond returns to its normal state. In its distorted form, the molecule is temporarily polarised. In other words, a temporarily induced dipole is created. It disappears upon relaxation and re-emission. The effectiveness of a bond toward scattering depends on how effectively the electrons of the bond can be distorted from their normal positions (that is the polarizability of the bonds). Polarizability decreases with increasing electron density, increasing bond strength and decreasing bond length [76]. Prakash Bhosale et al. measured carotenoid levels of a variety of agricultural products and juices using Resonance Raman Spectroscopy [77]. A rapid non-destructive estimation of carotenoid levels in intact fruits and vegetables and their juices could have great value when selecting nutritionally valuable crops for further propagation and commercial use. A sensitivity level up to 50 ng/ml was achieved using this method.

Figure 2.15 Raman scattering.

- **Surface plasmon resonance (SPR)**

Surface plasmon resonance is a phenomenon which occurs when light is reflected off thin metal films[78]. A fraction of the light energy incident at a sharply defined angle can interact with the delocalised electrons in the metal film (plasmon) thus reducing the reflected light intensity. The precise angle of incidence at which this occurs is determined by a number of factors, for example the refractive index close to the reverse side of the metal film, to which target molecules are immobilised and addressed by ligands in a mobile phase running through a flow cell. If binding occurs to the immobilised target the local refractive index changes, leading to a change in SPR angle, which can be monitored in real-time by detecting changes in the intensity of the reflected light, producing a sensorgram. Figure 2.16 represents

the schematics of the surface plasmon resonance unit. The size of the change in SPR signal is directly proportional to the mass being immobilised and can thus be interpreted crudely in terms of the stoichiometry of the interaction [79]. However, the major disadvantage of SPR for bioanalytical applications is that low concentration or low molecular mass analytes could not be detected directly. The sensor surfaces deteriorate very easily with re-use. The blocking of microfluidic channel by air bubble entrapment is very common and can lead to false-positive results [79].

Figure 2.16 surface plasmon resonance detection units. L: light source, D: photodiode array, P: prism, S: sensor surface, F: flow cell.

The two dark lines in the reflected beam projected on to the detector symbolise the light intensity drop following the resonance phenomenon at time = t_1 and t_2 . The line projected at t_1 corresponds to the situation before binding of antigens to the

antibodies on the surface and t_2 is the position of resonance after binding [79]. Muller – Renaud et al. used Biacore (A commercial SPR analyser) for the quantification of β -casein in milk and cheese [80]. The detection limit was 85ng/ml. Pei et al. proposed SPR immunosensing using a streptavidin-biotinylated protein complex [81]. Human IgG protein was detected with a sensitivity 10 μ g/ml.

- **Fluorescence technique**

Light travels through the optical fiber and the rays of light excite the fluorescent-tagged analyte. During excitation, the chemical dye emits fluorescent light, if the wavelength of the incident light is close to the excitation wavelength of the chemical dye. The fluorescence emission is usually at a lower energy level (higher wavelength) than the required excitation energy. Fluorescence emission belongs to one of the photoluminescence processes. The two common types of photoluminescence are fluorescence and phosphorescence. They are distinguished experimentally by observing the lifetime of the excited state [82].

In Figure 2.17, the lowest heavy horizontal line represents the ground state energy of the molecule, which is normally the singlet state, S_0 . The upper heavy lines are energy levels of the vibration states at three excited electronic states. S_1 and S_2 represent the excited singlet states while T_1 represents the energy of the first electronic triplet state. The first excited triplet state is normally lower than the energy of the excited singlet states. The lighter lines are vibrational energy levels of each electronic state.

Figure 2.17 Electronic and vibrational state showing fluorescence and phosphorescence excitation and emission [82].

The absorption of energy is usually from S_0 to S_1 or from S_0 to S_2 . The probability for S_0 to T_1 transition to occur is normally low. The absorption can fall in any of the vibration levels of the electronic state.

A molecular electronic state in which all electrons spin in a paired form are called singlet state. At singlet state, no splitting of the energy level occurs when the molecule is exposed to a magnetic field. The ground state for a free electron is called a doublet state because the odd electron can assume two orientations in a magnetic field thus giving rise to a splitting of an energy level. When one of the electrons of a molecule is excited to a higher energy level, a singlet or a triplet state

can result. In excited singlet state, the spin of the excited electron is still paired with the ground state electron. In the triplet state, the spins of both electrons become unpaired or parallel.

A singlet to triplet transition that involves a change in electronic state is significantly less probable than the singlet to singlet state. Thus, the average lifetime of an excited triplet state will be as long as a second or more as compared with the average lifetime of an excited singlet state of about 10^{-8} second. An excitation of a ground state molecule to an excited triplet state does not occur readily. The absorption peaks of a ground to triplet state process are several magnitudes less intense than the singlet to singlet process. A return from an excited triplet state to ground state is a phosphorescent behaviour. A return from excited singlet state to ground state is the fluorescent behaviour.

The evanescent wave can interact with molecules within the penetration depth, thereby producing a net flow of energy across the reflecting surface in the surrounding medium (i.e. that with refractive index n_2) to maintain the evanescent field. When the evanescent light selectively excites a fluorophore, the fluorescence emitted can be directed back into the fiber and guided to the detector. This principle is called total internal reflection of fluorescence (TIRF) and is widely applied in the design of biosensors.

Although fluorescence measurements can be used whenever a naturally fluorescent analyte is detected, this is not common in biosensor development and the technique

is usually applied in combination with artificially labelled compounds, for instance in competitive immunosensors, or with fluorescence quenching measurements. The measurement scheme for an extrinsic fluorescence sensor will be similar to that used in absorbance measurements. In this instance, the same or a different fiber will collect the light emitted by the sensing element containing the fluorescent indicator; this light will then be filtered and delivered to the detector. Alternatively, molecular recognition can occur at the surface of the fiber core, accompanied by binding or release of a chromophore or luminophore that can be excited by the evanescent wave. In all instances light intensity, decay time, polarisation, or phase of the emitted radiation can be selected as the analytical property used to evaluate the concentration of the analyte [83]. Examples for these fluorescence based sensors are summarised in Table 2.5 of this chapter.

The optical transduction systems, which frequently use optical fibers or waveguides, take advantage of the change produced in the optical properties after immunoreactions. The intrinsic transduction devices, which include evanescent wave spectroscopy and surface plasmon resonance (SPR), are those with higher potential of application. The SPR (Figure 2.15), a well-established method for real time biomolecular interaction analysis [84, 85] has been the basis for biosensors that have been extensively applied in immuno characterizations, in clinical analysis, and in environmental monitoring.

Other promising techniques, based on total internal reflection, have been proposed in the literature, such as the grating coupler immunosensors and interferometric

biosensors [86, 87]. Nellen *et al* developed a grating coupler instrument with planar waveguides as sensors were used for real-time monitoring of protein adsorption and of affinity and immunoreactions. The adsorbed proteins studied were avidin and immunoglobulins (h-IgG). The adsorbed monolayers of these proteins formed the receptors on the waveguide surface for the corresponding ligand in the sample. The formation of complexes between avidin and biotinylated protein A and biotinylated bovine serum albumin (BSA) between h-IgG and anti-hIgG, and between h-IgG and protein A were investigated [87].

Heideman *et al* did a study on the immunoreactivity of adsorbed α hCG molecules as a function of substrate hydrophobicity and protein coverage using an interferometric immunosensor [88]. The substrate hydrophobicity and the antibody density were found to be of major importance for the immunoreactivity of the adsorbed antibodies. However, the immunoreaction affinity constant of the active antibodies is found to be essentially independent of the substrate hydrophobicity.

Schlatter *et al* investigated the application of an interferometric sensor technology for determining the concentration of biologically and diagnostically relevant analytes with high sensitivity and investigating biological recognition reactions such as antibody-antigen reactions. The interaction between an anti-human IgG and a human IgG, a broad dynamic concentration range extending over six orders of magnitude and a detection limit of 1×10^{-11} dissolved antibody were demonstrated [89].

A porous silicon-based optical interferometric biosensor was reported by Lin *et al.* The optical interferometric transducer was based on inexpensive and readily available optically flat thin films of porous silicon. Using biotin immobilized on the porous silicon substrate, binding of streptavidin resulted in a change in the refractive index of the layer medium and was detected as a corresponding wavelength shift in the interference pattern by a charged-coupled device (CCD) camera. The lowest concentration used was 10^{-14} M of streptavidin [86].

An optical biosensor was reported for detection of label-free molecules based on optical resonances in a transparent dielectric microparticle as physical transduction [90]. The system detected the binding of streptavidin to surface immobilized–biotinylated BSA. The microparticle surface was modified with biotinylated BSA as the recognition element, capable of specific binding to streptavidin. The shift in resonance wavelength was due to the binding of streptavidin to the surface immobilized biotin.

In fluorescence immunosensors, the immunoreagents are immobilized onto the optical surfaces where the immunoreaction takes place, and a fluorescent labeled molecule provides the quantitative signal of the measurement, as in fluoro-immunoassays which were reviewed in the earlier part of this chapter.

2.4 Critical review

Currently Hepatitis B virus detection is carried out using laboratory-based immunoassays. The Enzyme Linked Immunosorbent Assay (ELISA), although commonly used for Hepatitis B virus detection, has got some limitations. It is time consuming (typically 6-7 hours) and requires skilled personnel [91]. Since the conjugate is an enzyme-linked molecule, accuracy of the test results depends on the activity of the enzyme [91]. Hepatitis displays multiple clinical features and current diagnostic methods do not give complete profiling of the different serological variables with a single detection device [1].

The fiber optic sensor concept is a simple, sensitive and accurate one. However, translation of this technology to a realistic, practical and clinically functional biosensor is far from simple. In most cases, its dependence lay on the key factors that enable optical component fabrication, preparation of a sensitive biochemical sensing element and final development of the system assembly.

Over the years, several methods based on standard colorimetric assay have been developed to detect and quantify protein concentration in biological samples [75, 92]. The Lowry's method helps to measure protein concentration ranging from 50 to 1000 $\mu\text{g/ml}$ and in Bradford's method protein samples ranging from 50 to 200 $\mu\text{g/ml}$ of protein can be measured. However, each of these methods presents certain limitations pertaining to its sensitivity, accuracy and reproducibility. Bradford's assay is less sensitive to basic or acid proteins. Lowry's method is

sensitive to pH and salt concentration [93, 94]. The recent trend to improve biochemical analysis is to provide a platform, which is more sensitive, accurate, portable, rapid and economically viable. The fiber optic sensing technology is a promising tool to achieve this [95].

