ARRAY BIOSENSOR FOR THE DETECTION OF ORGANOPHOSPHATES

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ARRAY BIOSENSOR FOR THE DETECTION OF ORGANOPHOSPHATES

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ARRAY BIOSENSOR FOR THE DETECTION OF ORGANOPHOSPHATES

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THESIS ABSTRACT

ARRAY BIOSENSOR FOR THE DETECTION OF ORGANOPHOSPHATES

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The aim of the current study was to develop an optical-based array biosensor for enzyme kinetics monitoring by fluorescence spectroscopy. The developed biosensor was used for the detection of Organophosphates (OPs), which are a group of chemicals that irreversibly inhibit the activity of an enzyme called acetyl cholinesterase, responsible for the synaptic nerve impulse transmission. The planar waveguide, which is the sensing surface is modified physically and chemically to facilitate better immobilization of bio-molecules. The array biosensor platform developed by the Naval Research Laboratories was adapted to suit the detection needs. The OP used in this study is paraoxon. The resulting sensor surface developed is reusable and is able to detect concentrations of paraoxon below its lethal dosage level.

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

The prevailing precarious picture of terrorism has attracted the use of Organophosphate (OPs) neurotoxins as chemical warfare agents. In addition, persistent minute quantities of OPs in soil, water and food, attributed to their use in agriculture as insecticides, have adverse ecological effects. The imminent danger posed by OPs has thus resulted in the need for the development of strategies for detecting these compounds to enable the first responders to immediately evacuate and detoxify the place of exposure and forestall causalities.

In this study, an optical-based array biosensor was developed for enzyme kinetics monitoring by fluorescence spectroscopy and applied for the detection of OPs. The array biosensor detection platform developed at Naval Research Laboratories was adopted here and significantly modified to suit the needs of enzyme-based detection. The bio-recognition element employed in this study was Organophosphorus Hydrolase (OPH), a highly specific enzyme for catalyzing the hydrolysis of OPs. The generation of protons resulting from the catalytic hydrolysis, decreases the microenvironment pH and increases the measured fluorescence intensity of the pH sensitive fluorophore conjugated to the enzyme. The planar waveguide was coated with TiO_2 and functionalized with (3-aminopropyl) triethoxysilane. OPH and BSA bound to the silanized glass slide using

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glutaraldehyde was further conjugated with carboxynaphthofluorescein (CNF), to detect the pH changes in course of the enzymatic hydrolysis of the P-O bonds of the organophosphates. The application of this system can be extended for the detection of many other agents by employing specific enzymes that, in course of catalytic breakdown, generate pH changes.

1.2 OVERVIEW

The main objective of this study was to develop a fluorescence based array biosensor for the detection of organophosphates using organophosphorus hydrolase. The current chapter gives a brief introduction and overview of the whole study. Chapter 2 gives the literature review on organophosphates, conventional analytical techniques for detecting organophosphates, the biosensors for detecting organophosphates based on the principle of inhibition and direct detection of OPs using organophosphorus hydrolase. Reviews on the multi-analyte sensing methods with array biosensor are also presented. Chapter 3 mentions the objectives of the study along with a detailed description of the methods and materials used for developing the sensor in order to achieve the objectives. Chapter 4 covers the results and the discussions of the study. Chapter 5 summarizes the conclusions and future work.

CHAPTER 2

LITERATURE REVIEW

2. 1 ORGANOPHOSPHATES:

Organophosphates (OPs) are a class of compounds, containing a phosphorus atom in the structure with four side chains. Synthesized as early as the 1800s, their widespread use as pesticides began in the 1930s after Gerhard Schrader in Germany and B. C. Saunders in England (Chambers et al., 2001) synthesized many OPs like diazinon, disulfoton, azinphos-methyl, fonofos, parathion and methyl-parathion. The most notable discoveries used as insecticides included tetraethyl pyrophosphate (TEPP, also known as Bladan), schradan (also known as OMPA) and parathion (Costa, 1987). Use of less toxic pesticides like malathion and parathion replaced Schradan in 1964(Caccia, 2000).

The current annual agricultural and non-agricultural use of organophosphates in Unites States is estimated to be around 60 million pounds. (http://www.epa.gov/pesticides/op/primer.htm). Apart from insects/pests, these chemicals above certain concentrations are highly toxic to mammals as well. Currently, they are first in the priority group of pesticides to be reviewed under Food Quality protection Act (http://www.epa.gov/pesticides/op/primer.htm). It has been reported that the more lethal

and notorious OPs like sarin, soman and tabun are stockpiled for use as chemical weapons (Costa, 1987).

2. 1. 1 Chemical Structure

These synthetic chemicals are products of reaction between alcohols and phosphoric/ phosphonic acids. **Figure 2.1** shows the general structure of an OP compound. "Z" is the leaving group that is displaced when an OP phosphorylates, the cleavage of which results in the hydrolysis of OP. "Z" can be an oxygen, sulfur, fluorine or nitrogen atom. The R and the R' may be an alkoxy (most common), alkyl, aryl, alkyl-thio or alkyl-amino group. **Table 2.1** gives the list of some OPs used as insecticides.

```
        (O/S)                    Z= O or S or F or CN
          ||                     R & R' = Alkyl group
   R —— P —— Z
          |
         R'
```

Figure 2.1: The general chemical structure of an Organophosphate compound

Chlorpyrifos methyl	Fonofos	Pirimiphos methyl
Chlorthiophos	Isazophos methyl	Profenofos
Coumaphos	Isofenphos	Propetamphos
Dialiflor	Malathion	Sulfotepp
Diazinon	Methamidophos	Sulprofos
Dichlorvos (DDVP)	Methidathion	Temephos
Dicrotophos	Methyl parathion	Terbufos
Dimethoate	Mevinphos	Tetrachlorvinphos
Dioxathion	Monocrotophos	Tribufos (DEF)
Disulfoton	Naled	Trichlorfon

Table 2.1: List of Organophosphates used as pesticides

The classification of the OPs that were developed for use as chemical weapons as the V (for venomous) agents and the G (for Germany) agents may comprise the VE, VG, VM, VX and the GA (tabun), GB (sarin), GD (soman), and GF (no common name) respectively (Wiener and Hoffman, 2004). The V agents are more potent and persistent than the G agents (Ellison, 2000). **Table 2.2** lists the LD50s of some of the OP insecticides and warfare agents. **Figure 2.2** shows the chemical structures of some of the OPs.

OP Compounds	LD50 dermal (mg/kg)	LD50 oral (mg/kg)
Paraoxon (P-O)	5	1
Parathion (P-O)	100	42
Demeton-S (P-S)	10	2
DFP (P-F)	100	4
Sarin (P-F)	1.5	0.1
Soman (P-F)	1	0.1
VX (P-S)	0.1	0.05

Table 2.2: List of the LD50s of some of the OP insecticides and warfare agents

Chemical Warfare Agents:

$$H_3C-\underset{\underset{CH_3}{|}}{\overset{\overset{H_3C}{|}}{C}}-\underset{\underset{CH_3}{|}}{HC}-O-\underset{\underset{CH_3}{|}}{\overset{\overset{O}{\|}}{P}}-F \qquad \textbf{Sarin}$$

$$OH_5C_2-\underset{\underset{CH_3}{|}}{\overset{\overset{O}{\|}}{P}}-S-CH_2-CH_2-\underset{\underset{\underset{H_3C \quad CH_3}{CH_2}}{|}}{\overset{\overset{CH_2}{|}}{\overset{\overset{H_3C \quad CH_3}{}}{N}}} \qquad \textbf{Vx}$$

$$H-\underset{\underset{CH_3}{|}}{\overset{\overset{CH_3}{|}}{C}}-O-\underset{\underset{CH_3}{|}}{\overset{\overset{O}{\|}}{P}}-F \qquad \textbf{Soman}$$

$$OH_5C_2-\underset{\underset{CN}{|}}{\overset{\overset{O}{\|}}{P}}-\underset{\underset{CH_3}{}}{\overset{\overset{CH_3}{}}{N}} \qquad \textbf{Tabun}$$

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Common Organophosphate pesticides:

Paraoxon

Malathion

TEPP - Tetraethylpyrophosphate
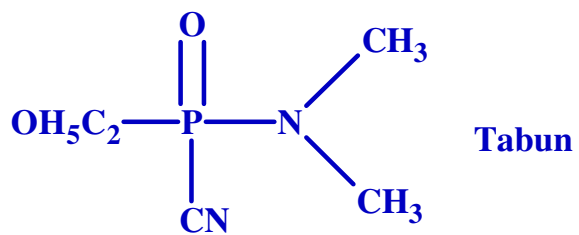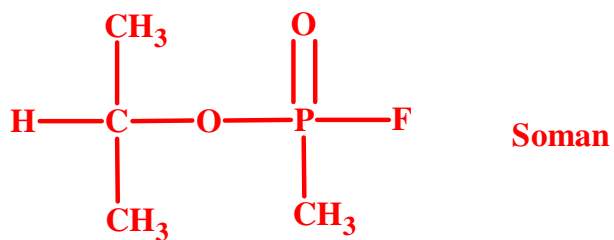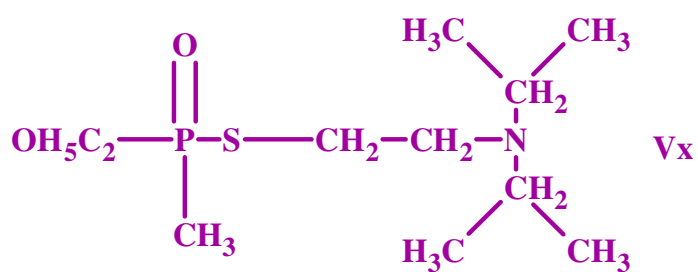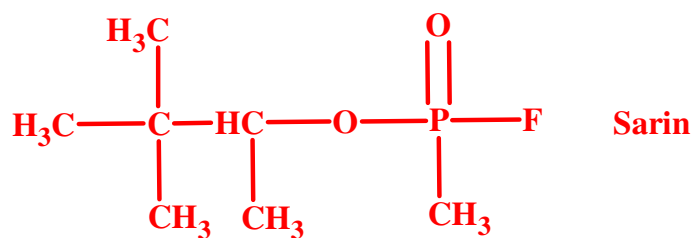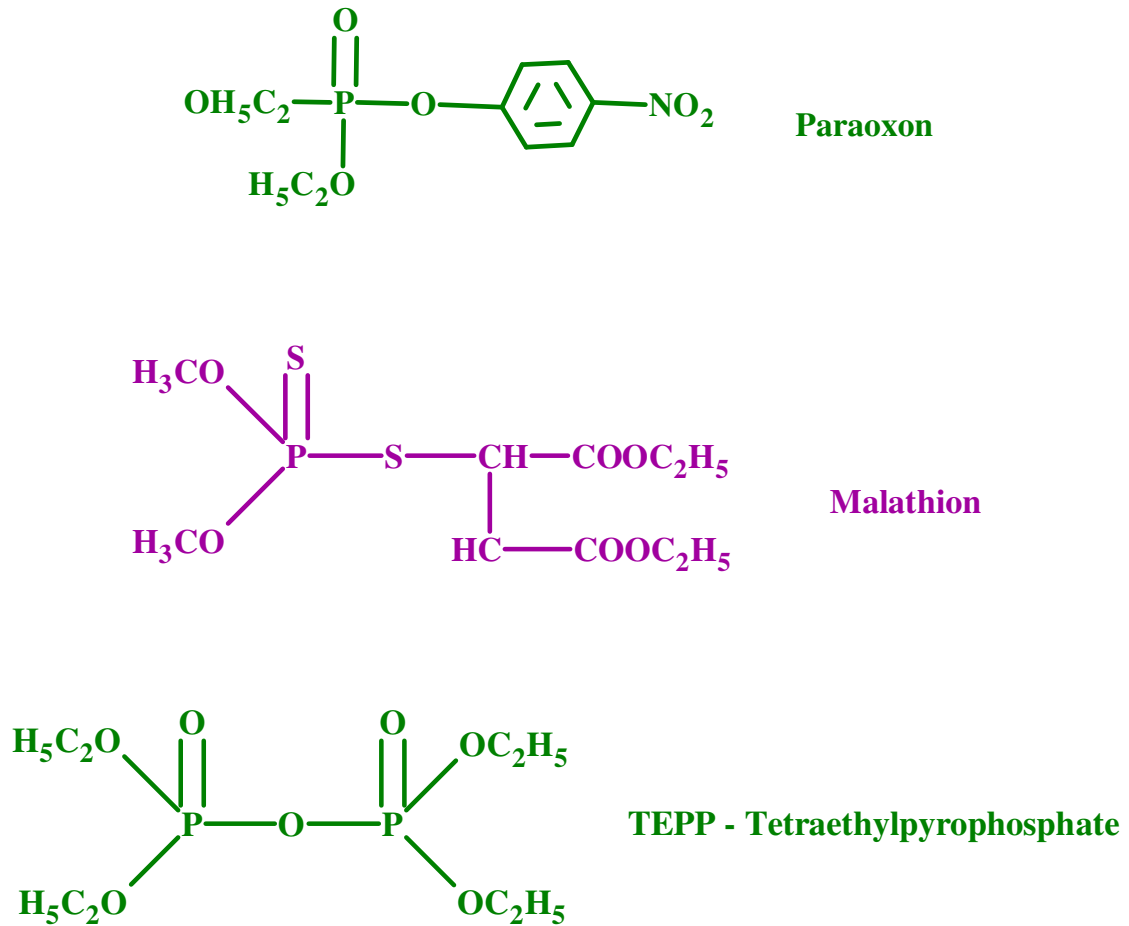
Figure 2.2: Chemical structures of some of the OPs: The different colored structures show the different leaving groups that undergo hydrolysis. Red: P-F, Blue: P-N, Green: P-O and Violet: P-S

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2. 1. 2 Organophosphate Toxicity