From reviews discussed in this chapter, it is evident that biosensor technique is a rapidly growing analytical technology for the detection and quantification of biomolecules. However, to date, there is no known mutiparametric biosensor system that can perform a rapid, quantifiable, multiple sensing of different biochemical variables for the detection of Hepatitis B virus. A multi -sensing fiber optic sensor will give better profiling of the serological markers, which will enable the physician to obtain better understanding of the liver damage. Moreover, it would allow a distinction to be made amongst persons who might benefit from antiviral therapy ($\text{HBsAg}^+ \text{HBeAg}^+$), those who are less prone to respond to antiviral therapy ($\text{HBsAg}^+ \text{HBeAg}^-$) and those who are in need of vaccination ($\text{HBsAg}^- \text{HBeAg}^-$). The development of the assay promises potential use of the biosensor for the rapid diagnosis of infection. Thus, the HBsAg/eAg test is a useful and appropriate instrument in the proactive management of the Hepatitis B disease. This will aid the physician to perform more successful treatments. Such a system will find tremendous application in health care sciences. It should be noted that hepatitis is a rapidly progressive condition, and occurs in geographical locations that usually lacks even a clinical diagnostic laboratory [5].

This project therefore aims to investigate and characterise a multi-parametric biosensor to detect cardinal clinical variables of HBV infection. The main advantages of the sensor system will be the ability to detect multiple parameters rapidly and sensitively. Towards this end, optic fiber technology was chosen, keeping in mind their geometric versatility, as well as their ability to provide sensitive and accurate information [61].

CHAPTER 3

A FIBER OPTIC EVANESCENT WAVE BIOSENSOR TO DETECT TOTAL PROTEIN

In this study a fiber optic-based protein sensor was developed to detect total protein content (immunological and non-immunological) in a liquid sample. The biosensor applies a dye-immobilised porous glass coating on a multi-mode optical fiber. The evanescent waves at the fiber optic core-cladding interface are used to monitor the protein-induced changes in the sensor element. The biosensor offers a single-step, method for quantifying protein concentrations without destroying the sample. This work was carried out as part of the study by considering the fact that during chronic hepatitis, there will be a drastic reduction of serum protein content. This sensor in conjunction with an immunosensor will give a better profile of the disease stage. Optimization of the fiber characteristics for this purpose includes: processing steps for the fiber, sol-gel preparation and fabrication of the fiber contouring device to improve profile of the fibers.

3.1 Introduction

The fiber optic total protein sensor developed in this study is based on the variation in the evanescent wave phenomenon at the core-cladding interface. Over the years, several methods based on standard colorimetric assays have been developed to detect and quantify protein concentration in biological samples [75, 90, 92, 96-100]. However, each of these methods presents certain limitations pertaining to sensitivity, accuracy, and reproducibility. There is thus a basic need to measure protein concentration simply and efficiently without large wastage. The recent trend to improve biochemical analysis is to provide a platform, which is more sensitive, accurate, portable, rapid and economically viable.

Optical fibers are versatile due to their geometrical versatility, remote-sensing capability, small dimension, low weight, availability and low cost [101]. The fiber optics-based biosensor can also facilitate the sensing of multiple parameters using a single optical fiber, without any crosstalk in real-time. The fiber optic total protein sensor introduced in this study is based on the variation in the evanescent wave phenomenon at the core-cladding interface. The theoretical formalization of these concepts such as evanescent wave, total internal reflection, V number etc can be found elsewhere [102] and is discussed in Chapter 2. In the biosensor design, an optical fiber is used as the transduction element. Concomitantly, a fluorescent recognition unit is used to generate an analyte-dependent, spectroscopically detectable signal within the sensing region of the optical fiber. The Nano orange, a fluorescent dye which is virtually nonfluorescent in aqueous solution, becomes

strongly fluorescent (emission ~ 610 nm) upon interaction with protein, when excited about 480 nm, was used in this study. The chemical change that occurs because of the interaction between the analyte and immobilized indicator are measured by monitoring the electromagnetic radiation that returns from the sensing unit. In this work, a fiber optic evanescent wave-based protein sensor to determine the total protein concentration in a liquid sample is reported.

3.2 Materials and methods

Optical fiber (600 μm diameter) (Thorlabs, USA), Nano orange, serum albumin, tetra ethyl ortho silicate (TEOS), ethanol, hydrofluoric acid (HF) (50%), formamide, borate buffer (pH 9), were used in this study. Reagents were purchased from Sigma Aldrich, USA. All chemicals were of analytical grade.

3.2.1 Fabrication of the Fiber optic total protein sensor

Five plastic clad silica fibers of length 1 m each were used to fabricate the protein sensor. They had a core diameter of 550 μm and a numerical aperture of 0.22. For each fiber, a length of 0.05 m of the outer protective sheath of the optical fiber was removed from the tip region using a microstripper (a mechanical stripper to remove plastic sheath from optical fibers). The uncovered region of fiber was then soaked in a 50 % (v/v) HF for ~ 10 to 20 minutes. The tip of the fiber was contoured using a contouring machine (Figure 3.1). The fiber contouring machine manufacturing was subcontracted to *Ultra Industrial Automation Pte Ltd.*

Singapore, based on our specific requirements. This was done mainly to avoid the V number mismatch as described in Chapter 2. The surface-prepared portion of the optical fiber was then rinsed with deionised water and air-dried at room temperature. Once dried, the etched portions of the optical fibers were treated with 1N HNO₃ for 10 min. This step activated the –OH groups on the surface of the glass core and, consequently improved the adherence of sol-gel layer with it.

Figure 3.1 (a) The setup for fiber contouring process.

Figure 3.1 (b) Photograph of the fiber contouring machine.

Figure 3.1 (c) Photograph of the fiber contouring machine.

3.2.1.1 Polishing stage

1. A 30 cm long optical fiber was cut from the main roll of fiber supplied by *Thorlabs* using a pair of pliers.
2. About 2 cm of the fiber was stripped from both ends using a microstripper (mechanical stripper).
3. Both ends were then polished using *Handimet®* 2 roll grinder to flatten the rough ends due to the cutting process in step 1.

4. An optical microscope was used to check the condition of the end surfaces. If rough surfaces were not obvious, the polishing process would be continued using a 6 micron *Mecaprex* self-adhesive polishing dish placed on the *Ecomet*® 6 variable speed grinder-polisher.
5. After 15 minutes, the fiber ends were again checked using the optical microscope at 400 times magnification. The polishing process would be repeated until no scratches were observed.
6. It is to be noted that water was used as the lubricant and the fiber was held in a jig throughout the polishing process.

3.2.1.2 Etching stage

In order to use optical fiber as biosensors, the fiber tip has to be exposed. The method used to treat the fiber is by hydrofluoric acid (HF) etching. Fibers were etched using a drawing machine with a draw speed control unit.

Procedure

1. The fiber was clamped to the contouring machine's clamp.

2. The drawing speed was set to 5mm/s initially. A total of 6 cycles were performed and the diameter of the fiber recorded for every cycle. A cycle is defined as a single downward-upward movement of the clamped fiber.
3. Step 2 was repeated with new fibers at the drawing velocity of 1.5mm/s, 2.5mm/s, 3mm/s and 4mm/s accordingly.
4. Tabulated data were used to plot the relationship between the diameter of fiber and the number of cycles at the 3 different positions.

3.2.1.3 Optimization of fiber fabrication parameters

Using the following equation, the value for the matching radius, r_{match} was calculated.

$$r_{match} = r_{cl} \sqrt{\frac{n_{co}^2 - n_{cl}^2}{n_{co}^2 - n_{aq}^2}} = 0.408 \text{ mm} \quad (15)$$

where, the radius of cladding, $r_{cl} = 0.600 \text{ mm}$

refractive index of core, $n_{co} = 1.457$

refractive index of cladding, $n_{cl} = 1.440$

refractive index of aqueous solution, $n_{aq} = 1.420$

The RI (refractive index) was obtained from the manufacture's data sheet.

As mentioned earlier, the radius of immersed fiber optic core has to be smaller than r_{match} in order to prevent the loss of fluorescence signal i.e. $r_{taper} < r_{match}$. In this work, the radii at position 1 and 3 are set to 0.350 mm and 0.396 mm respectively. The fiber probe shape was modelled after the continuous profiling.

Figure 3.2 Optimized dimensions (in mm) for the profiled fiber.

3.2.1.4 Continuous etching followed by profiling of fibers (Method #1)

Procedure

1. The optimized dimensions of a profiled fiber to counter V-number mismatch is given in Figure 3.2.
2. The time taken to etch a bare fiber to a diameter of about $400\text{ }\mu\text{m}$, t_e , was estimated by extrapolating the graph obtained in Figure 3.4.

3. After polishing and etching for t_e min, the fiber was fabricated at speed 5 according to the number of cycles that were required to obtain the specified diameters at the fiber end.
4. The appropriate data was graphically plotted.

3.2.1.5 Profiling followed by continuous etching of fibers (Method #2)

Procedure

1. After the initial 10 -20 min of etching and stripping off the thin film, the fiber was contoured for 1 cycle at the speed 5.
2. The diameters at all the 4 positions on the fiber were measured by a micrometer and tabulated.
3. The fiber was then etched for t_e min (the same time needed in Method #1) to obtain a bare fiber diameter of about 400 μm . Its diameter was measured and recorded at every 30 min interval.
4. The appropriate data was graphically plotted.

3.2.2 Sol-gel processing

The sol-gel technique was utilized to form a porous glass, thin-film coating around the cladding denuded portion of the optical fiber. Sol-gel is a process that enables materials to mix on a molecular level from a sol (liquid solution) into a colloidal gel. The moisture in the colloidal gel is removed and after drying the gel will become a solid with a high level of fine porosity. These fine porosity solids possess very high surface area and thus high surface free energy and this property enables the solid to be sintered/densified at much lower temperatures than normal processing of the same material. This process is used to coat the liquid chemical onto a sample surface (optical fiber or glassplate). After drying, the solidified chemical will be immobilised on the sample surface [103, 104].

Sol is defined as a stable dispersion of colloidal particles in an aqueous or organic liquid medium. When the particles in the sol are aggregated, the particles cross-link and form a continuous network structure with the solvent. The sol-gel helps to give a micro encapsulation to the dye, if we deposit dye directly dyes might get detached into the surrounding sample environment. The sol loses its mobility and ability to maintain its shape without a mold, this means the sol has become a gel. Sol is different from a solution as sol is a state of two different phases whilst a solution is a one-phase system. The gel is solid; usually soft with low elastic modulus. The composition and solid-to-liquid ratio of a gel is quite the same as the parent sol.