The widespread use of OPs as insecticides can be reasoned to the fact that they are inexpensive; they have broad-spectrum applications and that the pests normally showing resistance to organo-chlorine pesticides are not resistant to OPs (Report of a working group of Experts prepared for the Commision of European Communities, 1977). OPs primarily inhibit the activity of Acetyl Cholinesterase (AChE), an enzyme that hydrolyzes the central and peripheral neurotransmitter, Acetylcholine (ACh) (Sultatos, 1994). The P=O bonded OPs phosphorylates the OH⁻ group on serine in the AChE active site thereby preventing it from hydrolyzing ACh. The resulting accumulation of ACh may lead to uncontrolled convulsions followed by paralysis and death of the exposed organism. Certain hydroxylamine derivatives such as oximes may attach to the anionic site of AChE and facilitate dephosphorylation. This may be used to treat OP poisoning. However, when phosphorylated, depending on the type of OP acting, reactivation of the aged enzyme may not occur. **Figure 2.3** shows the main metabolic pathways involved in the biotransformation of parathion.
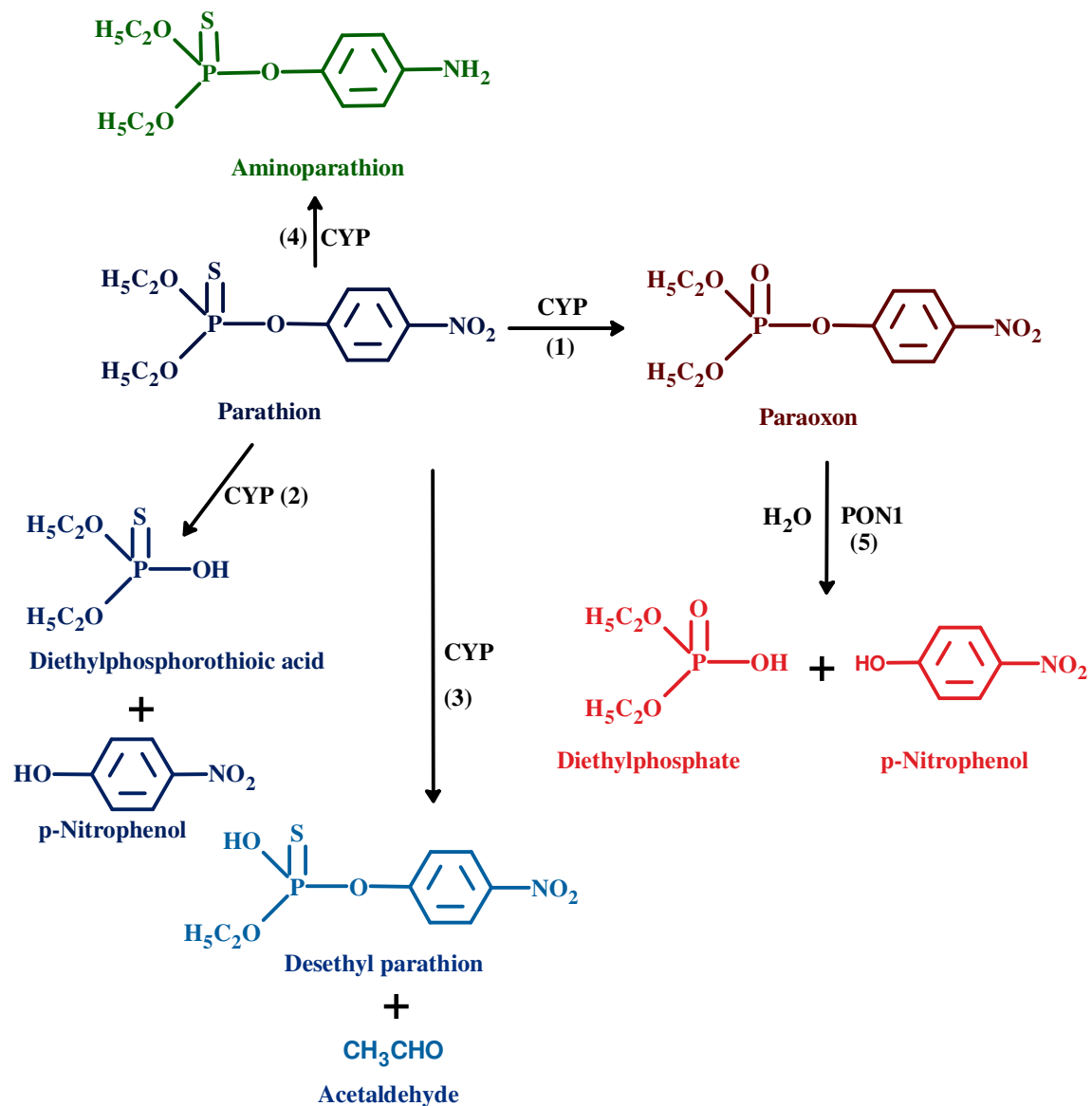
Figure 2.3: Metabolic pathways in biotransformation of parathion(Costa, 2005).

Reaction (1) – Bio activating reaction, Oxidative desulphurization

Reaction (2) – Oxidative De-arylation

Reaction (3) – Oxidative De-ethylation

Reaction (4) – Reduction of the nitro group

Reaction (5) – Hydrolysis

CYP – Cytochrome P450; PON1 – Paraoxonase

2. 2 NEED FOR DETECTION:

There are various analytical procedures for the diagnosis and determination of the extent of OP exposure. The determination of AChE and BChE activity, unbound nerve agent, decomposition products, analysis of fluoride induced reactivation of inhibited AChE and BChE with reconstitution of the inhibitor, phosphonyl-proteins-adducts in plasma are some of the ways to determine the levels of exposure (Worek et al., 2005). As both OPs and carbamates inhibit the AChE and BChE enzymes, diagnosis using AChE and BChE levels may suffer from drawbacks of specific determination. Treatment for exposures may include protection of airways, skin decontamination followed by administration of atropine sulfate or glycopyrolate or prolidoxime depending on the severity of exposure (Wiener and Hoffman, 2004).

With the increasing accessibility and synthesis of these compounds, there is also a proportional growth in the accidental exposures and fatalities due to the same. Many countries have signed the Geneva Convention in 1925 banning the use of chemical weapons. The governments worldwide have taken several steps to reduce accidents due to OP exposure since its discovery during the 1940s. Unlike the Geneva convention that only prohibits the use of chemical weapons, in 1997, 148 countries signed the treaty for Chemical Weapons Convention implemented by the Organization for the Prohibition of Chemical Weapons (OPCW) to completely ban any development, production, acquisition, stockpiling and retention of the chemical weapons(http://www.armscontrol.org/factsheets/cwcunderstanding.asp, July 2003).

The lethal effects of this broad spectrum of compounds that have agricultural applications have also been exercised for terrorist activities. Some of the cases include

the exposure of the general population to destroyed rockets containing sarin in March 1991 at Kamisiyah, Iraq(http://www.gulflink.osd.mil/dugway/low_lv_chem.htm), the gulf war illness of veterans due to exposure to OP nerve agents during war and the 1994 terrorist attack on Tokyo subway killing 5 people and injuring 5000 (http://en.wikipedia.org/wiki/Sarin_gas_attack_on_the_Tokyo_subway#External_links, 2006).

With the effortless accessibility, broad applications and production of OPs, the need to detect these compounds in the environment has increased drastically. The unchecked growing unrest and animosity among some factions has only added fuel to the existing fears of these chemicals getting into the wrong hands. All these events have propelled the need for quick, selective and sensitive detection of OP compounds in the environment to prevent further problems.

2.3 CONVENTIONAL ANALYTICAL DETECTION METHODS USED FOR ORGANOPHOSPHATES

Several analytical methods have been developed for the detection of organophosphates. The highly sensitive chromatographic techniques like Thin Layer Chromatography, Gas Chromatography and High Performance Liquid Chromatography (Mendoza, 1973), portable surface acoustic wave sensors and spectrophotometric sensors are some of them. Based on the four approaches for detection of chemical warfare agents that include the OPs, Gas Chromatograph coupled with mass spectrometer (GC/MS) is the only "approved equipment" to date for field verification (Hill and Martin, 2002). Some of the various analytical detection methods are described below.

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2. 3. 1 Acoustic wave sensors

The detection is based on the changes in the Surface Acoustic Wave (SAW) properties on adsorption of the analyte at the surface of the piezoelectric crystal. Advances have been made for selective adsorption of chemicals by applying thin film coats on the piezoelectric surface to create selective sensors (Ballantine et al., 1996, Nieuwenhuizen and Harteveld, 1997, Nieuwenhuizen and Harteveld, 1994). The Joint Chemical Agent Detector (JCAD) developed by the military used the SAW technology for detection of V and G type nerve agents with detection limits of $1mg/ml^3$ for each (Hill and Martin, 2002).

2.3.2 Spectrophotometric sensors

Color spot test is the most inexpensive method for detection of OP based chemical warfare agents. Here, the detection of the presence or absence of OPs are based on the color changes resulting from the interaction of pretreated test spot with the chemical agent on the addition of developer. Though the color-spot test method is convenient for initial screening, they are slow and are not selective. Combination of molecular imprinting techniques with spectrophotometers by using europium probe to measure the hydrolysis product of Sarin has better limits of detection (7ppt for Sarin) (Jenkins et al., 1999).

2. 3. 3 Chromatographic techniques

Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) are normally used for separation and detection of complex mixtures of liquid and vapor phase OPs respectively. Both the chromatographic techniques employ high resolution chromatographs in combination with element selective detectors like pulsed

Flame Photometric Detectors (FPD), Electron Capture Devices (ECD), Nitrogen Phosphorus Detectors (NPD), electrolytic conductivity detectors and microwave induced pulsed plasma atomic wave detection to reduce the individual times for separation, detection and analysis (Chen and Wang, 1996, Sanchez et al., 2003, Bardarov and Mitewa, 1989, Futagami et al., 1997). Solid-phase micro-extraction (SPME) methods in conjunction with GC has been found to particularly increase the selectivity of Sarin detection (Harvey et al., 2002). Development of Liquid Chromatography interfaced with Particle Beam (PB) and Mass spectrometry (MS) provided data comparable to Gas Chromatography interfaced with Mass spectrometry (GC-MS) detection (Aguilar et al., 1998). Others methods of detection include Gel Permeation Chromatography, Atomic Pressure Chemical Ionization Mass Spectrometry with Electron Capture Detectors, and Ultra Violet Absorbance Detectors. All the above analytical methods of sample extraction have provided valuable information on sample preparation from complex environmental matrices for quantitative analysis. However, these methods are not suitable for field applications because of their complex nature of operation that in turn requires skilled labor. Recently combined GC-Mass Spec (time of flight) instruments were developed, for detecting trace amounts of different substances. Although, they perform well in a controlled environment, they have been shown to lack specificity in true environmental situations, compromising their effectiveness(Asbury et al., 2000, Albert Robbat Jr, 1999). In addition, these instruments are expensive and difficult to deploy in the field.

2. 3. 4 Ion mobility spectrometry (IMS)

These instruments are used for field detection of OP chemical warfare agents. Here the vapor phase ionization of the neutral molecules creates gas-phase ions that have different velocities in an electric field. This method of detection is based on the separation of the gas phase ions according to their size-to-charge ratios. Some of the IMS instruments used for field detection like Chemical Agent Monitors (CAM) and improved chemical agent monitor (ICAM) require a minute of exposure time to detect G type and V type nerve agents (Hill and Martin, 2002). Though improvements in existing method by taking advantage of the multi-channel deflection of the ions under orthogonal electric field (Tuovinen et al., 2001) have been made, they have the disadvantage of low resolving power.

2. 3. 5 Immuno-Assays

The enzyme linked immuno-assay techniques developed for pesticide analysis is an additional technique that is sensitive and accurate with a reasonable speed of response. They are normally used for screening analysis for a single analyte. Immunosensors (IS), Immuno-labeling and Enzyme-Linked Immunoabsorbent (ELISA) techniques are the most commonly used immunoassays for OP analysis. In these methods, a protein binds specifically to an analyte chemical agent and produces an analytical, electrochemical or fluorescence response. The limit of detection for ELISA-based detection of Soman is found to be as low as 180ng/ml (Lenz et al., 1997). All the above methods, though highly sensitive, have intricate sample preparation methods like ligand binding interactions that in turn require skilled labor thereby posing accessibility restrictions of their use to the farmers and the first responders. Antibody based detection methods are confined to large

molecules and creation of antibodies for small chemicals is difficult. In addition, the constraints of one time usage of immuno-based detection, limited selectivity and expensive equipments are other problems faced.

2. 4 BIOSENSORS

These problems can be overcome by the use of easy to use, rapid, and portable biosensors. The history of biosensor may date back to as early as the 1960s. The first biosensor developed in 1962 by Clark and Lyons(Clark and Lyons, 1962) for the detection of glucose that consisted of soluble glucose oxidase entrapped in an oxygen electrode using a dialysis membrane revolutionized the field of medicine. In 1962, Gilbault and his colleagues built the first biosensor that had insoluble enzyme AChE, immobilized between two platinum electrodes to detect Organophosphates (Gilbault et al., 1962). Hicks and Updike developed the second glucose sensor in 1967 using immobilized glucose oxidase between platinum electrodes (Updike and Hicks, 1967). In 1969, Guilbault built the first potentiometric biosensor using immobilized urease for the detection of Urea (Guilbault and Montalvo, 1969). In 1986, Hill developed the second-generation biosensor using ferrocene mediator for glucose detection (Gleria et al., 1986). In 1995, wiring of enzyme directly to the electrode led to the third generation biosensors (McNeil et al., 1995). Thus, during the novice years of biosensor development, enzymes were the primary bio-recognition elements used.

Depending on the type of transduction mechanism applied and the bio-recognition element employed, the potential for these devices for detection can be enormous. The technological development and the success in single analyte detection propelled advances in the miniaturization of sensors along with multi-analyte detection with sensitivities

ranging in the nanomole to attomole range. With advances in techniques for biosensor construction, it has been possible to miniaturize the whole biosensor system on a chip. The main applications of biosensor may involve environmental monitoring of samples, for medical diagnosis of diseases or cure for the same.

2. 4. 1 Biosensors: Definition

According to the International Union of Pure and Applied Chemistry (IUPAC), a biosensor is "a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals" (IUPAC, 1997(1992)). **Figure 2.4** shows the general schematic of a biosensor. The biological component used for detection primarily differentiates a biosensor from a chemical sensor.

Specificity and selectivity are the most important features and requirements of a biosensor. The above can be met by suitable permutations and combinations of recognition and transducer elements depending on the application (**Figure 2.5**). Good speed of response; insensitivity to environmental interference, physical robustness are some of the other features of a good biosensor.
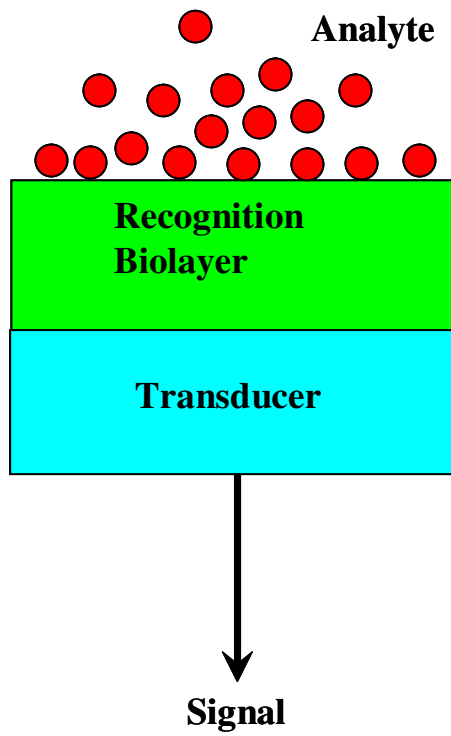
Figure 2.4: General schematic of a Biosensor

Enzyme/Substrate

Antibody/Antigen

Whole cells, tissue

Lipid Layer/Gas

DNA Fragments

SIGNAL TRANSDUCTION

Piezoelectric **Thermal** **Acoustic** **Optical** **Electrochemical**
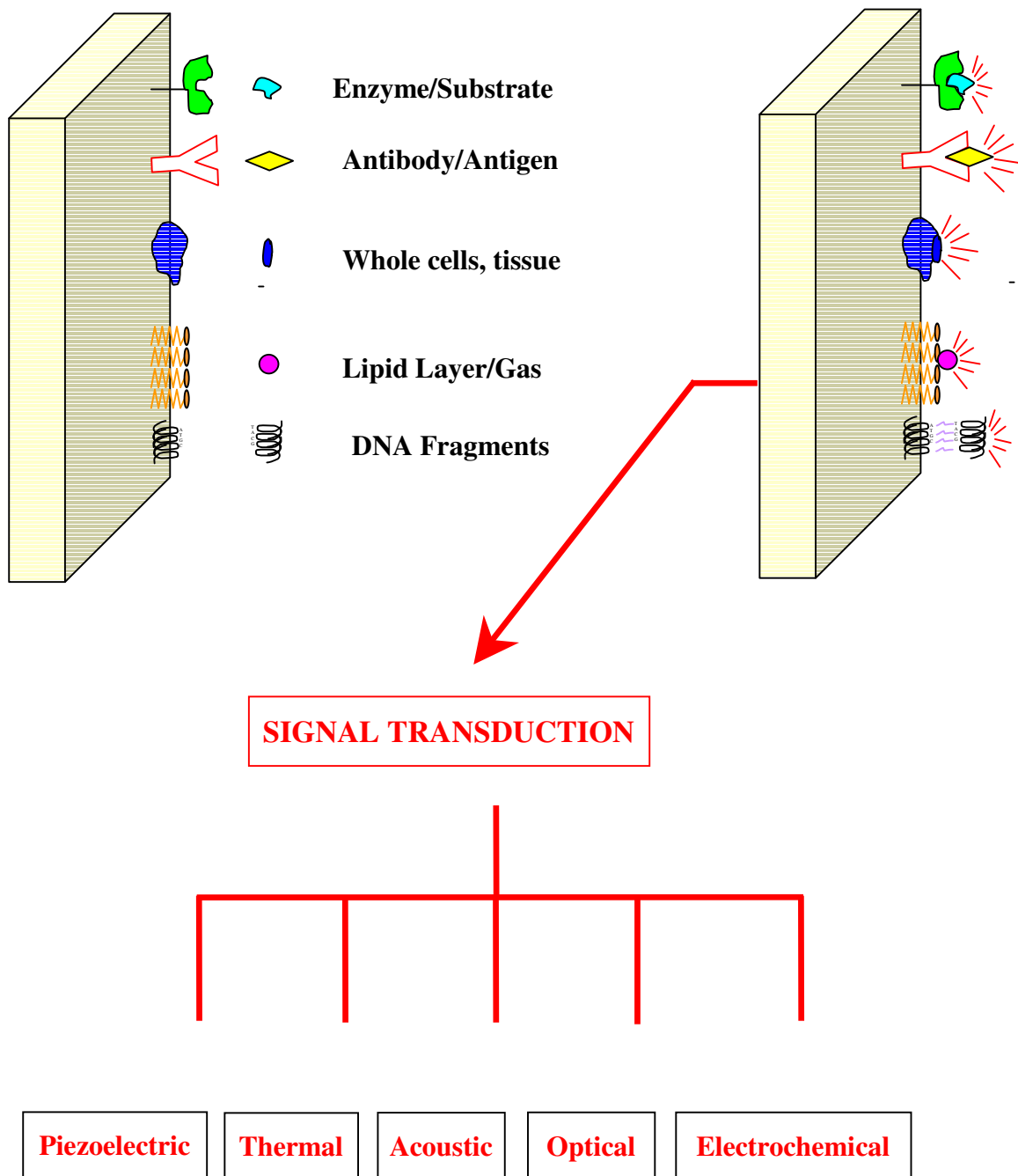
Figure 2.5: Pictorial/Schematic of working of a biosensor (Guilbault et al., 2004)

2. 4. 2 Classification of Biosensor

I.   Classification based on the method of transduction:

A. Optical Sensors (Ulber et al., 2002)

a. UV spectroscopy

Differences in absorption wavelength of different atoms in the UV region of electromagnetic spectrum form the basis of detection. Use of charged coupled device and diffraction grating enables multi-analyte detection. High sensitivity, scanning speed, compactness, low cost and robustness are some of the advantages of using this method.

b. IR spectroscopy

Changes in absorption wavelength of atoms in the IR region of the electromagnetic spectrum form the basis of detection. Low sensitivity and large volume sample requirements are some of the disadvantages that can be overcome by coupling Surface plasmon resonance with IR spectroscopy.

c. Raman spectroscopy

Scattering of radiation due to inelastic collision of photons in a molecule forms the basis of detection. Complex sample preparation is not required giving online field analysis. Problems with sensitivity due to low signal have been overcome by Surface Enhanced Raman Spectroscopy (SERS).

d. Surface plasmon resonance

Changes in refractive index of the absorbed material at the thin interface of metal form the basis of measurement. This method provides label free detection.

e. Fluorescence spectroscopy

Changes in fluorescence form the basis of detection. It is a rapid, non invasive and sensitive analytical technique.

B. Electrochemical Sensors

a. Amperometric

Changes in current as a result of changes in voltage form the basis of their detection. e.g. Clark Oxygen electrode(Clark and Lyons, 1962)

b. Potentiometric

Changes in voltage as a result of changes in current form the basis of detection. They normally use ion-selective electrodes/ Glass electrodes/ Ion-sensitive field effect transistor. e.g. Immobilized enzyme membrane surrounding pH meter probe

C. Piezoelectric Sensors

Changes in resonant frequency due to changes in the mass of the crystal form the basis of detection. e.g. detection of gaseous formaldehyde using immobilized formaldehyde dehydrogenase coating on quartz crystal (Guilbault, 1983)

D. Calorimetric Sensors

Heat changes due to thermo-chemical reaction forms the basis of detection. e.g. enzyme catalyzed reaction generates heat which is measured

E. Acoustic Sensors

Changes in acoustic wave properties due to mass/micro-viscosity changes form the basis of detection. The acoustic devices used may include Thickness shear mode (TSM), Surface acoustic wave (SAW), Surface transverse wave (STW), Flexural plate wave (FPW), Shear horizontal acoustic plate mode (SH-APM)

II.   <u>Classification based on the detection mode</u>

    A. Catalytic Biosensors

    This works on the principle of detection of the steady state concentration of the species formed, lost or inhibited by the transducer.Biocatalysts used may be enzymes, microorganisms, cells, tissues, organelles

    B. Affinity Biosensors

    Physiochemical changes due to non-catalytic irreversible binding of analyte to receptor molecules form the basis of their detection. Receptor molecules used may be antibodies, hormone receptors, nucleic acids

III.   <u>Classification based on the mode of delivery</u>

    A. Continuous/ Flow mode

    They involve the uninterrupted measurements of the analyte. Reactions take place during the flow of samples or bio-recognition element. Pumps are normally used to flow samples.

    B. Intermittent/ Batch mode

    They involve the introduction of the analyte into the flow stream.

2. 5 INHIBITION BASED BIOSENSORS FOR THE DETECTION OF ORGANOPHOSPHATES

Several biosensors capable of providing rapid and sensitive detection of different OPs have been developed based on inhibition of enzymes(Kuswandi et al., 2001). The measurement of the percentage of the enzyme inhibition against the initial enzyme activity is the general basis of detection for these analytical biosensors. Electrochemical transducers are most commonly integrated with the biological component for signal transduction. Optical, piezoelectric and calorimetric transducers have also been employed. Principles and experimental details of inhibition based biosensors for OP based pesticides have been reviewed by many authors (Solé et al., 2003, Kuswandi and Mascini, 2005).

Less invasive and more sensitive optical based transduction has also been exploited for OP detection using the principle of enzyme inhibition. The changes in optical properties, which may be that of absorbance, reflectance, fluorescence, phosphorescence, refractive index may correspond to the concentration of analytes. Fiber optic biosensor based acetyl cholinesterase immobilization on Langmuir Blodgett films by measuring the changes in absorbance of para-nitrophenol (Choi et al., 2001), cholinesterase immobilization in polyvinylidenefluoride membrane in contact with sol-gel layer incorporated with bromcresol purple (Andreou and Clonis, 2002), AChE conjugated with pH sensitive fluorophore in sol gel network (Doong and Tsai, 2001) are some of the methods developed. Recently, using the principle of competitive and discriminative inhibition of acetyl cholinesterase on planar waveguide, detection of OP (100 ppt of Sarin in solution and 250 picograms of Sarin in vapor) has been made

possible (White et al., 2003a, White et al., 2002, White et al., 2003b, White and Harmon, 2005).

Based on the cholinesterase inhibition principle, many commercial instruments like M8 and M9 detection papers (**Figure 2.6 a**) supplied with M256 kits, M256A1 kits (**Figure 2.6 b**) have been developed for OP chemical warfare detection. These kits have been used by the Metropolitan Medical Strike teams (MMST) and the Public Health Service (http://newton.nap.edu/html/terrorism/ch4.html, 1999).
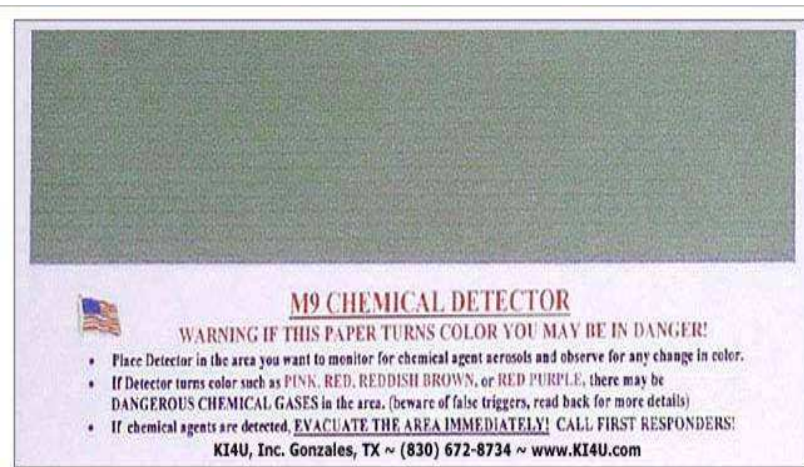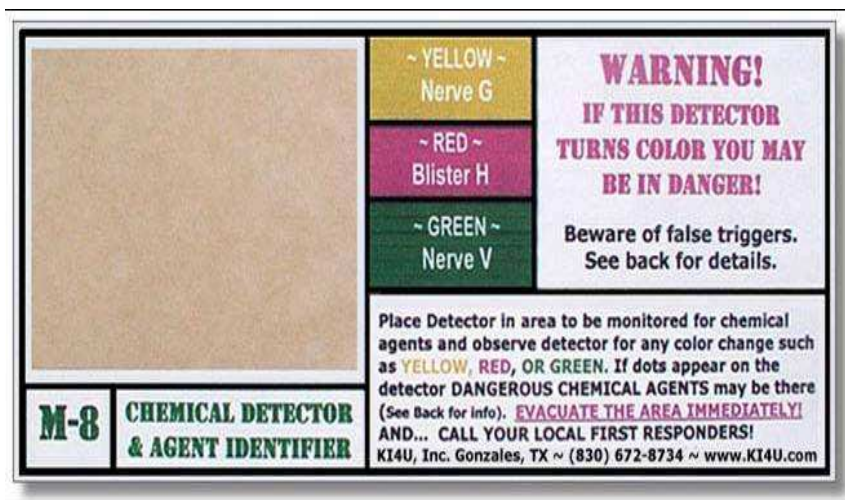
Figure 2.6 a: M9 Paper (http://www.ki4u.com/M9_Chemical_Detection_Paper.htm, 2003)M8 and M9 Detection kits
(http://www.ki4u.com/M9_Chemical_Detection_Paper.htm, 2003)

Figure 2.6 b: M256A1 detection kit (http://www.esgsafety.com/m256a1-def.htm, 2006)

The chief disadvantage of inhibition based detection principle is that the enzyme inhibition by other hazardous agents such as the mustard gas, carbamates and heavy metal ions, and the complex environmental matrices may give false positive alarms, which can create undesirable panic among the civilian population. In addition, the irreversible inhibition of the Cholinesterases by the OPs poses restrictions on the number of detection actions this system can perform. Thus, it is important for a detector to be able to specifically and selectively differentiate between carbamates and organophosphates, both of which target the Cholinesterases, in order to take appropriate measures and treatments in case of exposure.

2.6 DIRECT DETECTION OF ORGANOPHOSPHATES BY ORGANOPHOSPHORUS HYDROLASE:

Organophosphorus Hydrolase (OPH, EC 3.1.8.1) is an enzyme that can catalytically hydrolyze the P-S bond of OP substrates apart from P-O, P-F and P-CN bonds (Kolakowski et al., 1997, Lai et al., 1995, Chae et al., 1994, Dumas et al., 1990).
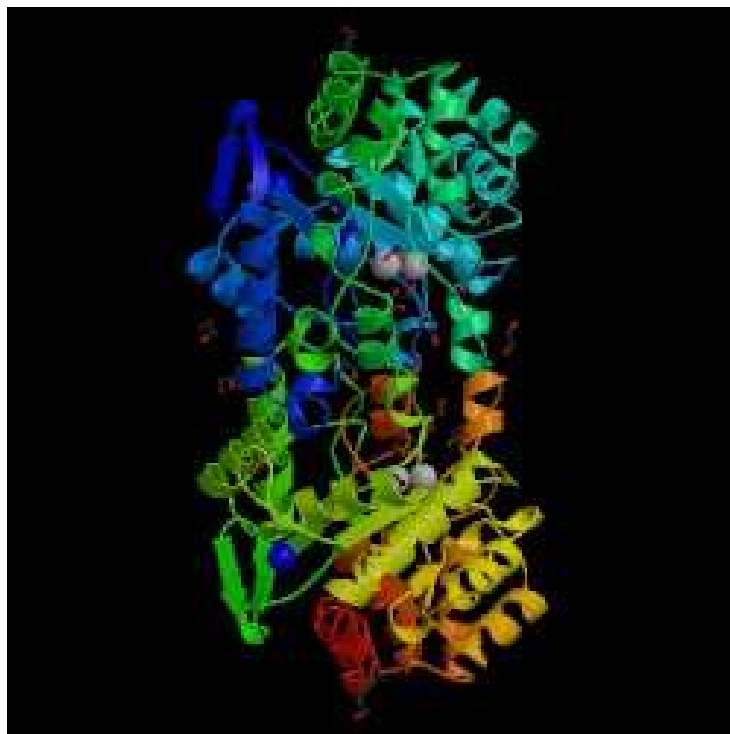
Figure 2.7: Structure of Organophosphorus Hydrolase (Holden et al., 2001)

OPH is a 72KDa, stable, dimeric, metalloenzyme with 336 residues present per monomer (McDaniel et al., 1988, Mulbry and Karns, 1989, Serdar and Gibson, 1985, Donarski et al., 1989, Lewis et al., 1988). **Figure 2.7** shows the structure of OPH. The binuclear metal center of wild type enzyme has two zinc ions present in the active site

that has structural and catalytic functions (Lai et al., 1994). Extensive studies were performed to modify and understand the structure of OPH with different metal ions like Co^{2+}, Cd^{2+}, Mn^{2+} and Ni^{2+} (Benning et al., 2001). It has been found that the catalytic activity can be increased nearly 3 times by replacing zinc ions with cobalt ions (Omburo et al., 1992). OPH has the highest catalytic activity for P-O bond OPs, with highest turnover number of $10^4 s^{-1}$ for paraoxon (Omburo et al., 1992). In the current study, we use OPH, wild type, as the bio-recognition element for the detection of OPs. **Figure 2.8** shows the binuclear metal center of OPH. From the studies, it has been found that the mechanism for the hydrolysis of the Organophosphates by OPH is through the nucleophilic attack on the phosphorus center of substrate binding to the active site (Aubert et al., 2004).
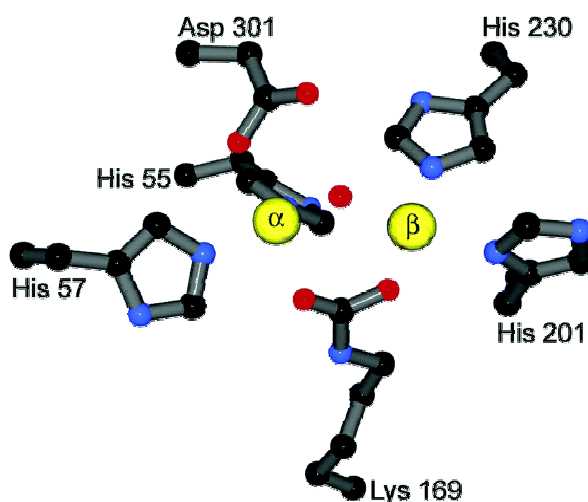
Figure 2.8: Structure of binuclear metal center of OPH (Aubert et al., 2004).

2. 6. 1 Organophosphorus Hydrolase based biosensors

Many biosensors have been developed using the OPH as bio-recognition element for the detection of OPs. The potentiometric biosensor based on immobilized bacterial cells with high OPH activity, pioneered at Texas A&M University in 1996 could detect concentrations as low as 1μM of paraoxon (Rainina et al., 1996). This method involved the cryo-immobilization of E-coli cells exhibiting OPH activity in polyvinyl-alcohol matrix. Measurements were made in flow through column reactor and stirred reactor with a pH glass electrode as the transducer. OPH immobilization on sol gel modified field effect transistors (pH-FET) was another potentiometric based biosensor (Flounders et al., 1999, Singh et al., 1999)). Though they are simple in operation, their drawbacks included low sensitivity and inability to discriminate between OPs and other pesticides like carbamates that also inhibit the activity of AChE. Discriminative detection of carbamates and Organophosphates was provided in amperometric biosensors that combined OPH and acetyl cholinesterase (Simonian et al., 2001). Many fluorescent based optical biosensors have been developed exploiting the principle of changes in micro-environmental pH due to OP hydrolysis. Encapsulation of OPH –SNAFL conjugates in polyethylene glycol (Russell et al., 1999) was one of them. Fluorescent based detection has the advantage over spectrophotometric detection that any organophosphate could be detected using the same detection method independent of the extinction coefficient of the products of OP hydrolysis.