3.2.3 Alkoxide gels

The chemical process of alkoxide gel formation is hydrolysis and polymerisation of metal alkoxides, tetraethyl orthosilicate (TEOS).

Water is added to hydrolyse tetraethyl orthosilicate (TEOS). Since they are immiscible, alcohol is added to allow them to form a solution and an acid or a base solution is added to catalyse the hydrolysis reaction. The final form of the gel (silica) is highly dependent on the pH of the solution. At $\text{pH} < 7$ and with water: TEOS molar ratio 2:4, reaction (1) is completed in 10 min with significant heat exchange, while reaction (2) happens slowly (~ 45 min). Because there is not enough water to complete reaction (1), the additional water released in the course of the reaction (2) will aid to complete reaction (1). In general, at $\text{pH} < 7$, the rate of reaction (2) is limited by the lack of OH groups. There, polymerisation proceeds slowly and this leads to the growth of a three-dimensional network which traps water-alcohol solution in spaces of the gel. At high pH levels, an abundance of the OH group causes rapid termination of network growth resulting in the gel not being able to coagulate.

The gel is a highly solvated solid because water and alcohol are trapped within the gel due to the chemical reactions. The drying stage is to remove the water and alcohol that are trapped in the gel. As the drying takes place, the gel shrinks by a large amount until it becomes a solid with a high level of porosity. The rate of drying depends on the thickness of the sol-gel coating, all else being constant.

The preparation of the sol-gel was carried out at room temperature (24⁰ C) by the hydrolysis and condensation of TEOS in an acidic environment to form siloxane polymer leading to gelation [103]. The hydrolysis reaction proceeded by the replacement of the ethoxy groups in TEOS by the OH groups from water. The TEOS solution was prepared by modifying a previously described procedure [105]. The ratio of TEOS, deionized water, formamide and ethanol in this work, however, is 1.2 :1: 1.17: 1.4 v/v. Formamide was used as a drying control chemical additive [106].

The polymer at this consistency was used to coat the prepared, uncladded portion of the optical fiber using dip coating equipment. The precursor solution containing the indicator was placed in a 20 ml pipette. The surface prepared optical fiber, dried in a desiccator overnight was dipped into the pipette and drawn upwards using the dip coating equipment at a rate of 50 mm/min, at room temperature (~23⁰ C). The coated fiber was then placed in a petri dish and allowed to dry for a period of 2 weeks in a dark place. Prior to the experimentations with protein samples, the sensor portion was also examined to determine whether the Nano orange could be washed out of the porous glass matrix.

The experiments in this study were conducted in two stages. In the first stage, experiments were carried out in order to characterize the sensor, while in the second stage; experiments were conducted to evaluate the performance and response time.

3.2.4 Characterisation of the fiber optic protein sensor

In developing a quantitative method for determining an unknown concentration of a given species by fluorescent spectrometry, the first consideration is the choice of the excitation band and emission band at which measurements are made. The fluorescence spectrum of the species to be determined can be obtained experimentally by means of a fluorometer. Fluorescence at a given wavelength is an inherent characteristic of the absorbing species. The numerical value of the fluorescence will determine the slope of the analytical curve and will demonstrate the concentration range over which the determination can be made.

Figure 3.3 Schematic diagram of the experimental set-up.

Figure 3.3 shows the schematic of the experimental system employed in this study. A tungsten halogen lamp (Ocean Optics, USA) was used as the light source. An objective lens with a numerical aperture similar to that of the optical fiber was used to launch the light from the source into one end of the optical fiber. A beam splitter was used in order to bifurcate the light. A broadband pass-filter (Chroma filter with emission peak at about 610 nm) was introduced between the beam splitter and detector. This was connected with the fiber optics spectrophotometer (USB2000-FL Ocean optics, USA) which has a sensitivity of 86 photons/count (spectral range of 200-1100 nm) using a patch code fiber with 600 micron diameter (Ocean Optics, USA).

The spectrophotometer was used in the monitoring of photon variations at the end terminal of the optical fiber. The dye-immobilized porous-glass coated portion of the optical fiber was secured at the base of the sample container (cuvette) using plastic wedges. The cuvette is a black polypropylene tube with a capacity of 0.25ml. A few drops (0.2 ml) of liquid sample would be sufficient to have the sensor portion immersed completely. A dark reference (reading without light source) was also taken. Commercially available light couplers (Newport, USA) were used to get the maximum coupling efficiency. The power source was used only after getting a stable intensity profile and cooling fan was used in order to avoid the temperature depended intensity variation. In order to evaluate the performance of the sensor, the following experiments were carried out. The details of the experimental set up in given figure 3.3.

3.2.5 Optimization of Nano orange concentration

Optimization of Nano orange concentration was carried out so as to take into account the time dependence of fluorescent emission. A working dilution of 10X (X refers to the value given by the manufacturer, 1X= 1 μ g/ml) was prepared from a 500X Nano orange stock in 15 nM borate (pH 9.0) buffer. Various volumes of working dilution were added in the cuvettes containing 15 mM borate buffer and 10 μ g/ml of serum albumin to obtain different dye concentrations. Total assay volume was 200 μ l. A total of 33 biosensors were used with multiple tests for each of the sample size.

3.2.6 Nano orange fluorescence with varying serum albumin concentration

A calibration curve for serum albumin was constructed as follows. Various concentrations of serum proteins ranging from 1 to 10 $\mu\text{g/ml}$ were prepared by the serial dilution of the stock solution in 15 mM borate buffer (pH 9.0). The specially constructed cuvette was filled with 180 μl of serum protein followed by the addition of 20 μl of 1X Nano orange, which is entrapped in the sol-gel. For each testing, multiple repeats were tested using individual biosensors. A total of 12 biosensors were made and multiple tests were conducted for each of the sample size.

3.2.7 Effect of salt concentration on fluorescence emission

This was examined by preparing 15 mM borate (pH 9.0) solution with 20 $\mu\text{g/ml}$ serum albumin and 0 to 100 mM NaCl. Fluorescence emission was measured immediately after 1 min of incubation. A total of 18 biosensors were prepared with multiple tests for each of the sample size.

3.3 Results and discussion

3.3.1 Fabrication of the fiber optic total protein sensor

3.3.1.1 Optimization of fiber profiling parameters

3.3.1.1 (a) Continuous etching followed by profiling of fibers

By extrapolating the graph from Figure 3.4 it can be concluded that ~65 min is required to get diameter of ~400 μm . It was determined that total time $t_e = \sim 90$ min was needed (by including initial thin film removal time) to obtain a diameter of about 400 μm . Table 3.1 shows the tabulated mean diameter and standard deviation of the profiled fibers.

Figure 3.4 Etching of fibers using hydrofluoric acid.

Table 3.1 Etching of fibers using Hydrofluoric acid

Time (minutes)	Mean Diameter (mm)	Standard deviation (±) n=9
0	0.6245	0.004
10	0.593	0.004
20	0.534	0.001
30	0.504	0.003
40	0.474	0.003
50	0.444	0.004
60	0.4135	0.004

Using this timing, the fiber was etched and profiled to obtain the theoretically predetermined fiber. To check for consistency in the result, between 3 to 5 fibers were etched and profiled using the same parameter for statistical comparisons.

Figure 3.5 The effect of initial 90 min of etching followed by 4 cycles of profiling.

Figure 3.5 shows the effect of etching followed by tapering at the 4 positions of the fiber as shown in Figure 3.2. This result shows that the estimated time and a total of 4 cycles are reliable in producing fibers with the optimized dimensions at each of the positions.

3.3.1.1 (b) Fabrication of the fibers followed by continuous etching.

Table 3.2 Effect of initial 1 cycle profiling followed by etching times.

Cycles	Diameter of Fiber 1 (mm)			
	Positions			
	1	2	3	4
0	0.606	0.605	0.606	0.606
1	0.588	0.599	0.605	0.606
Time (min)				
30	0.500	0.507	0.512	0.522
60	0.435	0.438	0.449	0.469
90	0.369	0.374	0.383	0.420
Standard deviation (90 th minute)	0.004	0.00208	0.00458	0.00265

Cycles	Diameter of Fiber 2 (mm)			
	Positions			
	1	2	3	4
0	0.604	0.603	0.603	0.603
1	0.574	0.591	0.599	0.603
Time (min)				
30	0.499	0.508	0.516	0.523
60	0.432	0.439	0.452	0.508
90	0.372	0.379	0.399	0.420
Standard deviation (90 th minute)	0.002	0.00306	0.00321	0.00361

Cycles	Diameter of Fiber 3 (mm)			
	Positions			
	1	2	3	4
0	0.603	0.605	0.602	0.603
1	0.556	0.582	0.600	0.602
Time (min)				
30	0.482	0.499	0.511	0.519
60	0.435	0.447	0.462	0.478
90	0.368	0.379	0.402	0.427
Standard deviation (90 th minute)	0.00208	0.00289	0.01021	0.00404

From Table 3.2, by etching the fibers without the polymeric thin film coating, the 3 fibers which were profiled over 1 cycle and followed by a total of 90 min of etching generally gave similar diameters for the 4 positions. The diameters are approximately 0.37, 0.38, 0.40 and 0.42 mm for positions 1, 2, 3 and 4 respectively.

Table 3.3 Comparison between the data obtained from the two different methods used to fabricate the optimized fiber probes

Position	Average Diameter (mm)		Difference (mm)	Overall Average Diameter (mm)	Standard Deviation n=5	% Error
	Method	Method				
	#1T	#2T				
1	0.357	0.370	0.0130	0.364	0.009	2.529
2	0.372	0.377	0.00500	0.375	0.004	0.944
3	0.391	0.395	0.00400	0.393	0.003	0.720
4	0.412	0.422	0.0100	0.417	0.007	1.70

The average diameter for each position and method were calculated and tabulated in Table 3.3. Generally, positions 2 and 3 have about the same average diameters while positions 1 and 4 have a difference of about 0.01 mm in diameter.

The photograph in Figure 3.6 shows the profiled section of a fiber obtained from an Olympus optical microscope. As expected, there is a gradual decrease in the diameter of fiber due to profiling process. The full length of the profiled region is shown in Figure 3.7.

Figure 3.6 Profiled section of a fiber end.

d_3 and d_4 show the progressive decrease in diameter.