2. 7 MULTI-ANALYTE SENSING

The first proposed work on multi-analyte immuno assay had biomedical applications (Ekins et al., 1990, Kakabakos et al., 1992). Many portable instruments based on evanescent waves were built for detecting signals from arrays (Herron et al., 1993, Herron et al., 1994, Herron et al., 1996). The use of total internal reflection to produce evanescent waves and harness that principle to build biosensor was first described in 1975 (Kronick and Little, 1976). Surface plasmon resonance, interferometers, resonant mirrors, fiber optic and planar array fluorescent sensors are the other biosensors that use evanescent wave excitation.

Total internal reflection fluorescence is a highly surface selective, sensitive phenomenon that can be used for real time, non-destructive sensing and interfacial bio-molecular interaction studies. Recently, significant progress was achieved in the sandwich immunoassays-based technology of simultaneous detection of multiple analytes using evanescent wave excited fluorescence based optical biosensors. Multi analyte immunoassays based on Surface plasmon resonance (Berger et al., 1998) and capillary flow systems(Narang et al., 1998, Ligler et al., 2002) have also been developed. Though very high sensitivity can be obtained in SPR, it suffers from poor selectivity and sensitivity due to fluctuations in refractive index of multi component systems.

There has been significant progress using Array Biosensor developed by the Naval Research Laboratory for simultaneous detection of multiple bio-threat agents like Ricin, Cholera Toxin, B. Anthracis, Campylobacter Jejuni, Staphylococcus Enterotoxin B, Ochratoxin and many others (Bakaltcheva et al., 1999, Wadkins et al., 1998, Benecky et al., 1998, Ligler et al., 1998, Rowe-Taitt et al., 2000b, Rowe-Taitt et al., 2000a, Rowe-Taitt et al., 2000c, Ngundi et al., 2006, Sapsford et al., 2006, Sapsford et al., 2004). Other groups have as well reported the development of fluorescence–based immunosensors systems (Barzen et al., 2002, Kartalov et al., 2006, Petrou et al., 2002).

In general, immuno-based assay, being extremely sensitive and selective for the detection of bacteria's, viruses, spores, etc., have two distinct drawbacks. Firstly, the use of immunoassays confines the detection to analytes that are primarily large molecules such as bacteria's etc., thereby barring the detection of smaller molecules such as chemical nerve agents. Secondly, the affinity-based immunoassay requires the substitution of the sensor bio-recognition part after detection action, because of potential problems in the regeneration of the bio-molecules on the surface.

For the detection of small chemical molecules such as OP neurotoxins, immunochemical methods are not appropriate because of problems in production of appropriate antibodies. Exploitation of enzyme- based "catalytic" process, where specific recognition of target that is the substrate for particular enzyme involved in catalytic transformation, is a more preferable approach. The major advantage of biosensor operating in "catalytic mode", in contrast to commonly used affinity-based "single action" mode, is its ability to continuously monitor any catalyzed cleavage using an

appropriate mode of signal transduction, such as fluorescence spectroscopy. Applications of enzymes in biosensors are well documented in many publications.

Although a variety of platforms such as electrochemical, thermal, etc. were successfully used in biosensors, recently significant attention was paid to optical fiber-based biosensors because of multiple advantages of optical platform and enlarged availabilit of optic fibers. Several detailed reviews are available on this subject (Hong et al., 1992, Monk and Walt, 2004, Epstein and Walt, 2003, Kuswandi et al., 2001). The detection of chlorinated herbicide atrazine base on enzyme Gluthathione S-Transferase and pH-sensitive reagent bromocresol green was recently reported (Andreou and Clonis, 2002). Several other authors reported on different optical biosensors based on monitoring of pH or oxygen changes in course of enzymatic reactions (Doong and Tsai, 2001, Ignatov et al., 2001, Issberner et al., 2002). All those systems were able to detect only one analyte in the single sample. Recently, several systems were described for simultaneous detection of multiple agents (Cho et al., 2002, Tsai and Doong, 2005).

2. 8 CURRENT WORK

In this study, array biosensor developed by the Naval Research Laboratories (NRL) is modified and is used for the direct detection of OP neurotoxins like paraoxon using OPH conjugated with pH sensitive fluorophore, Carboxynaphthofluorescein as the bio-recognition element. The principle of total internal reflection to generate evanescent waves is used for fluorophore excitation.

2. 8. 1 Principle of Total Internal Reflection

Total internal reflection is a phenomenon that occurs when light incident at an angle greater than the critical angle, traveling from a medium of higher index of refraction (e.g. glass) to a medium of lower index of refraction (e.g. an aqueous solution) gets totally internally reflected. The critical angle θ_c is defined as

$$\theta_c = \sin^{-1}(n_2/n_1) \tag{1}$$

Where

$\theta_{c=}$ Critical angle

n_2 = refractive index of liquid (aqueous solution)

n_1 = refractive index of solid (glass)

Although the incident beam is totally internally reflected at the interface, an electromagnetic field extends, that decays exponentially with distance "z" from the interface into the lower refractive index medium.

$$I_{(z)} = I_o\, e^{-z/d} \tag{2}$$

Where

$$d = \lambda_o / 4\pi \left[n_1^2 \sin^2 \theta - n_2^2 \right]^{-1/2} \tag{3}$$
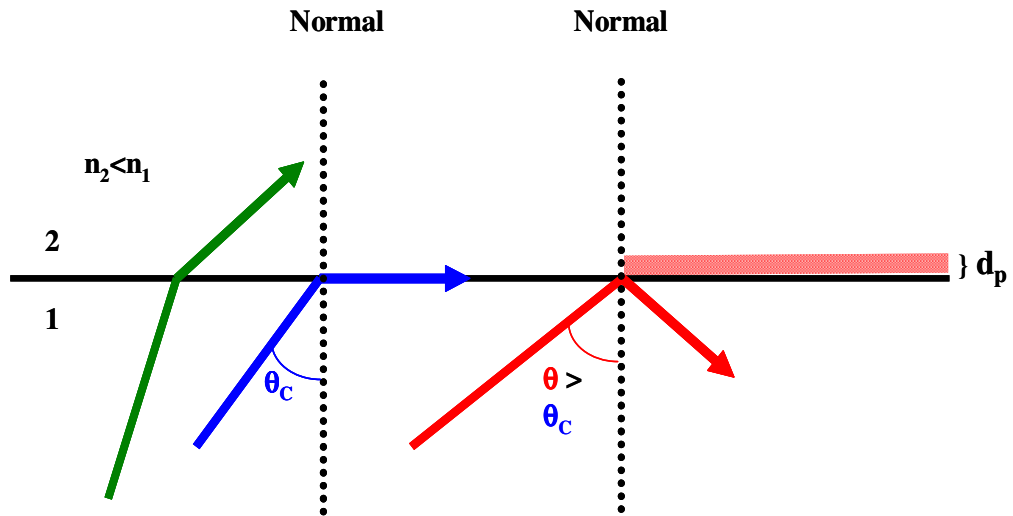
Figure 2.9: Schematic explaining Total internal reflection and evanescent waves

For multi-mode waveguides, this penetration depth d_p may be defined as the distance from the surface where the strength of the field is 1/e the value at the interface. **Figure 2.9** shows the phenomenon of total internal reflection and evanescent wave creation.

2. 8. 2 Array Biosensor Unit
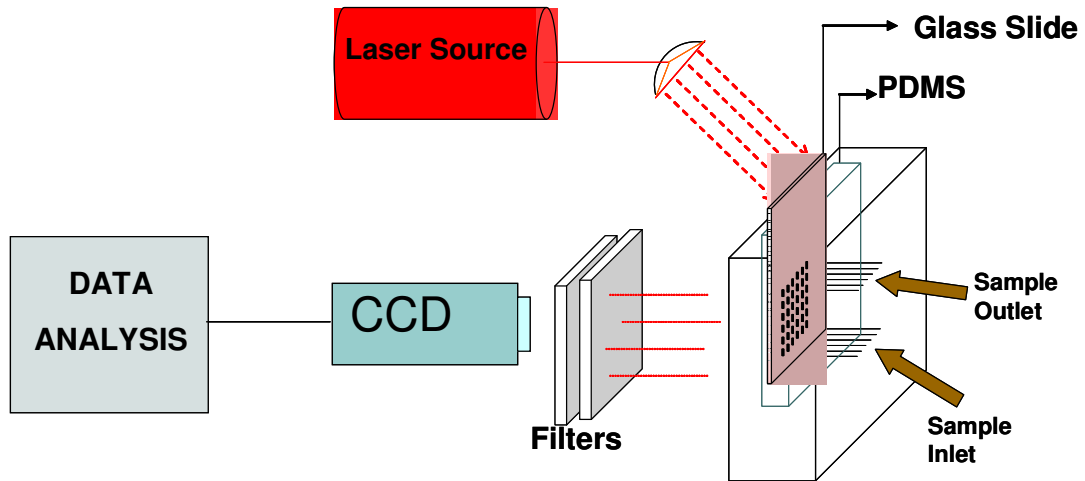
Figure 2.10: Schematic of the working of Array biosensor

The **Figure 2.10** shows the schematic of Array biosensor developed by the Naval Research Laboratories. The Array Biosensor can be primarily divided into the fluidics, waveguide cladding, optics and data processing units (Feldstein et al., 1998). This is an optical based sensing system that works on the principle of total internal reflection to create evanescent waves that excites the array of fluorophores conjugated to the Biorecognition element immobilized on the glass slide surface. The real time measurements of the fluorescent intensities can be monitored using the CCD camera and analyzed using the TIFF analysis program developed by the NRL.

CHAPTER 3

MATERIALS AND METHODS

3.1 OBJECTIVES

The objectives of this study are as follows

§ Modify the Array Biosensor instrumentation to make it amenable for OP detection

§ Engineer immobilization holders (IH) for sensor surface preparation

§ Chemically and physically characterize the surface of the planar waveguide to enable immobilization of OPH to in turn obtain good sensitivity for the detection of the Organophosphates

3.2 ARRAY BIOSENSOR INSTRUMENT – DESCRIPTION AND MODIFICATIONS

The Naval Research Laboratory group has extensively reviewed the instrumentation of array biosensor in many articles (Sapsford et al., 2006, Golden et al., 2005b, Golden et al., 2005a, Ligler et al., 1998, Feldstein et al., 1998). The **Figure 3.1** shows the array biosensor components. Here, a brief description of the various parts of the instrumentation is given. The modifications made in order to suit the needs for the study is also given.

3. 2. 1 Light source

The array biosensor has a 635nm diode laser with a line generator to spread the excitation beam over the edge of the glass slide held by the fluidics compartment. The beam incident on the edge of the slide provided even excitation of the fluorophore arrays attached to the slide surface(Feldstein et al., 1998). This set up had problems associated with the laser beam spreading over a wider range and extending further from glass slide. In this study, a 54mm long, hollow, cylindrical Aluminum tube with an 8mm diameter opening was fitted onto the laser diode in order to confine the laser beam to fall only on the line generator.

3. 2. 2 Imaging

Peltier-cooled CCD (charged coupled device) camera (Retiga 1300, Q imaging), with Q-capture software was used to capture the images and monitor the slide sensor surface reactions (Feldstein et al., 1998). In this study, more advanced software, namely Q-Capture Suite was used. The Suite software unlike Q-capture Pro could be programmed to take snaps at particular intervals of time, which is critical for kinetic assay based analysis. 660 nm bang pass filter and 700nm long pass filters were placed in between the

camera and flow chamber to eliminate the scattered excitation light from entering the CCD. A two dimensional graded index of refraction (GRIN) lens array was used to image the array onto the CCD (Feldstein et al., 1998).

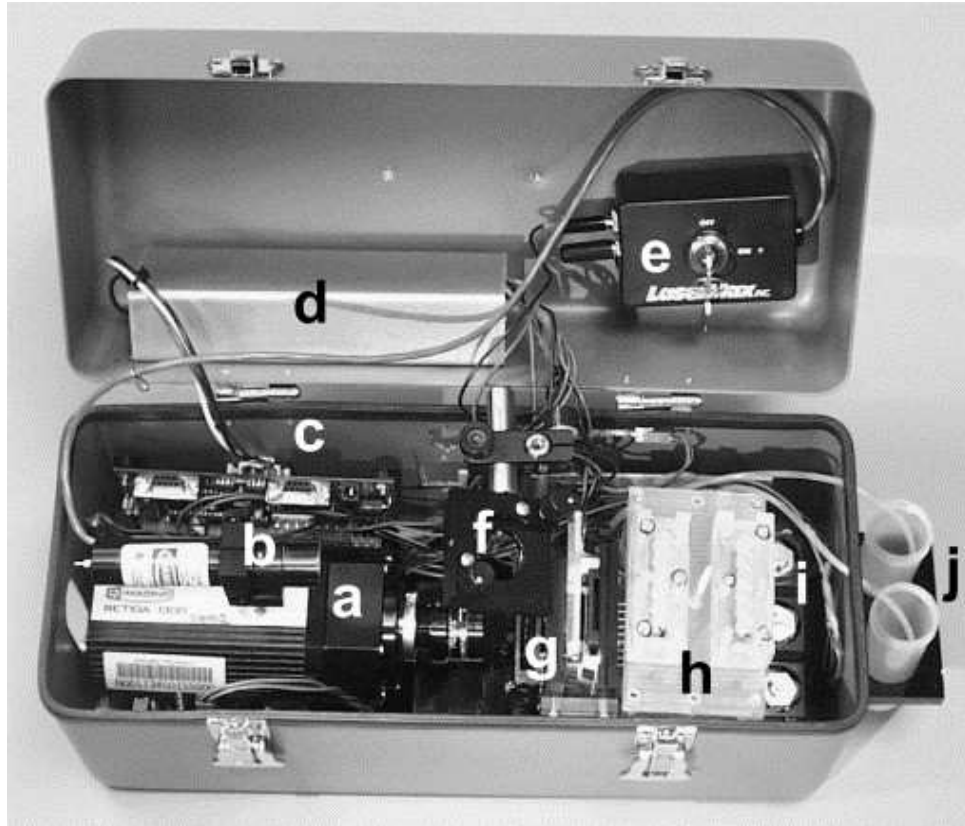**Figure 3.1**: The array biosensor components (a) CCD camera, (b) diode laser, (c) RS232 interface board, (d) power supply, (e) laser power switch, (f) mirror, (g) slide mount, (h) removable reservoir modules, (i) peristaltic pumps (j) waste and buffer reservoirs.

The electronic control boards, for the valves and pumps are not visible as they are underneath the reservoir modules (Golden et al., 2005b).In this study, a black cardboard

housing was made for the region around the CCD and flow chamber to eliminate any external light from entering the camera.

3. 2. 3 Data Analysis

Using the Tiff analysis program developed by the NRL, data analysis of the captured images was performed (Feldstein et al., 1998). This program created rectangular masks around fluorescent spots. Values of the background rectangular masks located at the sides of the fluorescent spots were averaged and subtracted from the average fluorescent spot value to give the net fluorescent intensity (Feldstein et al., 1998). These values can be exported to Microsoft Excel to perform the data analysis.

3. 2. 4 Fluidics compartment

The samples may be introduced through the two six chamber sample reservoirs that have the inlets located at the bottom of the chamber and the outlets at the top (Feldstein et al., 1998). The inlets are connected to six two way modular multi-position valves and eight channel peristaltic pumps that can be used to switch the flow direction of the samples (Feldstein et al., 1998). The whole flow system can be automated with precise control of timings using the Array CTRL software developed by the NRL. However, there were problems with interfacing the software with the valves and pumps and thus, in the current study, the Array CTRL software was not used since all the reservoirs, valves and pumps were removed and replaced by the ISMATEC pump. The pump was used to maintain and control the speed of sample introduction through the flow chamber. The fluidics cube holding the slide with sample inlets and outlets was made of thermoplastic with the flow chamber made up of PDMS (Feldstein et al., 1998). The flow

channels are 2.74mm wide x 38.1mm long x 2.54mm deep with an individual channel separation of 1.1mm (Feldstein et al., 1998).

To overcome the problems of low energy availability due to the light being coupled out and scattered by the flow cell, reflective silver based cladding of the glass slide, with a pattern of six channel flow cell in contact of the silver portion of the glass slides was used by the NRL. However, due to the problems of peeling and pitting of silver as a result of high salt concentrations in buffers used during sensor preparation, these silver cladded glass slides were not used as sensor surface in this study. The RS-232 interface with the help of a 50W power supply controlled the CCD camera, peristaltic pumps and valves (Feldstein et al., 1998). The ADR 2000 interface board converted the RS-232 signals into the digital/analog control voltages (Feldstein et al., 1998). The images from the CCD were acquired through a Fire wire connection (Feldstein et al., 1998).

3.3 DESIGN OF IMMOBILIZATION HOLDERS FOR SENSOR SURFACE PREPARATION

In order to obtain high-quality immobilization of the enzyme and fluorophore on the glass slide, it is essential that the sensor surface is cleaned and activated thoroughly. The immobilization employed (by NRL) for the array biosensor involved the used of physically isolated patterning (PIP) method. **Figure 3.2** shows the steps involved in the PIP method.
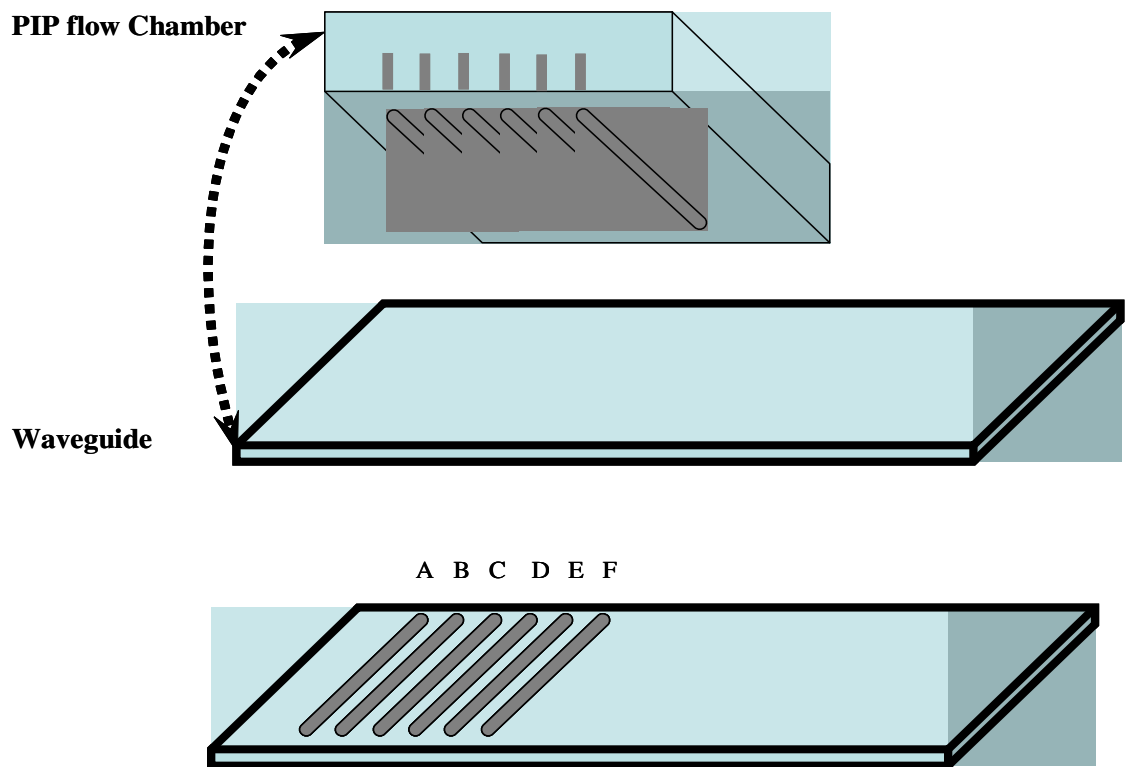
Figure 3.2: Schematic of Physically isolated patterning method (Feldstein et al., 1998).

This method is used to generate 1mm^2 array of bio-recognition elements on the planar waveguide (Feldstein et al., 1998). Here, the physically isolated patterning flow cell, made of Polydimethoxysiloxane (PDMS) is placed on the surface of the waveguide and the recognition elements that are antibodies in most cases are introduced in channels. After the incubation, the antibodies are removed and the slide is thoroughly rinsed resulting in an array of recognition elements patterned on the slide. A two dimensional array of rectangular recognition elements can be made by combining a multi-channel assay cell placed perpendicular to the described patterned elements as shown in **Figure 3.3**.

Figure 3.3: Schematic of Combination of multi-channel cell to create array of recognition elements (Feldstein et al., 1998).

Some of the problems faced with the above method of immobilization for the proteins employed in our study were the formation of bubble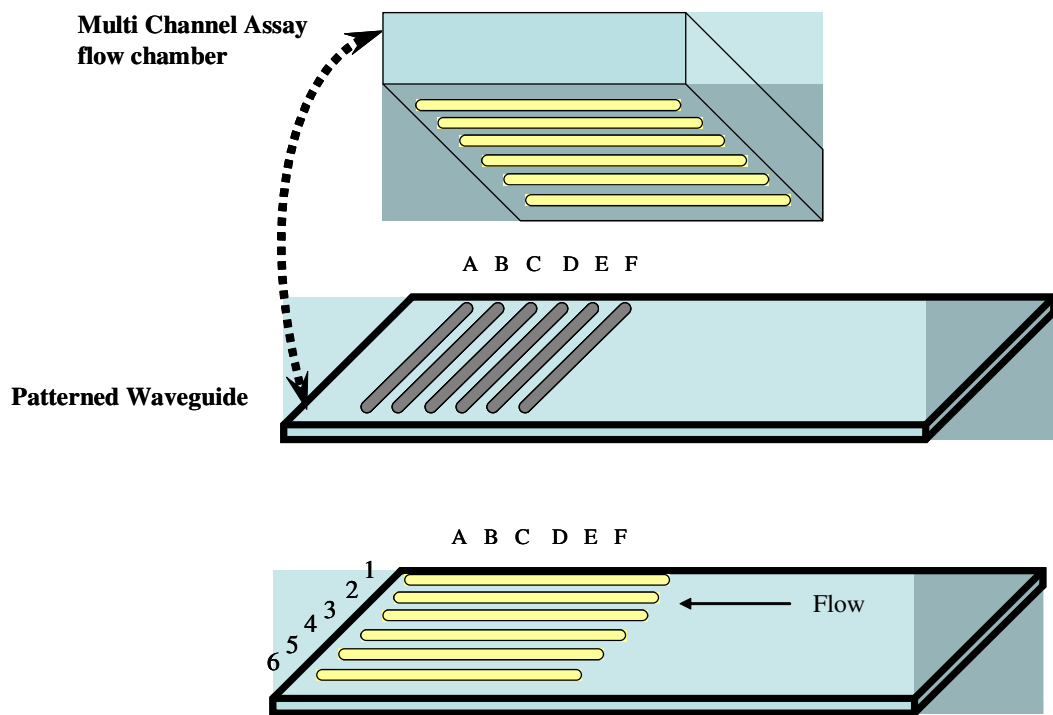s resulting in the non-uniform immobilization of the biomolecules resulting in response variation among different arrays. In order to avoid these problems, uncomplicated, reproducible and long-lasting, multiple-use incubation holders (IH) were designed and engineered using the principle of capillary action, as an alternative to the PIP flow chambers. Capillary action takes place when the forces between the liquid molecules are less than that between the liquid and the solid resulting in the pull of the liquid against gravity. The principal of capillary action is used here to direct the fluid flow to form rows of arrays of proteins with fluorophores on the waveguide surface. The incubation holder is composed of the glass capillary tubes and plexiglass platform.

3. 3. 1 Immobilization holders design and preparation

The plexiglass platform has a well for seating the glass slide and multiple wells for holding the capillary tubes. 9mm plexiglass obtained from Dillard was cut to rectangular shape having dimensions of 100x65mm using a cutter. The edges of the plexiglass were made smooth using a sand paper. Using the All Inch Mini Drill/Mill Machine manufactured by Microlux^{TM} present in the Materials Engineering Department at Auburn University, a well (for seating the glass slide) was milled at the center of the rectangular plexiglass having dimension of 76x26x3mm. The extra 1mm on the sides were provided to enable easy placement and removal of the glass slide to and from the holder. The extra 2mm depth was provided as a tolerance to place wet tissue to maintain the moisture conditions during the immobilization procedure. This is critical as it might otherwise result in evaporation of the protein/fluorophore leading to difficult-to-remove,

non-covalently bound particles that may compromise the sensitivity of biosensor. **Figure 3.4** shows the schematic of the plexiglass with wells milled for holding the glass slide and capillary tubes. The number of wells for the capillary tubes can be increased or decreased depending on the number of rows of bio-recognition elements required. The capillary tubes used are the hematocrit tubes purchased from Chase Scientific Glass Inc. They have an inner diameter of 1.1mm-1.2mm with the tube thickness of 0.2± 0.02mm. The length of the tubes used is 75mm.
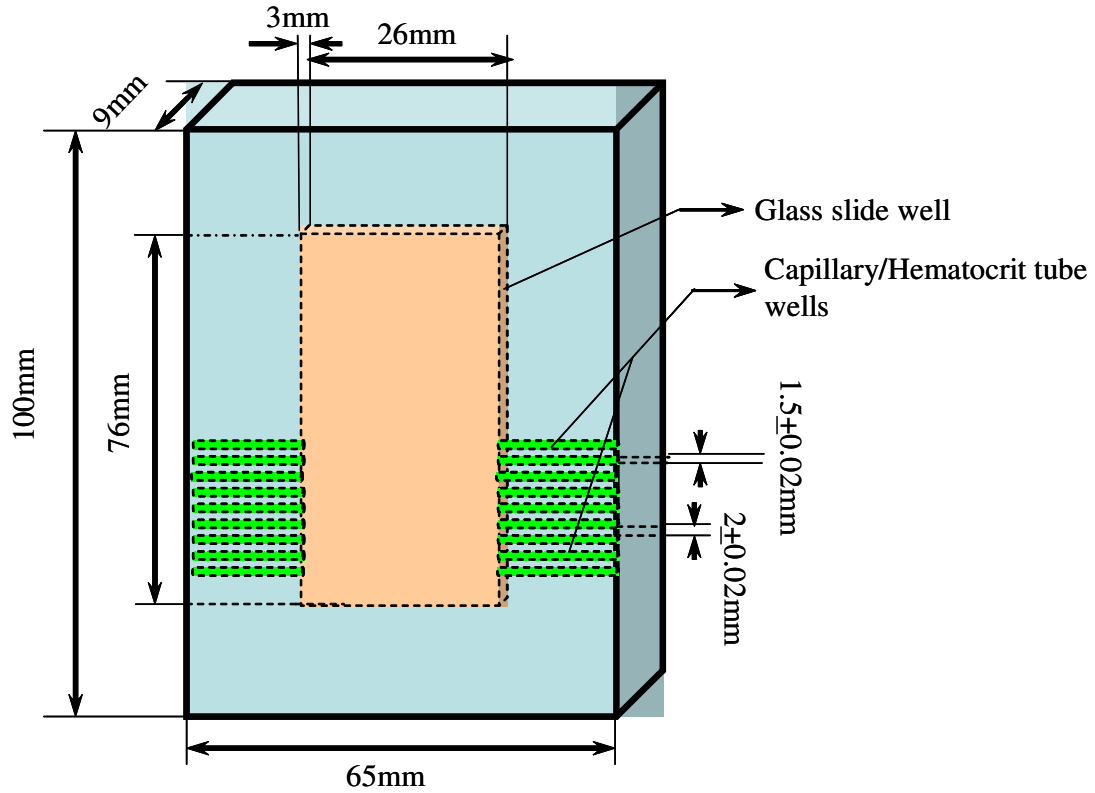
Figure 3.4: Schematic of the immobilization holder with dimensions.

Figure 3.5 shows the schematic of the procedure followed for incubating the slide with enzyme and fluorophore. The hematocrit tubes placed at a distance from the glass slide surface determines the amount of solution required to be placed on the slide-tube interface that directs the row immobilization resulting from surface tension. This method of immobilization required 5-10μl of solution in contrast to 50-100μl requirement in the case of PIP immobilization method. After the solution supply, the whole incubation holder with the slide was placed in a Petri dish with moistened tissue to maintain humid conditions.

Figure 3.5: Schematic of modified process for incubation using hematocrit tubes

3. 4 MATERIALS

Ti-Nanoxide T paste (Solaronix SA), Absolute Ethyl Alcohol (Florida Distillers Co.), De-Ionized water (Type I Millipore $\leq$ 18.2 mΩ), Phosphate Buffer (Fisher Scientific), CHES (2-[N-Cyclohexylamino] ethanesulfonic Acid) (99%, FW. 207.29, Alfa Aesa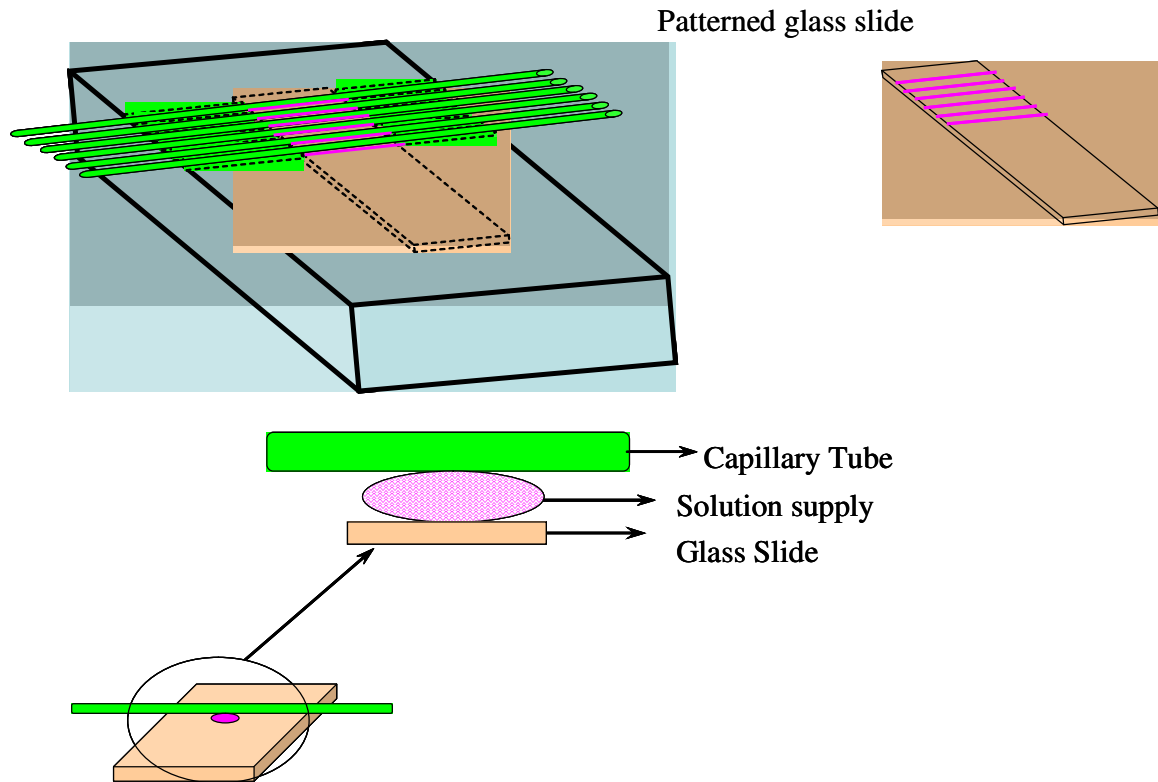r), N,N,-Dimethyl Formamide (Fisher), 3-Aminoproply Triethoxysilane (98%, FW. 221.4, Sigma), Nitrogen gas (Ultra High Purity, Airgas Inc.), Glutaraldehyde (Grade I, 50% Aqueous solution, FW. 100.1, Sigma), Bovine Serum Albumin (Fract V, cold alcohol precipitated, Fisher Scientific), CNF (5-(and-6-)-Carboxynaphthofluorescein-succinimidyl ester, mixed isomers, FW. 573.51, Molecular Probes), wild-type OPH (E.C.3.1.8.1) isolated from recombinant *Escherichia coli* strain obtained from Texas A&M (Dr. James Wild's Lab), Paraoxon (diethyl-p-nitro phenyl phosphate, FW. 275.22, Chem. Service), 12.1 N Hydrochloric Acid (Fisher Scientific), 32 N Sulfuric Acid (Fisher Scientific),

Glass slides (from VWR Scientific Inc.), Array Biosensor (Naval Research Laboratories), Ismatic Peristaltic pump (IDEX corporation), Silicone tubing (1.02mm ID, Cole Parmer).