Figure 3.7 Combined pictures of a profiled fiber end taken by an optical microscope. The starting region of the profiled segment is indicated by the arrow.

Figure 3.8 Comparison of fluorescence detection between a bare and profiled fiber.

Table 3.4 (a) Fluorescent intensity of the bare fiber.

Concentration of the fluorescent dye (µg/ml)	Mean Optical Intensity (au)	Standard Deviation (±) n=9
0	1060.56	6.61
1	1109.12	3.10
3	1113.22	3.03
6	1139.28	2.10
10	1128.32	4.09

Table 3.4 (b) Fluorescent intensity of the profiled fiber.

Concentration of the fluorescent dye (µg/ml)	Mean Optical Intensity (au)	Standard Deviation (±) n=9
0	1554.75	3.80
1	2096.90	5.78
3	2578.49	9.46
6	3097.71	1.20
10	3551.68	4.64

Figure 3.8 shows the graph shows the fluorescent intensity change in the profiled and unprofiled fiber, when it interacts with the dye. It is observed that the profiled probe has a steeper gradient of 188.14 compared to that of unprofiled bare fiber. The sensitivity of the profiled fiber compared to the unprofiled fiber can be calculated as,

$$\frac{\text{gradient of profiled fiber}}{\text{gradient of bare fiber}} = \frac{188.14}{5.39} = 34.9$$

As expected, the profiled probe gave a much better detection of fluorescence (about 35 times better) compared to that of the unprofiled one. Table 3.4 (a) and (b) shows the tabulated data of mean optical density and standard deviation of the bare and profiled fiber respectively.

3.3.1.2 Sample calculation for fiber profile angle

Using the optical microscope software, the diameters at positions 4 and 1 of fiber 1 were obtained as $d_4 = 453.75 \mu\text{m}$ and $d_1 = 366.25 \mu\text{m}$ respectively. These values were substituted into Eq. 11 to obtain the corresponding profile angle, β (as shown in Figure 2.7):

$$\begin{aligned}
\beta &= \tan^{-1} \left(\frac{r_0 - r_L}{L} \right) \\
&= \tan^{-1} \left(\frac{(d_4/2 - d_1/2)}{L} \right) \\
&= \tan^{-1} \left(\frac{(453.75/2 - 366.25/2)}{5 \times 10^4} \right) = 0.05^\circ
\end{aligned}$$

where $L = 5 \text{ cm} = 5 \times 10^4 \mu\text{m}$

The profile angle for another fiber was also calculated in the similar way (0.055°). It has to be noted that the profile angles for both fibers 1 and 2 are very similar as they are made using the same optimized conditions. The error was about $\pm 0.005^\circ$. The profiling angles may vary slightly from fiber to fiber. Thus, it is crucial that the parameters used for each profiling process are consistent in order to minimize errors.

3.3.2 Characterization of the optical biosensor

Figure 3.9 (a) Excitation spectrum of Nano orange

RFU= (Relative Fluorescent Intensity)

Figure 3.9 (b) Emission spectrum of Nano orange. RFU = (Relative Fluorescent Intensity)

Spectral signatures of Nano orange were analyzed with serum albumin to determine the spectral wavelength selection. Figure 3.9(a) illustrates the excitation spectra obtained upon binding with serum albumin. Figure 3.9 (b) shows the emission spectrum of the Nano orange when it binds with serum albumin. Emission spectrum is given at about 610 nm and excitation spectrum is at about 480 nm. The large stokes shift allows the efficient spectral discrimination between excitation source and fluorescent emission resulting in a significant enhancement of the fluorescent produced in the presence of serum albumin. Nano orange's large emission band allows the utilization of commercially available filters while still obtaining microgram levels of protein.

It was noted that the sol-gel immobilized dye differed from that in solution state. The difference in the maximum absorption peak value between the sol-gel immobilized dye and liquid dye can be attributed to the difference in the pK value of the dye in solution form and that in the sol-gel matrix [107]. In order to avoid the cracking problem, the immobilization matrix formamide was used as a drying control additive agent.

3.3.3 Optimization of Nano orange concentration

Figure 3.10 Optimization of Nano orange concentrations for protein detection.

Table 3.5 Optimization of Nano orange concentration for the protein detection.

Nano Orange Concentration (X)	Mean Optical density (au)	Standard deviation (±) n=9
0	0	0
0.5	403.33	15.28
1	1104.67	13.61
1.5	1077.33	14.19
2	993.33	30.55
2.5	881.67	40.53
3	800	20
3.5	813.67	15.18
4	785.67	21.83
4.5	769	11.53
5	736.33	11.85

Development of cost effective assays for the detection of protein concentration is highly important in clinical laboratory settings. It was thus important to find the optimal concentration of Nano orange that can be used with assay buffer. A 10 $\mu\text{g/ml}$ of serum albumin was taken and allowed to react with Nano orange concentration ranging from 0 to 5X (X refers to the value given by the manufacturer, 1X= 1 $\mu\text{g/ml}$). Figure 3.10 represents the binding curve for different concentrations of Nano orange. Table 3.5 shows the tabulated mean optical density and standard deviation of the fluorescent emission of different concentration of Nano orange.

A concentration of 1X showed the highest optical output. A higher concentration resulted in a reduction in fluorescence. Quenching and the inner filter effects are believed to play an important role at these higher concentrations, which resulted in a reduction in intensity. Since the Nano orange provided by the manufacturer was at a 500X concentration, it is not possible to express the binding stoichiometry in terms of mole fraction. A 1X Nano orange concentration was thus used for the fiber optic biosensor assay.

3.3.4 Nano orange fluorescence with varying serum albumin concentrations

Figure 3.11 (a) Spectral signatures of Nano orange fluorescence with varying serum albumin concentrations.

Figure 3. 11 (b) Calibration curve for serum albumin using Nano orange.

Table 3.6 The fluorescent intensity at different concentration of serum albumin.

Concentration (µg/ml)	Mean value of Normalised Optical density (au)	Standard deviation (±) n=9
25	0.58	0.02
75	0.74	0.01
150	0.87	0.01
250	1	0

Figure 3.11 (a) shows the spectral signatures that were obtained by Nano orange. A wavelength of 480 nm was used as an excitation source and an emission peak was

found to be at a wavelength of ~610 nm. It was observed that fluorescent emission increases with increase in concentration of the serum albumin and a standard curve was constructed (Figure 3.11 (b)). A non-linear curve fitting was done. This non-linear curve has an r^2 value of 0.9939. The P value is less than 0.05, which shows that results are statistically significant. Concentrations of the order of microgram levels per ml were determined with the profiled fiber optic biosensor. In order to prove the repeatability, 12 biosensors were tested for each of the multiple repeats of the sample size. Table 3.6 shows the tabulated value of different concentration and mean value of normalised optical intensity. The results show that tests are repeatable.

3.3.5 Effect of salt concentration on fluorescence emission

Figure 3.12 The effect of salt concentration on fluorescence emission.

Table 3.7 The effect of salt concentration on fluorescence emission.

Concentration (mM)	Mean Normalised Optical Density (au)	Standard Deviation(±) n=9
10	1	0
20	0.51	0.03
40	0.29	0.03
60	0.22	0.01
80	0.19	0.02
100	0.17	0.02

Since bio-fluids contain high amounts of salt concentrations, a study was conducted to determine the effect of increasing salt concentration on Nano orange

fluorescence emission (Figure 3.12). The serum albumin at a concentration of 20 $\mu\text{g/ml}$ was used with 15 mM borate (pH 9.0) buffer. The sodium chloride (NaCl) concentrations ranging from 0 to 100 mM were added in an assay buffer. After a minute of incubation with Nano orange, a maximum fluorescent emission was found to be at a concentration of 10mM. This decreased gradually forming a plateau in the region from 60 to 100 mM. The results from this experiment suggest that in the presence of a salt, an optimal concentration can be obtained by diluting the bio-fluid so as to reduce the salt concentration to a range of around 10mM. Individual sensors were used for each of the multiple repeats of the sample size. Table 3.7 shows the tabulated values of salt concentration and mean optical density value of fluorescent emission at different concentration

3.4 Conclusion

A new sensor technique, based on fluorescent fiber optic spectroscopy for the rapid and sensitive detection of total protein, has been introduced². The contoured fibers were used in order to enhance the fluorescent signal. The profiling of optical fibers were optimized. Two methods were selected. Comparing Methods #1 and #2 used in contouring the optimized dimensions for the fiber, there was very little difference (about ± 0.010 mm) in the final diameter obtained for the 4 positions. The advantage of Method #2, however, is that it requires only 1 cycle of profiling instead of 4 cycles as with Method #1. This means that Method #2 requires lesser total profiling time than Method #1 to obtain the same optimized diameter and shape of a fiber. The sensitivity of the profiled fiber compared to the unprofiled fiber was calculated. As hypothesized, the profiled probe gave a much better detection of fluorescence (about 35 times better) compared to the unprofiled one. The profiling angle may vary slightly from fiber to fiber. Thus, it is crucial that the parameters used for each profiling process are kept consistent in order to minimize variations in the diameter of the fiber. The fiber optic total protein sensor enables single-step detection and quantification of microgram levels of total protein directly without destroying the sample. The entire assay was completed in around 2 minutes time compared with the conventional protein detection method which might take 30 minutes or more. The highly sensitive Nano orange assay and the fiber optic spectroscopy were utilized in this study to develop the novel biosensor. The evanescent waves from the optical fibers excited and sensed the presence of

² The author received the Far Eastern Economic Review's Young Inventor's Award (Finalist) 2003 for his submission "Fiber optic total protein sensor".

protein via fluorescent enhancement in the dye-immobilized porous-glass film along the cladding denuded section. The dye concentration was optimized for assay and a calibration curve was generated. The effect of salt concentration on the sensor performance was also studied. In the final testing, 12 separate biosensor sensors were used for each of the multiple repeats of the sample size tested, in order to prove the repeatability of the sensor. The selection of appropriate standard together with a rigorous validation of the analytical process can, in principle, solve the problems presented by the protein composition. It is important to note that such sensing methods cannot be employed to differentiate various proteins. This fiber optic-based sensor can be used to determine the level of total protein content in Hepatitis B patients to serve as an indicator of extent of liver damage. It can also be used to determine the specific enzymatic activity, which is normally expressed in terms of activity per milligram of protein.

CHAPTER 4

A FIBER OPTIC EVANESCENT WAVE IMMUNOSENSOR

In this chapter a fiber optic-based immunosensor system to detect the surface and envelop antigens from Hepatitis B virus, the etiological agents for hepatitis, is described. In this sensor design, specific antibodies were immobilised at the surface of an optical fiber that binds to the surface and envelop Hepatitis B antigens. This antigen-antibody complex can be detected by adding fluorescent tagged anti Hepatitis B antigens at the sensor region. This forms the basis for a sandwich fluoro-immunoassay at the fiber surface and helps in the detection of fluorescent complex bound to the fiber sensor. This sensor provides for a rapid and specific detection of the virus for an efficient screening of the disease condition. One of the major advantages of this sensor is that it enables the simultaneous detection of Hepatitis B surface antigen and Hepatitis B envelop antigen which would allow a distinction amongst people who might benefit from antiviral treatment (HBsAg^+ and HBeAg^+), those who are less prone to respond to antiviral therapy (HBsAg^+ and HBeAg^-), and also those who are in need of vaccination (HBsAg^- HBeAg^-).

4.1 Introduction

The immunoassay technique is the fastest growing analytical technology for the detection and quantification of biomolecules [16]. It takes advantage of the affinity binding between antibodies and the corresponding antigens that allow the detection of one of these. This affinity between antigen and antibody can be utilised for the detection of any one of them, even if it is present at very low concentrations in a complex biological matrix such as whole blood, serum and other biological fluids [16]. The unique capacity of antibodies to bind specifically to the analyte of interest is the key factor in their usefulness in immunosensor design. The higher the affinity of the antibody for the analyte, the better the sensitivity achieved. The selectivity, on the other hand, is a specific property of the antibody applied. Target antigens can be of viruses, bacteria, toxins, or any other molecule, organic or inorganic, that is antigenic, i.e. that induces an immunological response and can thus be recognised by an antibody [16].

In the present study, investigations were carried out to introduce an immunosensor for detecting Hepatitis B surface and envelop antigens. A variety of serological assays may be employed to differentiate the type of viral infection as well as differentiate between chronic and acute Hepatitis B virus (HBV) infection [1]. The most sensitive and specific methods used commercially in diagnosis is immunosorbent assays. These assays make use of specific antibodies against various HBV proteins and can detect HBsAg/HBeAg.

Most of the immunoassay kits currently available in the market are single use tests and which merely provide a positive or negative response [108]. Biosensors, which perform the rapid detection of biological agents, are being developed to meet the increasing demand for highly qualitative immunoassay [74] and this has been reviewed and discussed in Chapter 2 of this report. The fiber optic immunosensor fulfils this objective by combining the sensitivity provided by fluoro-immunoassay [109] with advances in fiber optic spectroscopy [110].

Optical fibers are ideal transducers. The fluoro-immunoassays performed with this fiber optic biosensor are distinctive in that the assay occurs in the evanescent wave region of an optical fiber. Light propagates in optical fibers by being totally internally reflected because of the difference in the indices of refraction between the core and the cladding material [111]. However, the electromagnetic field does not fall instantaneously to zero at the core-cladding interface. The total internally reflected light extends just beyond the core surface into the surrounding cladding. This phenomenon is called the evanescent wave. Because of the limited penetration depth of the evanescent wave ($\sim 100\text{nm}$), detection is a surface-sensitive measurement [112]. Silica composition of the fiber enables macromolecular conjugation via silanisation, they transmit optical signal with minimal loss, and they can be easily adapted from one antigen-antibody system to another. Fiber optic biosensors can thus serve as a promising tool for the rapid detection of antigens.

4.2 Materials and methods

4.2.1 Reagents

Analytical-grade reagents and distilled water were used to prepare all solutions (Sigma Aldrich, USA). The Hepatitis B surface antigen, polyclonal antibody against Hepatitis B surface antigen, Hepatitis B envelop antigen, anti Hepatitis B immunoglobulin G and anti Hepatitis B envelop immunoglobulin were purchased from Chemicon (Chemicon International, USA). The fluorescent dye Alexa-Fluor was purchased from Molecular Probes (Molecular Probes, USA). The Chemical was handled in Fume hood and antigens were diluted in Biosafety cabinet.

4.2.2 Sensor fabrication

Five plastic clad silica fibers of length 1 m each were used to fabricate the immunosensor. The core diameters were 550 μm and have numerical apertures of 0.22. For each fiber, a length of 0.05 m of the outer protective sheath of the optical fiber was removed from the tip region of the selected optical fibers. The uncovered region of fiber was then soaked in a 50 % (v/v) HF for 45 min. The tip of the fiber was profiled using a controller determined profiling machine. This was done mainly to avoid V number mismatch [72]. The surface-prepared portion of the optical fiber was then washed and dried at room temperature. Once dried, the etched portions of the optical fibers were treated with 1N HNO_3 for 10 min. This step activated the $-\text{OH}$ groups on the surface of the glass core.

4.2.3 Profiling of fiber

The experimental setup shown in Figure 3.1 was carried out in a fume hood. A retort stand was used to hold the test tube which contained the HF acid. The direction (upward or downward) of the draw was set by a switch located near the motor speed dial. Fabrication of fiber was done as described in Chapter 3.

Capture antibodies were immobilised onto the sensing region as follows.

4.2.4 Immobilisation techniques

4.2.4.1 APTS- dextran immobilisation

The probes were cleaned with sulphuric acid/hydrogen peroxide mixture for 10 min, and rinsed with distilled water. This was followed by cleaning in hot distilled water (90°C) for 15-20 min. The fiber was then dipped in Nitric acid (1N) for 10 min to activate the –OH group. The surface was modified by incubating the probes in 0.025 % APTS (Aminoxy propyl trimethoxy silane; Fluka, NY) for different periods of time (2-24 hours). The probes were again rinsed with acetone and distilled water probes were then kept desiccated at room temperature until use. Amine groups on the sensor surface were determined by the Bromo Phenol Blue (BPB) dye binding method. In this method, a stock solution of BPB was prepared by dissolving 10mg/ml DMF, 500 μ l of the stock solution was diluted in 50ml DMF. The slides were incubated for 30 minutes in this solution rinsed with

ethanol. The BPB forms a complex with amine groups [113]. This complex is stable against ethanol wash but can be removed by 20 % piperidine in DMF for 10 minutes. The optical density (OD) of the eluent was measured at 605 nm and used to quantify amine groups on the sensor by using a modified Beer-Lambert law.

$$\text{SNH}_2 \text{ [nmol/cm}^2\text{]} = \frac{\text{OD}_{605} \times V \times 10^6}{\epsilon_{605} \times A \times d} \quad (16)$$

The molar excitation coefficient for BPB is 918000 per (mol/l)/ cm. V is the volume of piperidine solution in ml, A is the area of the sensing region and d is the length of the optical path in the cuvette. The value was multiplied by 10^6 to obtain a result in nmol per cm^2 .

For dextran immobilization, different amounts of dextran (40, 70, 100 mg/ml) were dissolved in carbonate buffer and mixed with ethyl-3-[1-dimethylaminopropyl] carbomide (EDC) plus *N*-hydroxysuccinimide (NHS) for activation [114]. The APTS modified sensor surface was dipped in this solution and incubated for about 45 min to increase the interaction time. The probes were rinsed with running buffer for at least 10 min. Non-specific binding to the sensor surface was blocked using Phosphate Buffered Saline (PBS) containing Tween-20 (0.1%), casein (2mg/ml) and BSA (2mg/ml). Finally, the probes were incubated with the Hepatitis B antibody at 50 $\mu\text{g/ml}$ in PBS. The fiber probes were placed in PBS–0.1% sodium azide at 4⁰C for storage until use.

4.2.4.2 MTS-GMBS reference method

Capture antibodies were immobilised onto the core by the modified procedure of Bhatia and colleagues [111]. The probes were cleaned with Sulphuric acid for 30 min, rinsed with distilled water and followed by cleaning in hot distilled water (90°C) for 15-20 min. The fiber was then dipped in Nitric acid (1N) for 10 min to activate the –OH group. The surface was modified by incubating the probes in 2 % thio-terminal silane in toluene (Mercapto propyl trimethoxy silane; Fluka, NY) for two hours and then incubating the probes with the heterobifunctional cross-linker 2mM GMBS (N-succinimidyl 4-maleimidobutyrate, Fluka) in ethanol for one hour. Crosslinking agent was first dissolved in minimum amount of DMF (dimethyl formamide) and then with absolute alcohol with a final concentration of 2mM. Finally, the probes were incubated with the capture antibody at 0.05 mg/ml in PBS. The fiber probes were placed in storage in PBS–0.1% sodium azide at 4°C.

4.2.5 Evaluation of surface binding kinetics

The functionalised glass slides of 3mm thick by 9 mm wide and 28 mm long were used for both immobilisation methods. For each immobilisation method, two sets of slides were prepared; one with hepatitis antibody and the other with the control antibody which does not specifically bind to the Hepatitis B antigen (Immunoglobulin-G). Different concentrations of fluorescent conjugated antigens were used. For each concentration, fluorescent signals were measured using silica slides with Hepatitis B antibody and control antibodies. This difference, determined by spectrofluorometric measurements is proportional to the amount of fluorescent tagged antigens. Each measurement was performed on five slides and repeated thrice. This method was used to evaluate the amount of the active sites that specifically bind the antigen, and hence get a functional surface density. A bio-receptor has to be densely packed but more importantly it has to be active in target recognition.

4.2.6 Experimental set up

Figure 4.1 Schematic of the experimental set up.

Figure 4.1 represents the schematic of the experimental set up used in the study. A tungsten halogen lamp was used as the light source (Ocean Optics, USA). An objective lens with a numerical aperture similar to the optical fiber was used to launch the light from the source into one end of the optical fiber. The distal end of the optical fiber was connected to a fiber optic spectrophotometer (USB2000-FL, Ocean Optics, USA), which has a sensitivity of 86 photons/count (spectral range of 200-1100 nm). The CCD (Charged Coupled Device), along with the

spectrophotometer, aided in the monitoring spectral variation at the end terminal of the optical fiber. An emission filter (Band pass filter with 535/40nm, Chroma technologies, USA) was placed between the tip of the sensing end and the spectrophotometer in order to reduce the excitation light. A base line correction was done using phosphate buffered saline (PBS). A dark reference (reading with out light source) was also taken. Commercially available light couplers (Newport, USA) were used to get the maximum coupling efficiency. The power source was used only after getting a stable intensity profile and cooling fan was used in order to avoid the temperature depended intensity variation. A cuvette was placed at the tip of the sensing region. This was done in such a way that a few drops (0.25 ml) of sample would be sufficient to have the sensor portion immersed completely. The antibody was immobilised on the profiled region of the fiber.