3. 5 SENSOR PREPARATION

The proteins may be immobilized on the glass slide by covalent binding (Weetall, 1969). In some cases, covalent bonds may be formed during the physical adsorption of the enzyme. Depending on the various factors like the ionic strength of the solution, quantity of the enzyme and temperature conditions that affect the quality of immobilization, a suitable method is chosen such that the functional properties of the enzyme are maintained. In the current study, the enzyme OPH is further conjugated with a fluorophore, carboxy napthofluorescein.

The waveguide in our study is a microscopic 27x75x1mm glass slide. Microscopic glass slides are ideal for fluorescence based optical sensing as they have low intrinsic florescence, high chemical inertness, transparence, resistance to high temperatures, easy to handle and inexpensive. The composition of the glass slide obtained from the manufacturer (Eerie Electoverre) is given in **Table 3.1**.

SL. No	Compound	Amount (%)
1.	Silicon Dioxide	72.20
2.	Sodium Oxide	14.30
3.	Calcium Oxide	6.40
4.	Magnesium Oxide	4.30
5.	Aluminum Oxide	1.20
6.	Potassium Oxide	1.20
7.	Sulfur Trioxide	0.03
8.	Sulfur Trioxide	0.03

Table 3.1: Glass composition obtained from the manufacturer

The procedure describing the sensor surface preparation and an explanation of the agents used is given below

a) **Numbering the glass slide**

The glass slides were numbered using a diamond point tip on the bottom right corner. The numbered face of the glass slide is the face on which the surface activation and immobilization of bio-recognition elements is done. After numbering the glass slide, any left over glass powder is wiped out.
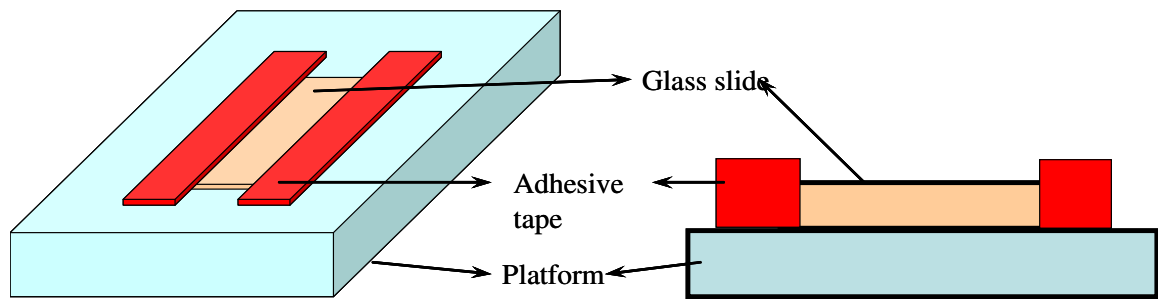
b) Cleaning the glass slides

Before silanization, the glass slides were cleaned thoroughly to remove any dirt and oil that may be present, for the complete activation of the surface. Plain microscopic glass slides are used instead of the silver coated slides developed by the Naval Research Laboratories to avoid the problems of silver peeling out under the highly concentrated environments of the buffers used. Many different groups use different cleaning procedures. Studies have been performed comparing the different cleaning methods of glass to gain good silanization (Cras et al., 1999). In this study, the glass slides were incubated in concentrated hydrochloric acid (12.1N) for 30 minutes. It was followed by drying under nitrogen atmosphere.
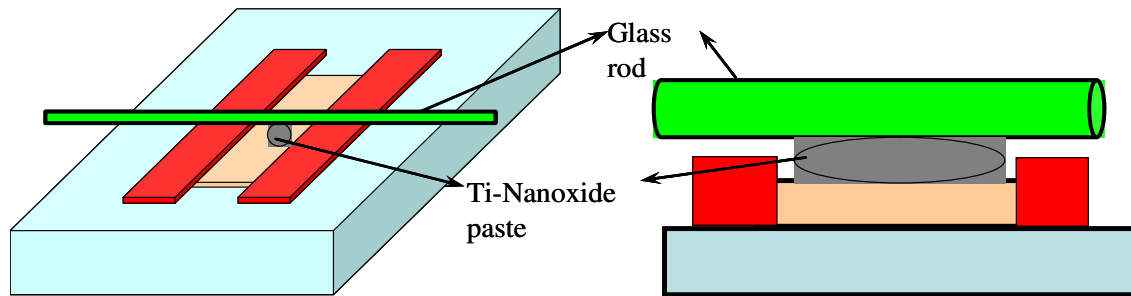
c) Coating the glass slide with Ti-Nanoxide paste

The surface of the glass slide was coated with Ti-Nanoxide paste, to enhance the surface area and its optical properties due to the large refractive index (refractive index of the Ti-Nanoxide paste is 2.34) and optical transparency. The Ti-Nanoxide paste obtained from the manufacturer (Solaronix, Switzerland) has ~11% weight of nanocrystalline titanium oxide anatase particles, water, less than 20% ethanol and polyethylene oxide. The procedure followed for coating the glass slide involves the "Dr. Blade squeegee printing" method as recommended by the manufacturer. The **Figure 3.6** shows the schematic of the coating process. It involves the use of 3M Scotch adhesive tape. The glass slide was strapped by the sides using an adhesive tape onto a flat platform. The Ti-nanoxide paste was dropped at the center and by using a glass rod, the paste was spread evenly on the whole slide. The slide was allowed to dry and the adhesive tape was removed. This was followed by sintering the slide at 450º C, with further cooling down to

room temperature. The sintering process enables better bonding of the titanium oxide to the glass surface. The resulting glass slide has a bluish haze.

STEP: 1

STEP: 2

STEP: 3

STEP: 4

Coated Ti-
Nanoxide glass
slide
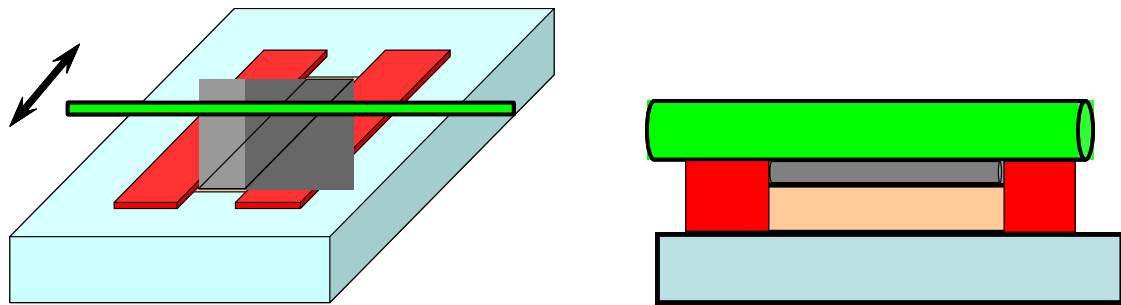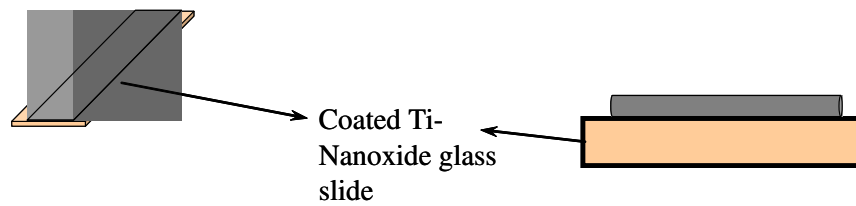
NOT IN SCALE

Figure 3.6: The steps showing the procedure for coating the glass slide with Ti-Nanoxide paste. **Step 1**: Strap the glass slide with adhesive tape **Step 2**: Place Ti-Nanoxide paste **Step 3**: Spread the Ti-Nanoxide using a glass rod **Step 4**: Remove the adhesive to get Ti-Nanoxide coated glass slide.

It has been experimentally found that dilution of the Ti-Nanoxide paste in the ratio of 3:2 Ti-Nanoxide to absolute ethanol is more suited for the experimental conditions, taking into account diffusion problems associated with the products of OP hydrolysis onto the slide. This in turn simplifies the washing steps after detection action.

d) Cleaning the sintered glass slide

The sintered glass slide was cleaned with 1N hydrochloric acid. This was done to remove any dirt accumulation due to handling during the sintering process in furnace. After cleaning, the slides were thoroughly rinsed in de-ionized water, dried in nitrogen atmosphere and placed on clean, moisture free coplin jar.

e) Silanization of the glass slide

Silanization enables the covalent immobilization of the proteins on the glass slide surface. It helps provide a chemical bond between the organic and inorganic support material. Silanes have a generic structure of Y-R-Si-X$_3$. The X is the hydrolysable alkoxy group like methoxy, ethoxy or acetoxy groups that bind to the inorganic support material. The Y is the organo-functional group which may be an amino, vinyl, epoxy or methacryl groups. They may bind to the organic part. The organo-functional group is attached to the silicon atom through an alkyl bridge "R".

Various types of silanes have been used to immobilize proteins on glass slides. In this study, aminosilane was used to silanize the glass slide. Aminopropyltriethoxy silanes (APTS) are the most popular silanes used for producing glass slides with amine rich surface. **Figure 3.7** describes the general principle involved in silanization (Witucki, 1993). In the first step, the slides were incubated for an hour at room temperature in 5% APTS prepared in dry acetone. During this step, the hydroxyl groups present on the

51

surface of the metal oxide react with the ethoxy groups present in the silane to from

silanols. This was followed by thoroughly rinsing the slides in dry acetone to remove any

unbound silane formed. The glass slides were then cured at 120º C for at least two hours

for the condensation reaction to occur resulting in the formation of siloxane layers.

Hydrolysis of Alkoxy (X) groups and formation of alcohols

$$H_5C_2O-\underset{\underset{OC_2H_5}{|}}{\overset{\overset{R}{|}}{Si}}-OC_2H_5 \;+\; 3H_2O \longrightarrow 3C_2H_5OH \;+\; HO-\underset{\underset{OH}{|}}{\overset{\overset{R}{|}}{Si}}-OH$$
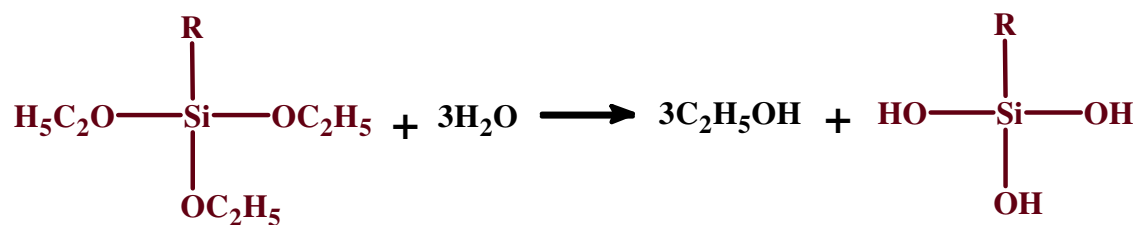
Condensation to Oligomers

$$HO-\underset{\underset{OH}{|}}{\overset{\overset{R}{|}}{Si}}-OH \;+\; HO-\underset{\underset{OH}{|}}{\overset{\overset{R}{|}}{Si}}-OH \;+\; HO-\underset{\underset{OH}{|}}{\overset{\overset{R}{|}}{Si}}-OH$$

$$\downarrow$$

$$HO-\underset{\underset{OH}{|}}{\overset{\overset{R}{|}}{Si}}-O-\underset{\underset{OH}{|}}{\overset{\overset{R}{|}}{Si}}-O-\underset{\underset{OH}{|}}{\overset{\overset{R}{|}}{Si}}-OH \;+\; 2H_2O$$
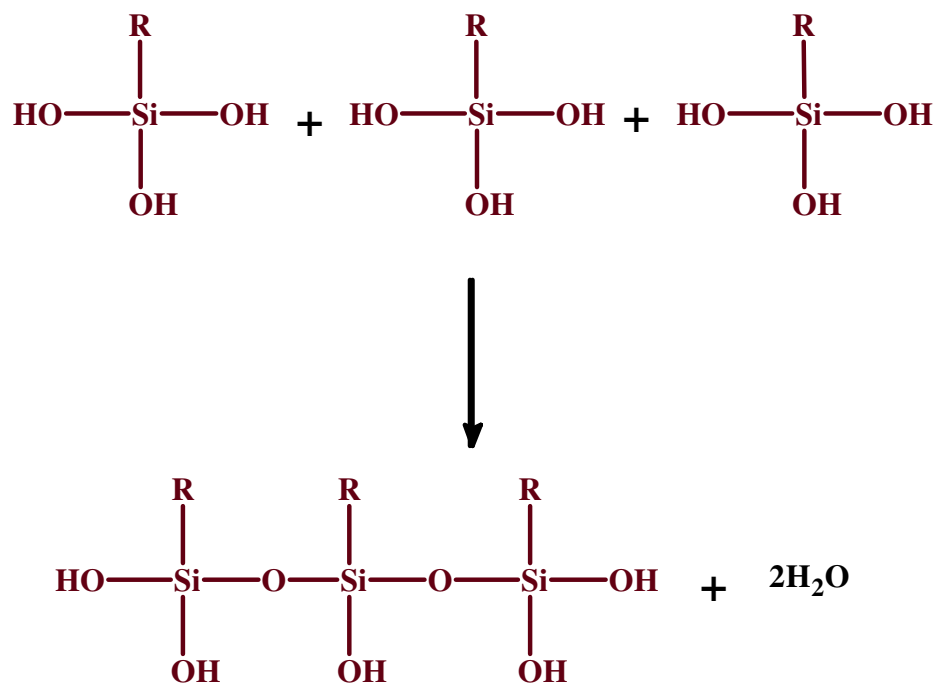
Covalent bond formation during Curing
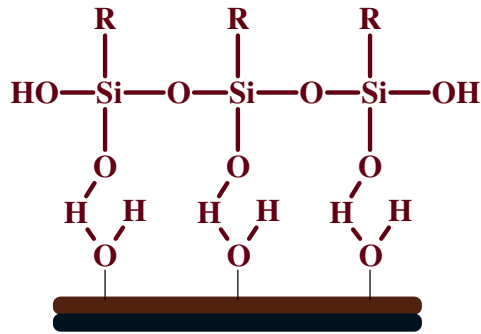
Condensation
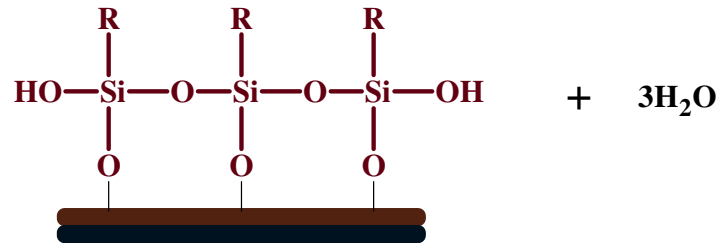
Figure 3.7: Chemistry of the general steps involved during silanization of the glass slide (Witucki, 1993)**.**

Figure 3.8 shows the glass slide surface chemistry treated with APTS. Silanization plays a critical role in determining the quality of bio-recognition elements immobilized. The presence of water in even minute quantities can result in the neighboring siloxane layers of the silane to react with each other and form a three dimensional polymerized network that is rough and inhomogeneous (Kinkel and Unger, 1984).