4.2.7 Immunoassay

The sensing area of the fiber was immersed for 15 min in PBS containing 2 mg of casein per ml, 2 mg of BSA per ml, and 0.1% Tween-20 to minimise non-specific absorption. During this time, the fiber probe was continuously exposed to the excitation light in order to bleach the autofluorescent components of the fiber. To prevent photo-bleaching of the fluorescent complexes, the excitation beam was blocked at all times except during signal collection.

Fiber coated with capture antibody (50 $\mu\text{g/ml}$) was first immersed in blocking agent (Tween-20) for 15-20 min. This will prevent the non-specific binding. The

fiber was then incubated with successively increasing concentrations of hepatitis antigens in PBS (5 to 500 ng/ml) for 5-10 min. The fiber was then washed with PBS containing 0.1% Tween-20 for 5 min.

To prepare fluorescently labelled antibodies, 1 mg of each antibody (1mg/ml) was dialysed again in 50mM borate (pH9.3) 50 mM NaCl and was then dialysed overnight in the same buffer containing Alexa-Flour (Molecular Probes Eugene, Oregon) (0.01mg/ml). Free dyes were removed by gel filtration on Bio-Gel P10 (Bio Rad) equilibrated with PBS-0.1% sodium azide.

For signal generation, Alexa-Flour -IgG (5 μ g/ml) was added and incubated for 15 min. After that, the fiber was washed with PBS in order to wash out the unbound conjugate. The protocol described above constituted one test. Separate sensors were used for each test. A total of 27 biosensors were made for Hepatitis B surface and envelop antigen with multiple repeats for each of the sample size. Each assay required a 250 μ l sample volume, which was transferred into the cuvette with a pipette. The samples positive for Hepatitis B were also analysed in the same way.

4.3 Results and discussion

4.3.1 Fiber characterisation

Figure 4.2 SEM photograph of the capture antibody distribution on the glass surface.

Figure 4.2 shows the immobilised immunoglobulin distribution on the glass surface by the silanisation method. Captured antibodies were immobilised on the sensor surface by silane coupling.

4.3.2 APTS–dextran immobilisation.

To obtain a reactive surface for modification by amino group, sensor tips were treated with an amine group containing silane. Increasing the incubation time of APTS leads to an increase in the amine group on the sensor surface.

Figure 4.3 The effect of APTS incubation on reactive group formation.

Table 4.1 The effect of APTS incubation on reactive group formation.

Time (hours)	Mean Concentration (nmol/cm ²)	Standard Deviation (±) n=9
0	0	0
2	0.12	0.01
5	0.22	0.02
10	0.26	0.02
15	0.36	0.02
20	0.44	0.02
24	0.57	0.01
30	0.66	0.01
35	0.69	0.03

Figure 4.3 shows the effect of APTS incubation on reactive group formation. According to Vandenberg *et al* [115], silanisation of silicon samples with APTS and reaction conditions are comparable with the method used in this study. In this study an attempt was made to modify the surface with amine groups that allow covalent attachment of dextran. High amine coverage could lead to frequent links between the surface and dextran resulting in inflexible layers with low mesh size. This could prevent an easy access and exchange of buffer and samples. Therefore, a low APTS concentration of 2 hours resulting in low amine coverage was chosen. Blocking the free amine groups on the surface of the sensor by dextran could eliminate the positive charges, which could increase unspecific binding events. By using coupling reagents EDC/NHS more dextran could be immobilised. However, increasing in dextran concentration did not lead to a further increase in surface coverage. It can therefore be concluded that higher concentrations of dextran lead to different conformation of the dextran layer resulting in a higher inhibition of

unspecific binding. Table 4.1 shows the mean amine group concentration and standard deviation.

4.3.3 Evaluation of surface densities

Five different concentrations were used. At each concentration, the surface density of the occupied binding sites were evaluated using Langmuir adsorption model proposed by Eddowes [116]. In this model surface density of the occupied binding site or the antigen surface density is given by the product of the total number of antibody binding sites, τ and the fraction of the sites occupied by antigens θ .

We can consider the reaction between antigens, A, in a solution and its complementary antibody immobilized on a surface. The rate, ν , in its units of moles/ms of the adsorption is given by :

$$\nu_a = K_a [A] \Gamma_{\text{lim}} (1 - \theta) \quad (17)$$

And desorption is give by

$$\nu_d = K_d \Gamma_{\text{lim}} \theta \quad (18)$$

where k_a ($\text{M}^{-1} \text{s}^{-1}$) and k_d s^{-1} are the rate constants for adsorption and desorption respectively, $[A]$ is the concentration of antigens in the solution, Γ_{lim} moles/ m^2

represents the density of the binding sites on the surface and θ is the fractional coverage of the surface. At equilibrium the adsorption and desorption rate balance, by equating (17) and (18), an expression relating to the equilibrium fractional surface coverage θ_{eq} , to the dissociation constant, $K = k_d/k_a$ is obtained. The antigen concentration is therefore,

$$\theta_{eq} = \frac{[A]_{eq}}{([A]_{eq} + K)} \quad (19)$$

Or in reciprocal form:

$$1/\theta_{eq} = 1 + \frac{K}{[A]} \quad (20)$$

This is the Langmuir adsorption isotherm for the equilibrium surface coverage, θ_{eq} as a function of normalized concentration, $[A]/K$.

If an amount of antigen, $\theta_{eq} \Gamma_{lim} S$ (moles), adsorbs on an area S (m^2) from a volume, V (l) to leave an equilibrium concentration, $[A]_{eq}$, in solution, the depletion of the solution concentration will be simply $\theta_{eq} \Gamma_{lim} S / V$. The initial and equilibrium $[A]_i$ will be related by:

$$[A]_i = [A]_{eq} \left[1 + \frac{\Gamma_{lim} S}{V([A]_{eq} + K)} \right] \quad (21)$$

Total quantification approach may be appropriate if the total amount of antigen in the sample is less than that required for complete surface coverage, $[A]_i V < \Gamma_{\text{lim}} S$. Provided the initial antigen concentration is much higher than the dissociation constant, $[A]_i \gg K$, the antigen will bind to the surface until effectively all the antigen initially in solution is equivalent to the fractional surface coverage. Thus, the region where $\theta < 1$ will be given by:

$$\theta = [A]_i \frac{V}{S \Gamma_{\text{lim}}} \tag{22}$$

The surface densities of the antibody sites available for binding with antigens are tabulated in Table 4.1.

Table 4.2 The surface densities of the antibody sites available for binding with antigens.

Immobilization method	Density of the binding site τ (pmol/cm^2) $n=3$
APTS-dextran	0.48
APTS	0.31
MTS-GMBS	0.40

The APTS-dextran silanisation method was the most promising method and it was adopted for further studies. The immobilisation in dextran gave very good results

and can be attributed to the fact that antibodies were able to be organised in a polymeric network of hydro-gel formed on the sensor surface, instead of arranging in a surface monolayer subjected to steric hindrances. The value τ for the reference method (MTS-GBMS) is higher than those reported in literature (0.39 pmol/cm^2) [111]. This might be due to the surface irregularities and many other considerations such as impurities in the silica slides, which could result in a different geometrical area for binding antibodies.

4.3.4 Immunoassay

Figure 4.4 Spectral signatures obtained using different surface antigen concentrations and Hepatitis B positive sample.

Figure 4.5 Standard graph for the surface antigen.

Table 4.3 The fluorescent intensity at different concentration of surface antigen.

Concentration (ng/ml)	Mean Intensity (au)	Standard Deviation (±) n=9
5	57.78	1.45
50	69.27	1.08
200	87.63	1.16
350	111.02	1.75
500	121.61	1.73

Figure 4.6 Spectral signatures obtained using different envelop antigen concentrations and Hepatitis B positive sample.

Figure 4.7 Standard graph for the envelop antigen.

Figure 4.4 shows the fluorescent intensity obtained when measuring the different concentrations of the surface antigens. There was a significant increase in the fluorescent intensity corresponding to the increase in the concentration of the antigen. Figure 4.5 shows the standard graph obtained from the spectral signatures.

This relationship fitted well with the Michaelis-Menten equation, $y = \frac{A * X}{X + B}$ where A and B are constants. This non-linear curve has an r^2 value of 0.97. The Michaelis-Menten constant B has a value of 5.791ng/ml and intensity constant A, got the value of 104.4 au. The P value is less than 0.05, which shows that results are statistically significant. The concentration of surface antigen in the test sample was at ~ 300ng/ml. Similarly, Figure 4.6 shows the spectral signatures obtained for different concentrations of envelop antigens and Hepatitis B positive test samples. Table 4.3 shows the mean optical intensity and standard deviation of different surface antigen concentration.

Table 4.4 The fluorescent intensity at different concentration of Envelop antigen.

Concentration (ng/ml)	Mean Intensity (au)	Standard Deviation (±) n=9
5	54.06	1.65
50	67.91	1.41
250	87.61	0.95
350	107.51	0.87

The standard graph in Figure 4.7 was used to find out the concentration of envelop antigen. This also fitted well with the Michealis-Mention equation and this non-linear curve had an r^2 value of 0.96. The Michaelis-Menten constant B has a value of 3.646 ng/ml and intensity constant A, got the value of 90.4 au. The P value is less than 0.05, which shows that results are statistically significant. The concentration of envelop antigen in the test sample was at ~ 157 ng/ml. Table 4.4 shows the mean fluorescent intensity at different concentration of envelop antigen.

The deviation from the Michaelis-Menten equation fit line and the experimental results in the graph 4.5 and 4.7 could be because of the background antibody absorption on the sensor platform. Although, blocking buffers (0.1% tween in PBS) were used to reduce the background absorption present at an intensity of 0ng/ml of the antigen, there could be some background absorption intensities due to passive physical adsorption on the sensor surface. It could contribute an intensity variation compared with an ideal antigen-antibody complex intensity [91]. Such an effect can be minimised by the selection of appropriate standard range and dilution factors of antigens [9].