Ti-Nanoxide coated glass slide with APTS

Figure 3.8: Chemistry of Ti-Nanoxide coated glass slides silanized with APTS

f) Cross linking the Ti-Nanoxide coated silanized glass slide

Cross-linkers with specific functional groups at their ends enable Cross-linking different molecules though a covalent bond. These functional groups may be primary amines, sulfhydryls, carbonyls, carbohydrates, carboxylic a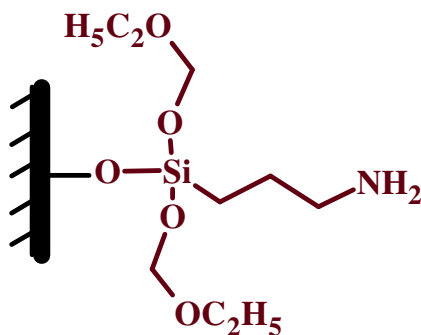cids etc (Hermanson, 1995). The determination of croslinker for a particular application depends on its chemical specificity, spacer arm length, water solubility, nature of the functional group and cleavability (Hermanson, 1995). The protein to cross-linker molar ratio should be such that the functional activity and stability of the protein is maintained. Cross linking reagents may be either homo-bifunctional or hetero-bifunctional (Hermanson, 1995). Homo-bifunctional cross linkers have identical functional groups at their reactive ends. They are used for single step reaction of cross linking proteins. The hetero-bifunctional cross linkers have different reactive groups. The cross linkers used in this study for APTS silanized slide was Glutaraldehyde. **Figure 3.9** shows the structure of Glutaraldehyde (http://www.piercenet.com/files/Cross-LinkingTechHB.pdf, 2006).

Glutaraldehyde is a homo-bifunctional crosslinker whose one functional group reacts with the amine end of the APTS and the other end reacts with the primary amine of the protein in consideration. The glass slides silanized with APTS was incubated in 2% Glutaraldehyde for one hour at room temperature (0.1M phosphate buffer, pH 8.0). After an hour, the slides were thoroughly rinsed with buffer. The color change of the slides from yellow to light pink was observed after incubation. **Figure 3.10** shows the Ti-Nanoxide coated silanized glass slide with glutaraldehyde.

55

Glutaraldehyde

Figure 3.9: Chemical structure of Glutaraldehyde

Ti-Nanoxide coated APTS silanized glass slide with Glutaraldehyde
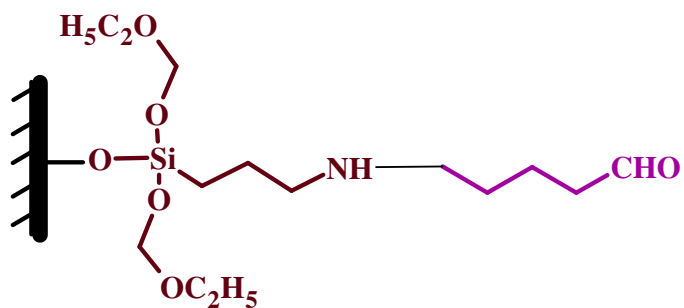
Figure 3.10: Chemistry of the Ti-Nanoxide, APTS silanized glass slide with Glutaraldehyde

g) Immobilization of Biorecognition elements

The bio-recognition elements used in this study is Organophosphorus Hydrolase (OPH). **Figure 3.11** shows the structure of OPH. The 7 lysines present on the surface of the enzyme shown in yellow, enable covalent immobilization to other biomolecules or reagents. The structural and functional characteristics of OPH have already been described in the literature review section.

Figure 3.11: Structure of OPH. The yellow regions represent the lysines present on the enzyme surface

OPH was immobilized on the working channel, and the reference channel contained BSA as a scaffold protein for CNF immobilization, in order to differentiate between the enzymatic and non-enzymatic based pH changes. BSA is a 67KDa protein that in found in abundance in plasma. It has 59 primary amines with 30-32 of them present on the surface of its structure.

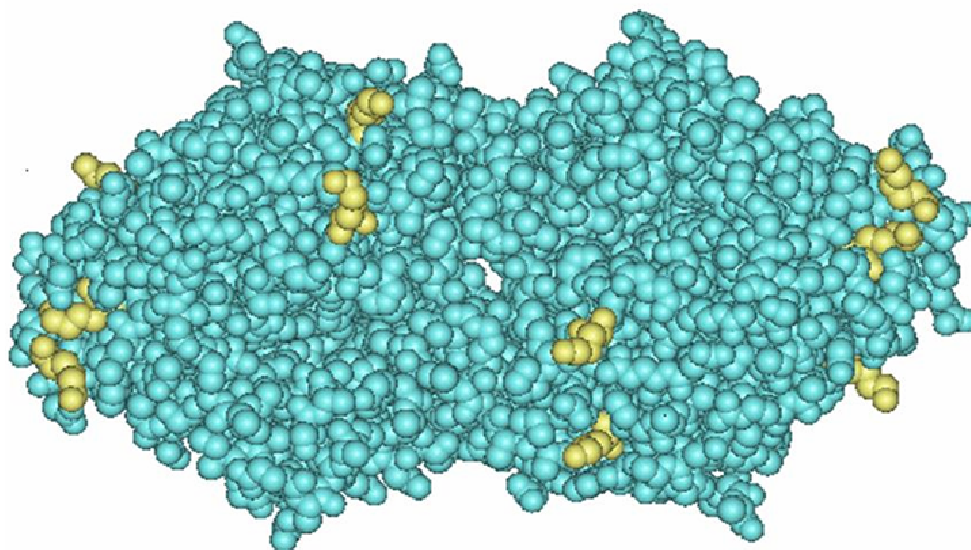Before immobilization, the enzymatic activity of OPH was checked by performing an OPH-Paraoxon assay using the UV spectrophotometer. The extinction coefficient of the product of enzymatic reaction, Para-nitrophenol used for calculation was 17000 at absorbance of 405nm. The enzyme obtained from Dr. Wilds' lab at Texas A&M normally has a concentration ranging from 1-2.5mg/ml. Its concentration in this study was increased to 3.5-4.0mg/ml by centrifugation process using centrifugal filter units having a nominal molecular weight cut-off limit of 30KDa. The concentration of the resulting enzyme was checked using the UV spectrophotometer. The extinction coefficient of OPH is 58000. The concentration of bovine serum albumin prepared in 0.1M phosphate buffer with pH 8.0 was kept similar to that of OPH.

The slides prepared with Glutaraldehyde were placed on the incubation holders (IH) that were described in section 3.3 of this chapter. The hematocrit tubes were placed on the wells of the IH. The activated sensor surface were incubated with 2 rows of working channel and reference channel each for at least 36 hours at 4° C. Humid environment was created to prevent any evaporation of the proteins. BSA with CNF was immobilized on the lower rows of the slide while the OPH with CNF was immobilized on the upper rows. This was done to avoid any false positive response of the sensor as the analyte flow direction was from the bottom to the top of the slide. The total protein

volume (OPH/BSA) required for incubation per row was less than 10µl. After 36 hours, the slides were thoroughly rinsed in de-Ionized water, air dried and placed on the IH for immobilization of the fluorophore.

f) Fluorophore conjugation

The pH sensitive fluorophore used in the study was carboxy napthofluorescein (CNF). It has an excitation wavelength of 598nm and emission wavelength of 668nm(http://probes.invitrogen.com/servlets/structure?item=652, 2006). **Figure 3.12** shows the structure of CNF.

Carboxynaphthofluorescein
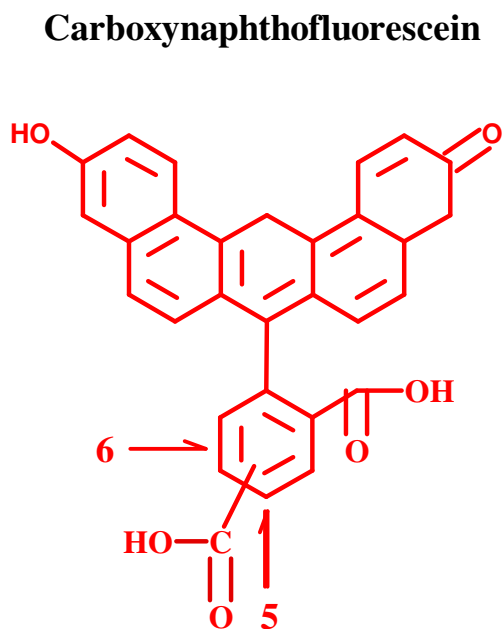
Figure 3.12: The chemical structure of CNF

(http://probes.invitrogen.com/servlets/structure?item=652, 2006)

The CNF used was functionalized with NHS ester to enable its conjugation to the Biorecognition elements immobilized on the glass slide. The reaction between the CNF having NHS ester with the primary amine of the proteins is shown in **Figure 3.13**.
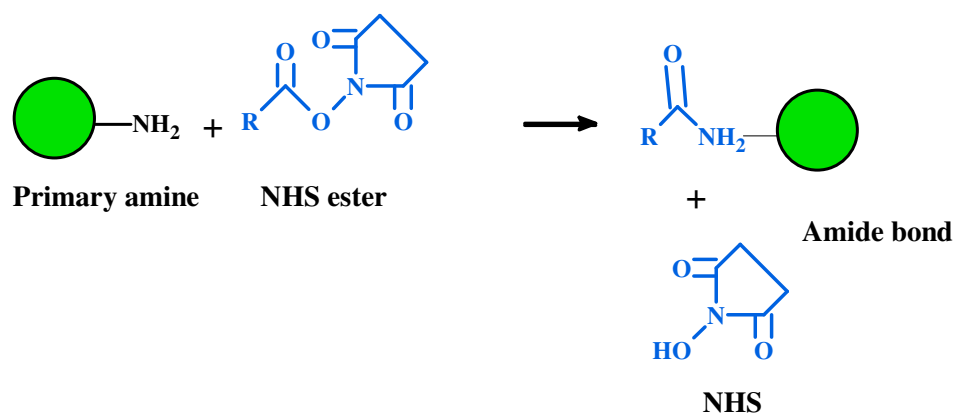
Figure 3.13: Reaction between the primary amine and the NHS ester of the CNF

The slides with the proteins were thoroughly rinsed in de-ionized water and air-dried. They were placed on the IH with the hematocrit tubes. The immobilization procedure for CNF was similar to that used for proteins. 1mg/ml solution of CNF was prepared in 10mM, 8.0 pH Ches buffer. DMF was used as the organic solvent to dissolve the CNF. The slides were immobilized with CNF for not more than 8 hours at 4º C. After 8 hours incubation, the slides were rinsed with DI water and stored in phosphate buffer at 4º C with cobalt chloride present to maintain the activity of OPH.

3. 6 BUFFERS

Ches was the buffer used for rinsing purposes and for sample preparation. The strength of the buffer was chosen such that the pH response of the sensor is not compromised, while at the same time, the pH stability of the system is maintained. In this study, stock solution of 10mM Ches was prepared and diluted to 1mM for experimental purposes. The recipe of the 10mM CHES buffer in 1L is given in the **Table 3.2**.

Name of the Salt	Weight (g)	Concentration (mM)
Ches	2.07	10
Potassium Chloride	2.04	2.7
Sodium Chloride	70.2	120
Cobalt Chloride	0.1189	0.05

Table 3.2: Constituents of Ches buffer

3. 7 SAMPLE PREPARATION

Stock solutions of Paraoxon purchased from the Chem. Service Inc. were prepared using the protocol provided by the manufacturers. These stock solutions were stored in the freezer (-20°Celsius) for further experimental purposes. Before the experiment samples of required concentrations were prepared from the stock solution, using 1mM Ches buffer in pH 8.2-8.3. This was done to keep the pH variations between the sample and the rinsing buffers as low as possible.

3. 8 EXPERIMENTAL PROCEDURE:

The patterned slide was rinsed with water, air-dried and placed in the 6 channel flow chamber of array biosensor. A 54mm, long, hollow, cylindrical aluminum tube with an 8mm diameter opening was fitted onto the laser diode in order to restrict the laser beam to fall only on the line generator. From the line-generator, the laser-beam was directed at the edge of the slide to create evanescent waves that were used to excite the fluorophores on the waveguide surface, creating a fluorescence array. A CCD camera was used to monitor the changes in florescence intensity during the assay performed under room temperature conditions. Q-Capture Suite software (Q-Imaging, USA) was used for taking the snaps, and a TIFF analysis program developed by the NRL, was used for data analysis. The rinsing buffer, 1mM Ches, pH 8.2 was introduced at the flow rate of 4ml/minute using an ISMATEC peristaltic pump, and the fluorescence array pattern was monitored with an exposure time of two seconds. After the background signal level was established, the OP samples prepared in buffer, was flowed for 15 s and the flow was stopped. Six consecutive snaps were taken with a ten second time interval between snaps. The slopes were calculated for the initial 30 second period after the sample introduction in order to give the true representation of first order catalytic reaction.

3.9 SURFACE CHARACTERIZATION STUDIES:

The uniformity of the Ti-Nanoxide coating on the glass slides were studied using Scanning Electron Microscopy (SEM, JEOL JSM-840). In order to make the glass slide surface conductive, gold sputtering was performed. The topographical studies on the glass slide coated with Titanium dioxide paste was done using (AFM, Pacific Nanotechnology, Nano-R), non-contact mode.

CHAPTER 4

RESULTS AND DISCUSSIONS

4. 1 SEM STUDIES

SEM studies were performed on the slides coated with Ti-Nanoxide in order to know the following

a. uniformity of Ti-Nanoxide coating

b. difference between hand coated and spin coated slide

c. difference between diluted and non-diluted slide

Absolute ethanol was used for the dilution of Ti-Nanoxide. The **Figures 4.1, 4.2** and **4.3** shows the dilute (3:2) Ti-Nanoxide hand coated slides, the Non-Diluted Ti-Nanoxide Hand coated slides and the diluted (1:10) spin coated Ti-Nanoxide glass slides respectively. All the slides studied were sintered at 450°C for 30 minutes and cooled down. They were sputtered with gold in order to give a conductive surface required for SEM studies.

The **Figures 4.1, 4.2** and **4.3** appeared to show uniform and similar coating of Ti-Nanoxide. However, the ten times diluted spin coated slide appears to look denser when compared to the hand coated 3: 2 diluted and non-diluted slides.

The thicknesses of the slides were measured using a profliometer. It was determined that the coatings on the slide were thicker by nearly 50nm on the edges than

on the center. Also, the center thickness variation was approximately 10-20nm. The thickness of non-diluted Ti-nanoxide was approximately 2μm while for the 3: 2 dilute Ti-nanoxide, it was approximately 800nm. The spin coated Ti-nanoxide slide with 10 times dilution had a thickness of approximately 500nm. The spin coated slide had a mask during spin coating to have regions on the glass slide without Ti-nanoxide to enable thickness determination. Spin coating was performed at a speed of 3000rpm for 30s with 400ml of paste placed on the slide.