The detection of hepatitis antigens by the fiber optic biosensor was due to the formation of fluorescent complexes on the fiber's surface. These complexes were formed when immobilised antibody was bound by hepatitis antigen, which was subsequently bound by a fluorescently labelled antibody. The Alexa-Flour was used as a fluorescent label, which displayed an emission peak at around 521 nm. Alexa-Flour exhibited brighter fluorescence and greater photostability compared to the conventional organic dyes [117]. The dye is water soluble and pH insensitive from pH 4-10, which will aid us to work with almost all biological environments [117]. In this assay, a minimum level of detection at 5 ng/ml was achieved. A diagnostic procedure which allows the discrimination between infected and non-infected persons within minutes would facilitate and add to the success of a proactive approach toward Hepatitis B disease management. Table 4.4 shows the mean optical intensity and standard deviation of different envelop antigen concentration. Separate sensors were used for each of the tests, with multiple repeats of each of the sample size to prove the repeatability. A total of 27 biosensors were made for measuring Hepatitis B surface and envelop antigens. The results presented in table 4.3 and 4.4 shows that tests are repeatable.

An assay that enables the simultaneous detection of Hepatitis B surface antigen (HBsAg) and Hepatitis B envelop antigen (HBeAg) would allow a distinction to be made amongst persons who might benefit from antiviral therapy ($\text{HBsAg}^+ \text{HBeAg}^+$), who are less prone to respond to antiviral therapy ($\text{HBsAg}^+ \text{HBeAg}^-$) and those who are in need of vaccination ($\text{HBsAg}^- \text{HBeAg}^-$). The development of the assay promises a potential use of the biosensor for the rapid diagnosis of

infection. Thus, the HBsAg/HBeAg test is a useful and appropriate instrument in the proactive management of the Hepatitis B disease. Although in this method, multiple reagent additions lengthen the assay time to almost one hour, it was still significantly lower than the conventional method which takes about 6-7 hours to complete the entire reaction [91]. Similarly, if one were to screen mostly negative samples, each one could be analysed fairly quickly with no need to replace the optical probe. In a practical application, this would decrease the sensing operation time and cost.

4.4 Conclusion

A novel immunosensor system was developed for the simultaneous detection of Hepatitis B surface and envelop antigens, which showed itself to be a fast and sensitive method. This assay enables simultaneous detection of Hepatitis B surface antigen (HBsAg) and Hepatitis B envelop antigen (HBeAg). It would allow a distinction to be made amongst persons who might benefit from antiviral therapy ($\text{HBsAg}^+ \text{HBeAg}^+$), those who are less prone to respond to antiviral therapy ($\text{HBsAg}^+ \text{HBeAg}^-$), and those who are in need of vaccination ($\text{HBsAg}^- \text{HBeAg}^-$). The development of the assay promises potential use of the biosensor for the rapid and single step diagnosis of the infection. Thus, the HBsAg/HBeAg test is a novel, useful approach in the proactive management of the hepatitis. Fluorescent optrodes have a wide variety of prospective applications in clinical fields, which often require greater sensitivity or speedy diagnosis. The Alexa-Flour was used as a fluorescent label, which has an emission peak at around 521 nm. Alexa-Flour exhibits brighter fluorescence and greater photostablility compared to the

conventional organic dyes. The dye is water soluble and pH insensitive from pH 4-10, which will aid us to work with almost all biological environments [117]. A sensitivity level down to 5 ng/ml was achieved with the sensor. This concentration of surface antigen was within the range that is normally present in Hepatitis B positive blood samples. In the initial phase of infection, the surface antigen will be of the order of ng/ml (4th week onwards). In the later phase, it will be of the order of $\mu\text{g/ml}$ (12th week onwards) [1], this re-emphasises that the novel system meets the ideal sensitivity for the Hepatitis B diagnosis. Multiple repeats for each sample were tested using individual biosensors and results were repeatable.

Novel sensor characterisation methods were carried out and compared with current methods. A simple and fast process for coating antigen on the sensor surface was introduced. The most time consuming step was silanisation (around 2 hours). However, this can be done for several fibers at a time. This can be stored in a desiccator at room temperature without further treatment. Surface binding kinetics of the antibody was examined and APTS-dextran based method was found to be promising for antibody immobilisation via silanisation. The entire assay experiment was completed within an hour's time. This is significantly lower than the conventional time taken with ELISA methods, which typically require about 6 to 7 hours [91].

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS FOR FUTURE STUDIES

This work describes a rapid, simple to use fiber optic-based biosensor system to detect different serological variables associated with Hepatitis B virus infection. There are 350 million people worldwide who are Hepatitis B carriers. Hepatitis is an inflammatory disease of the liver that can present serious complications ranging from partial liver damage to liver cancer. Early diagnosis of this disease condition has been established as vital to prevent irreversible liver damage and to save the lives of patients. It has been well established that Hepatitis B infection causes cardinal serological changes in blood, which include the presence of Hepatitis B surface antigen (HBsAg), Hepatitis B envelop antigen (HBeAg) and serum protein level. Current diagnostic methods for Hepatitis B are laboratory-based assays, expensive, time consuming and require skilled personnel. A novel fiber optic-based fluoro-immunosensor system to detect the antigen from Hepatitis B virus, the etiological agent for hepatitis, has been investigated. A method of immobilizing the polyclonal antibodies onto Dextran-APTS modified optic fibers was introduced in this work. A detection sensitivity level in the order of 10^{-9} g/ml was achieved with the sensor. The detection time was less than 1 hour, which is significantly lower, compared to the conventional sensing methods that take about 6 to 7 hours. A total of 39 biosensors were made, with multiple repeats for each sample size prepare, in order to prove the repeatability of the sensor.

The conclusions drawn from the experiments conducted in this research are listed as follows:

Conclusions made from protein sensor work:

A new sensor technique, based on fluorescent fiber optic spectroscopy for the rapid and sensitive detection of total protein, has been introduced³. The fluorescence signal was enhanced by fabricating the fibers so as to overcome V-number mismatch, thereby providing increased sensitivity of one to two orders of magnitude.

The profiling of the optical fiber was optimized. Two methods were selected and compared. Methods #1 and #2 used in contouring the optimized dimensions for the fiber, did not result in much difference in the final diameter (only about ± 0.010 mm). The advantage of Method #2, however, is that it requires only 1 cycle of etching instead of 4 cycles in Method #1. This means that Method #2 requires lesser total profiling time than Method #1 to obtain the same optimized diameter and profile of the fibers.

The sensitivity of the profiled fiber compared to the unprofiled fiber was calculated. As expected, the profiled probe gave a much better detection of fluorescence (about 35 times better), compared to the unprofiled one.

³ The author received the Far Eastern Economic Review's Young Inventor's Award (Finalist) 2003 for his submission "Fiber optic total protein sensor".

The fiber optic total protein sensor enables single-step detection and quantification of microgram levels of total protein directly without destroying the sample. The highly sensitive Nano orange assay and the fiber optic spectroscopy were utilized in this study to develop the novel biosensor. Sensitivity levels down to 25 μ g/ml were obtained.

The dye concentration was optimized for the assay and a calibration curve was generated. Sol-gel technique was used to immobilise dye at the sensor region. The effect of salt concentration on the sensor performance was also studied, as most biological fluids would contain salts. Influence of other buffer systems can be studied as a future research to expand the scope of this method in other areas of protein research.

This protein sensor was developed as part of the study by considering the fact that during chronic hepatitis, there will be a drastic reduction of serum protein content. This sensor in conjunction with an immunosensor will give a better psychological profile of the disease stage.

The selection of an appropriate standard together with a rigorous validation of the analytical process can, in principle, solve the problems presented by the protein composition. This fiber optic-based sensor can be used to determine the level of protein content of Hepatitis B patients, which is an indicator of extent of liver damage. It can also be used to determine the specific activity of enzymes, which is normally expressed in terms of activity per milligram of protein.

The adaptation of Nano orange method in an automated platform may extend the use of this sensor in high through put screening. The investigation of Nano orange in lab on a chip can be done to extend its scope in proteomics research.

Conclusions drawn from fiber optic immunosensor study:

The utilisation of immunosensors in clinical analysis could help to improve the quality of analytical information due to the high precision and reliability provided by direct determination, without any prior separation of the analyte. The selection of transducer and immobilisation procedure plays an important role in the precision of measurement, sensitivity and selectivity.

A novel immunosensor system was introduced for simultaneous detection of Hepatitis B surface and core antigens. The system showed itself to be a fast and sensitive method. This assay enables simultaneous detection of (HBsAg) and (HBeAg). The new system would allow a distinction to be made amongst people who might benefit from antiviral therapy ($\text{HBsAg}^+ \text{HBeAg}^+$), those who are less prone to respond to antiviral therapy ($\text{HBsAg}^+ \text{HBeAg}^-$), and those who are in need of vaccination ($\text{HBsAg}^- \text{HBeAg}^-$). The development of the assay promises the potential use of the biosensor for the rapid diagnosis of this infection. Thus, the HBsAg/eAg test is a useful and appropriate instrument in the proactive management of the Hepatitis B disease.

Sensor characterisation methods were carried out and compared with current methods. A simple and fast method for coating antigen on the sensor surface was researched. The most time-consuming step was silanisation, which is around 2 hours, and can be carried out on several fibers at a time. Surface binding kinetics of the antibodies were examined and the APTS-dextran-based method was established to be a promising tool for antibody immobilisation via silanisation.

Since fluorescent-tagged conjugate antibodies were used, problems associated with the enzyme conjugate antibodies can be avoided. In enzyme conjugated antibody, the specificity of the reaction depends on the activity of the enzyme and the substrate. Fluorescent optrodes have a wide variety of prospective applications in clinical fields, which often require greater sensitivity or speedy diagnosis. The Alexa-Fluor was used as a fluorescent label and it exhibits brighter fluorescence and greater photostability compared to the conventional organic dyes [117]. The dye is water soluble and pH insensitive from pH 4-10, which will aid us to work with almost all biological environments [117].

A sensitivity level down to 5 ng/ml was achieved with the sensor. This concentration of antigens was within the range that is normally present in Hepatitis B positive serological samples. This is stated on the clinical basis that in the initial phase of infection, the surface antigen will be of the order of ng/ml (4th week onwards). In the later phase, it will be of the order of $\mu\text{g/ml}$ (12th week onwards) [1].

The novel sensor could detect the antigens rapidly within one hour. This compares very well to the conventional methods such as ELISA, which may take about 6 to 7 hours. Future studies can be done to further reduce the assay time by adapting automated assay platform.

A total of 39 sensors were made, with multiple repeats of each sample size for each biosensor. Results show that sensor is repeatable.

Non-specific binding of the antibodies, which can result in false positives and cross linking, can be prevented. This is possible because of the use of the site-specific binding in this work. Blocking the free amine groups on the surface of the sensor by dextran could eliminate the positive charges, which in turn could increase unspecific binding events. The immobilisation in dextran gave a very good result compared to the current ELISA method where simple physical adsorption is used. This is due to the fact that the antibodies can arrange themselves in a polymeric network of hydro-gel formed on the sensor surface as part of this work, instead of arranging themselves in a surface monolayer subjected to steric hindrances.

The assay of analytes in biological fluids other than serum samples such as plasma, whole blood, saliva etc can be studied as there are concerns due to their complex matrix, this would further open up the scope of the sensor. The use of non-organic dyes such as quantum dots can be studied as secondary antibody conjugate for the further sensitivity enhancement of the immunosensor. The other immobilisation matrices such as conducting polymers can be studied as a future extension of the

project and a comparison with current method can be made in terms of sensitivity. The sensitivity of the immunosensor can be further enhanced by using monoclonal antibodies compared with the polyclonal antibody which was used in this study. One of the major disadvantages of monoclonal antibody is its high production cost, but mass production could bring down the product cost. So depending on the end user's requirement, choice of the antibody can be made.

Mass production of cheap disposable chips, the possibility for real-time measurements and the option for multianalyte analysis, will probably bring this technique into the realm of practicability for diagnostics. This will help to focus on areas where the commercialization will probably be successful.

The traditional pathogen detection methods, although sensitive enough, are often too slow and laborious. The more rapid detection of etiological agents, such as Hepatitis B virus, would increase the chances of successful therapy. Detection of both immunological and non-immunological markers seems to be promising in the diagnosis of Hepatitis B which exhibits multiple serological markers. A successful method for the detection of serum protein and hepatitis antigens were investigated in this research. Immunoreactions are recognized for their high sensitivity and selectivity. This is the main reason to select immunochemical methods for clinical analysis. The utilization of immunosensor in the place of other immunochemical methods simplifies the analysis considerably, making it rapid and reliable. Thus, the role of biosensor in medical diagnostics is important and challenging. The

following are some of the suggestions for future studies which can be used to extend the scope of research work described in this thesis.

Some of the future works are suggested as follows:

1. The successful adaptation of the Nano orange method in total protein sensing to the automated platforms will likely lead to more rapid procedures for both medical diagnostic and research laboratories. This will help to screen more number of samples in one lot.
2. Possibility of using Nano orange in a microchip with nanotechnology can be explored. This extends its scope as it can be used for other biological assays, for example, enzymes for specific activity measurement, etc.
3. Additional protocols can be exploited in order to expand the scope of Nano orange as a universal labelling agent, for example, on column labelling of the dye in capillary electrophoresis. This would further open up its scope to do a real-time detection of proteins in capillary electrophoresis.
4. Further studies can be carried out to reduce the sol-gel preparation time. Investigations can be made to see any further catalytic agents can be applied to reduce sol-gel formation and dye encapsulation time.

5. The biomolecule immobilisation step is critical in the development of any sort of biosensor. It provides the core of the biosensors and gives its identity. More over immobilised biomolecule need to keep its original functionalities far as possible in order for the biosensor to work. The other types of surface characterisation of the sensor element can be explored, for example, the use of conducting polymers such as poly-pyrrole in the immunosensor to make a comparison with the current method. Special attention can be focused on improving the specific and regenerable bioaffinity interfaces.

6. Other immunoassay formats such as competitive immunoassay can be studied apart from the sandwich model, for comparison in terms of sensitivity and selectivity. The use of monoclonal antibodies against HBsAg and HBeAg can be investigated to study the sensitivity and selectivity enhancement.

7. Apart from the conventional organic conjugate that was used in this study, further investigations can be done on utilisation on inorganic dyes such as quantum dots and its sensitivity studies can be compared with the current conjugate used in this research.

8. The testing of the samples from different geographical regions can be carried out in order to validate the assay.

9. Complete automation of assay can be explored as it could help in the mass screening of the disease state in an epidemic breakout. The automation will also

help to get rapid results in scenarios such as bedside or chairside monitoring of patients.

10. Recognition elements other than antibodies can be investigated as future studies, such as molecular beacons (Oligonucleotide probes) against Hepatitis B genome. Immobilisation methods, detection platforms etc. have to be optimised for this study. This would help to further enhance the sensitivity of the sensor to genomic level.

Advancements in nanotechnology will help for a further development of biosensors particularly in proteomics and cellomics [118, 119]. A sophisticated immunosensor systems may be successful in the clinical laboratory [120, 121]. There is a great opportunity for introducing biosensor devices, since clinical diagnostics represent a huge, well-established and important analytical field. The mass production of sensor chips and the possibility for real time measurements will bring this technology into the realm of practicability for diagnosis. The future goals for immunosensor include immunological assays for patients in ambulatory settings, laboratory automation therapeutic vaccine effectiveness testing and also endocrinological monitoring. Further improvement of the laboratory services and also cost effectiveness are to be considered while implementing modular-designed laboratory automation [122].

During the research candidature, the author also made the following contributions:

Journal papers:

1. P. V. Preejith, C S. Lim, A. Kishen, M. S. John , A. Asundi, “Total protein measurement using a fiber-optic evanescent wave-based biosensor”, *Biotechnology Letters* **25**, 105–110, (2003).
2. P. V. Preejith, C. S. Lim, T F Chia “Serum albumin measurement using a tapered fluorescent fiber-optic evanescent wave-based biosensor.” *Measurement science and Technology* **17**, 3255-3260, (2006).

Conference paper:

3. P. V. Preejith, C. S. Lim, A. K. Asundi and T. F. Chia (2004). “Biomass determination using a fiber optic-based technique”. International Conference: Optics & Photonics in Technology Frontier, Chiba, Japan.

Awards:

4. The author received the Far Eastern Economic Review’s Young Inventor’s award (Finalist) 2003 for his submission “Fiber optic total protein sensor”. This is an international award where 780 submissions were selected to represent their respective institutions worldwide.

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APPENDIX

Raw Data

1. Etching of Fibers using Hydrofluoric acid.

Time	Diameter (mm) Data 1 n=5	Diameter (mm) Data 2 n=5
0	0.627	0.622
10	0.59	0.596
20	0.535	0.533
30	0.506	0.502
40	0.476	0.472
50	0.447	0.441
60	0.416	0.411

2. Effect of initial 90 minutes of etching followed by 4 cycles of profiling.

	Cycles	Diameter (mm)	Diameter (mm)
Position 1	1	0.383	0.385
	2	0.375	0.376
	3	0.365	0.366
	4	0.357	0.357
Position 2	1	0.384	0.382
	2	0.38	0.38
	3	0.379	0.375
	4	0.374	0.37
Position 3	1	0.392	0.391
	2	0.392	0.39
	3	0.392	0.39
	4	0.392	0.389
Position 4	1	0.416	0.411
	2	0.414	0.41
	3	0.415	0.41
	4	0.413	0.41

3. Comparison of fluorescence detection between a bare and a tapered fiber.

3.1 Bare Fiber

Concentration of the fluorescent dye($\mu\text{g/ml}$)	Optical Intensity (au) Data 1 n=3	Optical Intensity (au) Data 2 n=3	Optical Intensity (au) Data 3 n=3
0	1067.615	1059.54	1054.52
1	1111.879	1109.83	1105.79
3	1116.255	1110.2	1113.2
6	1140.956	1139.95	1136.92
10	1132.698	1127.67	1124.6

3.2 Tapered Fiber.

Concentration of the fluorescent dye($\mu\text{g/ml}$)	Optical Intensity (au) Data 1 n=3	Optical Intensity (au) Data 2 n=3	Optical Intensity (au) Data 3 n=3
0	1557.42	1556.42	1550.4
1	2100.294	2100.18	2090.22
3	2589.18	2575.12	2571.18
6	3098.406	3096.32	3098.4
10	3554.396	3554.32	3546.32

4. Optimization of Nano Orange concentration.

Nano orange concentration (X)	Optical Intensity (au) Data 1 n=3	Optical Intensity (au) Data 2 n=3	Optical Intensity (au) Data 3 n=3
0	0	0	0
0.5	420	400	390
1	1094	1120	1100
1.5	1062	1080	1090
2	1020	1000	960
2.5	880	842	923
3	820	800	780
3.5	800	830	811
4	790	762	805
4.5	770	757	780
5	750	730	729

5. Standard graph for Nano orange.

Concentration of the protein (µg/ml)	Normalized Optical Intensity (au) Data 1 n=3	Normalized Optical Intensity (au) Data 2 n=3	Normalized Optical Intensity (au) Data 2 n=3
25	0.59921	0.58605	0.56872
75	0.73449	0.75424	0.74141
150	0.88072	0.85136	0.86411
250	1	1	1

6. Effect of salt concentration on fluorescence emission.

Salt concentration mM	Normalized Optical Intensity (au) Data 1 n=3	Normalized Optical Intensity (au) Data 2 n=3	Normalized Optical Intensity (au) Data 2 n=3
10	1	1	1
20	0.5	0.4871	0.543
40	0.28846	0.26	0.3088
60	0.22596	0.2202	0.21
80	0.18269	0.1611	0.1911
100	0.19231	0.1723	0.1561

7. Effect of APTS incubation on reactive group formation.

Time (hours)	Amine group (nmol/cm ²) Data 1 n=3	Amine group (nmol/cm ²) Data 2 n=3	Amine group (nmol/cm ²) Data 3 n=3
0	0	0	0
2	0.11	0.13	0.12
5	0.22	0.23	0.2
10	0.26	0.28	0.25
15	0.37	0.34	0.36
20	0.45	0.42	0.46
24	0.57	0.55	0.58
30	0.64	0.66	0.67
35	0.68	0.7	0.71

8. Standard graph for surface Antigen.

Concentration (ng/ml)	Optical (au) Intensity Data 1 n=3	Optical (au) Intensity Data 2 n=3	Optical (au) Intensity Data 3 n=3
5	58.01	56.2	59.11
50	69.29	68.18	70.34
200	88.9	87.4	86.6
350	109.66	113	110.4
500	122.64	122.57	119.61

9. Standard Graph for Envelop Antigen.

Concentration (ng/ml)	Optical (au) Intensity Data 1 n=3	Optical (au) Intensity Data 2 n=3	Optical (au) Intensity Data 3 n=3
5	55.23	52.9	55.7
50	67.43	66.8	69.5
250	87.24	86.9	88.7
350	107.14	108.5	106.9