The 10 times diluted Ti-nanoxide coating did not produce any good results for paraoxon assays (lowest detectable concentration for paraoxon was 0.1mM). The non-diluted and the diluted (3:2) coatings had better sensitivities. However the problems of diffusion were experienced with the non-diluted slides and hence, the 3:2 dilution was used to prepare the sensor surface for all experimental purposes.
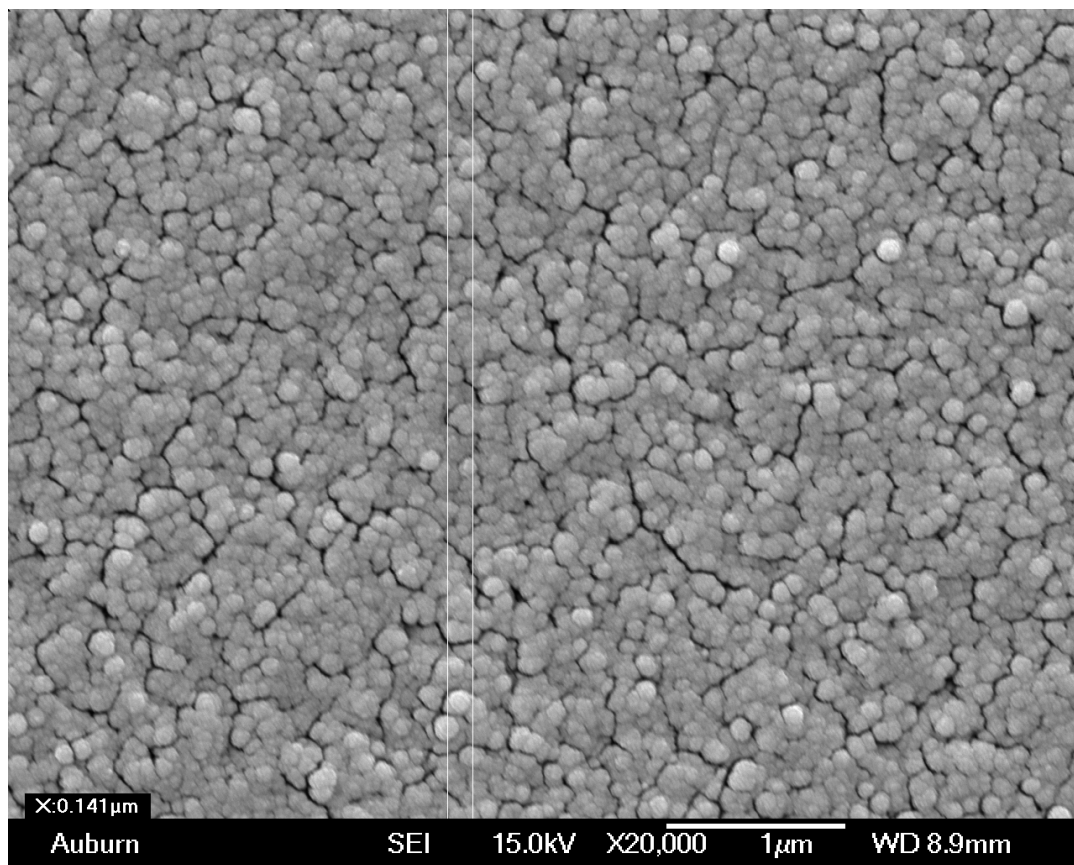
Figure 4.1: SEM image of glass slide hand-coated with diluted Ti-Nanoxide paste. The ratio of Ti-Nanoxide to absolute ethanol was 3: 2. The slide after coating was dried and sintered at 450°C for 30 minutes and cooled. Gold sputtering was performed on the slide to give it a conducting surface. The size of nano-particle is 0.141µm.
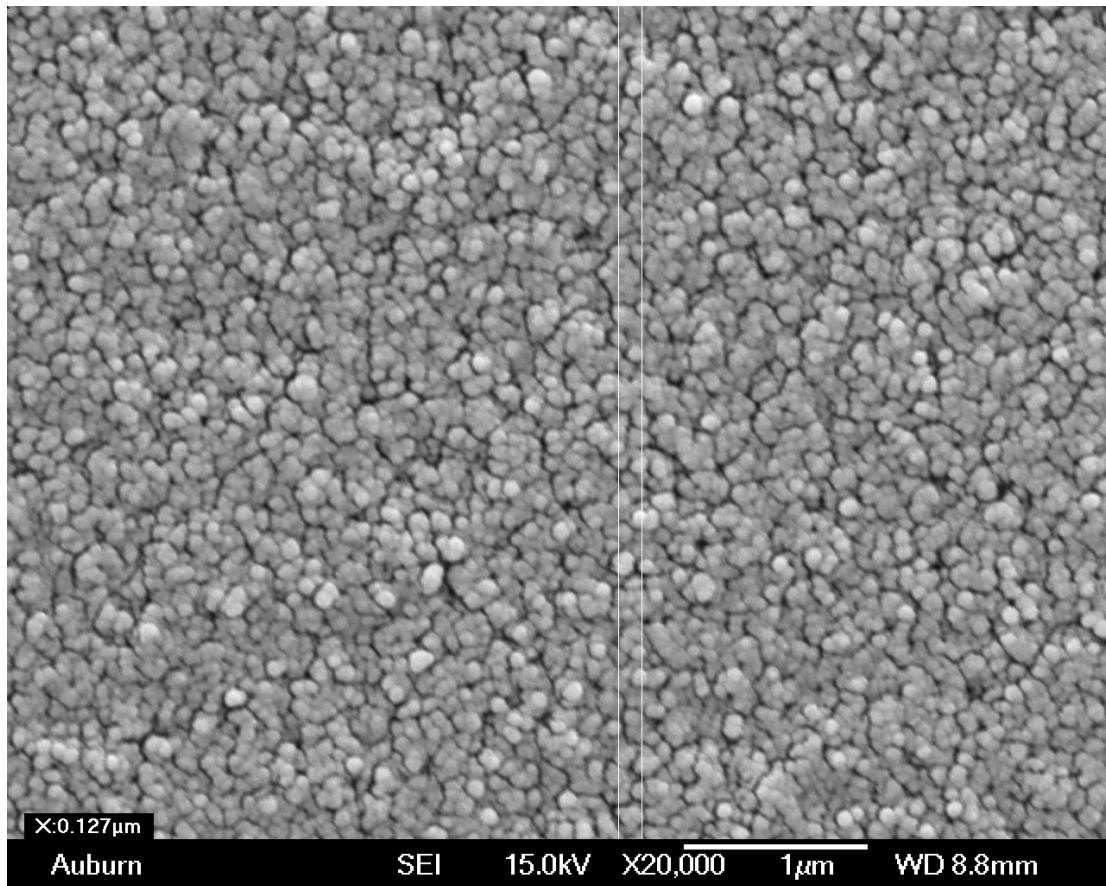
Figure 4.2: SEM image of the Non-Diluted hand coated Ti-Nanoxide glass slide. The slide after coating was dried and sintered at 450°C for 30 minutes and cooled. Gold sputtering was performed on the slide to give it a conducting surface. The size of the nano-particle is found to be 0.127μm.

Figure 4.3: SEM image of ten times Diluted spin coated Ti-Nanoxide glass slide. The slide after coating was dried and sintered at 450 deg Celsius for 30 minutes and cooled. Gold sputtering was performed on the slide to give it a conducting surface. The size of the nano-particle is found to be 0.127μm
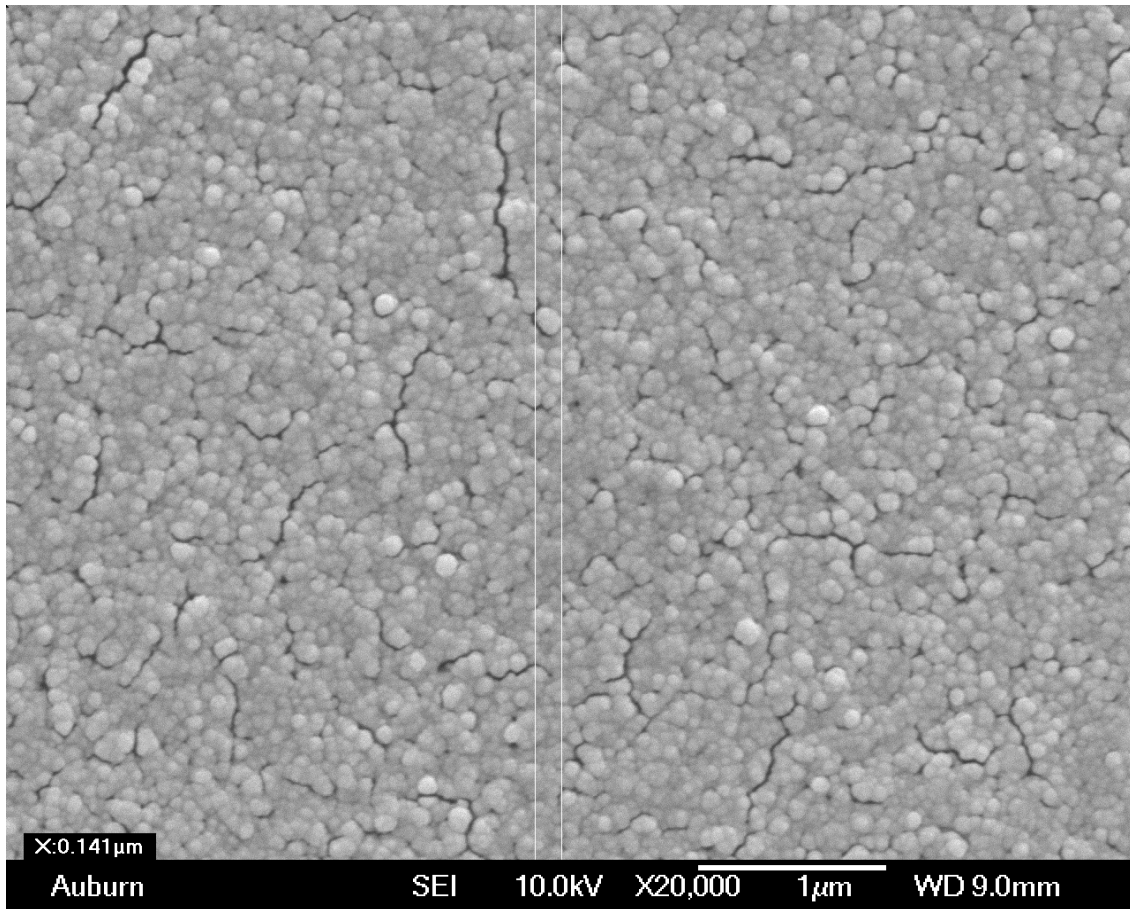
4.2 AFM STUDIES:

The topographical studies of Titanium nanoxide coating on the waveguide was studied using Atomic Force Microscopy (AFM, Pacific Nanotechnology, Nano-R), in non-contact mode. Glass slides hand coated with Titanium nanoxide paste that was not diluted, diluted with ethanol in the ratio of 3:2 and spin coated when diluted in the ratio of 1:10 with ethanol were studied for surface roughness determination. **Table 4.1** details the root mean square (RMS) values of these glass slides coated with Titanium nanoxide pastes. **Figures 4.4**, **4.5** and **4.6** show the AFM images of these slides.

Coat	Coating method	Root mean square value (nm)	Total Area (μm2)
TiO$_2$ paste	Hand Coat	58	96
TiO$_2$: Ethanol 3:2	Hand Coat	20	96
TiO$_2$: Ethanol 1:10	Spin Coat	20	96

Table 4.1: Root mean square values of the glass slides coated with Titanium nanoxide.
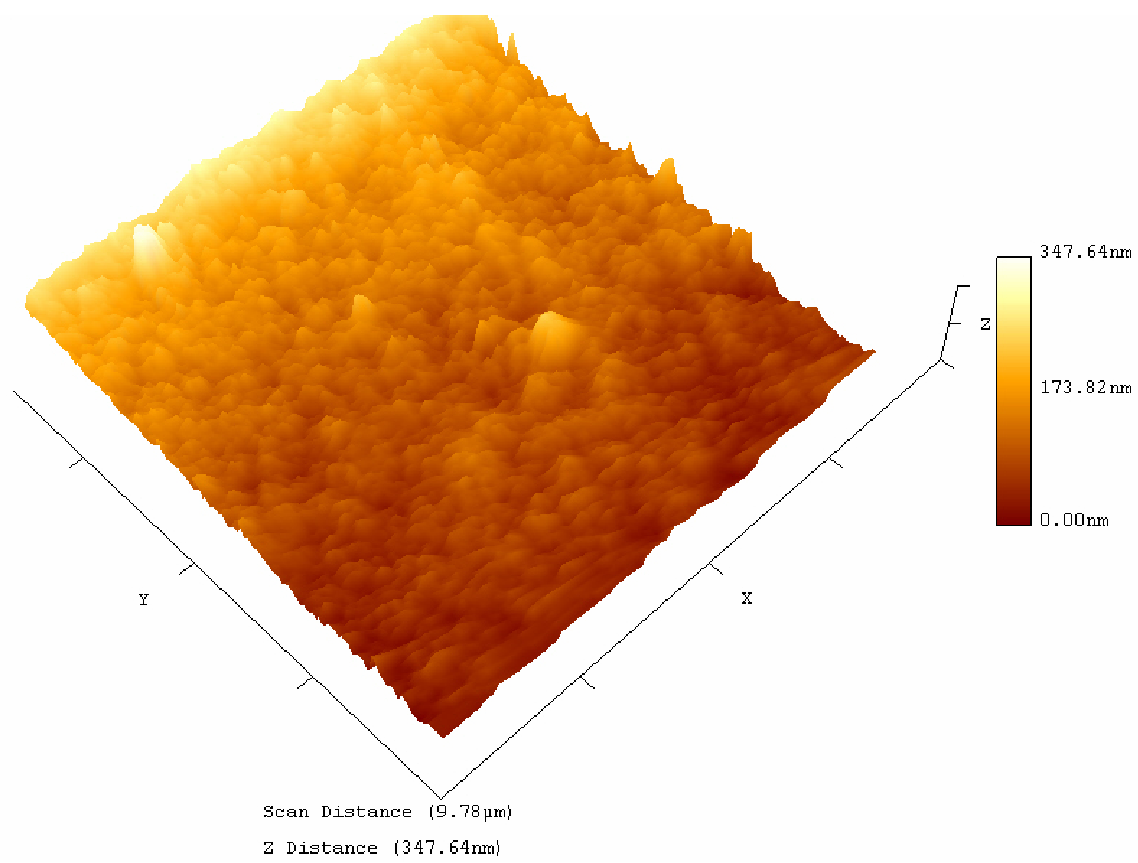
Figure 4.4: AFM image of glass slide hand coated with Titanium nanoxide in non-contact mode.
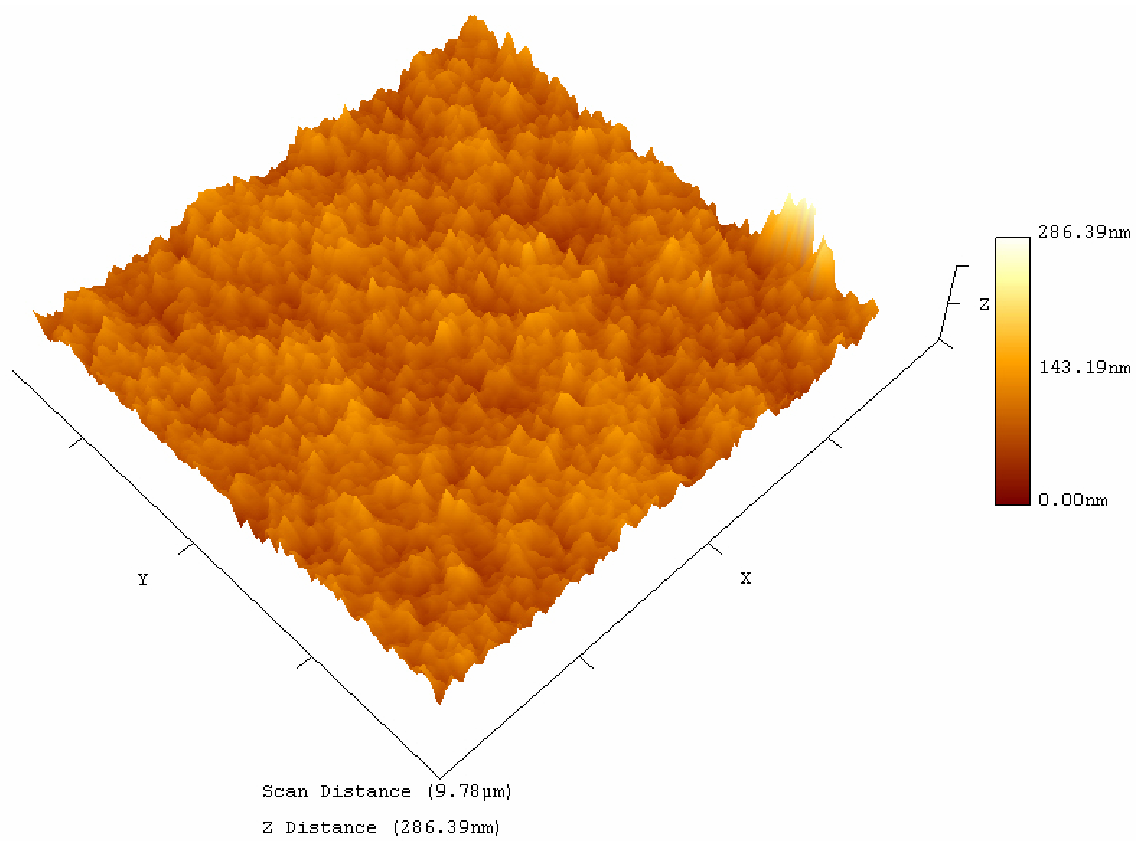
Figure 4.5: AFM image of the glass slide hand coated with Titanium nanoxide paste diluted with ethanol in the ratio of 3:2 (TiO_2: ethanol)

225.68nm

Z

112.84nm

0.00nm

Y

X

Scan Distance (9.78μm)

Z Distance (225.68nm)
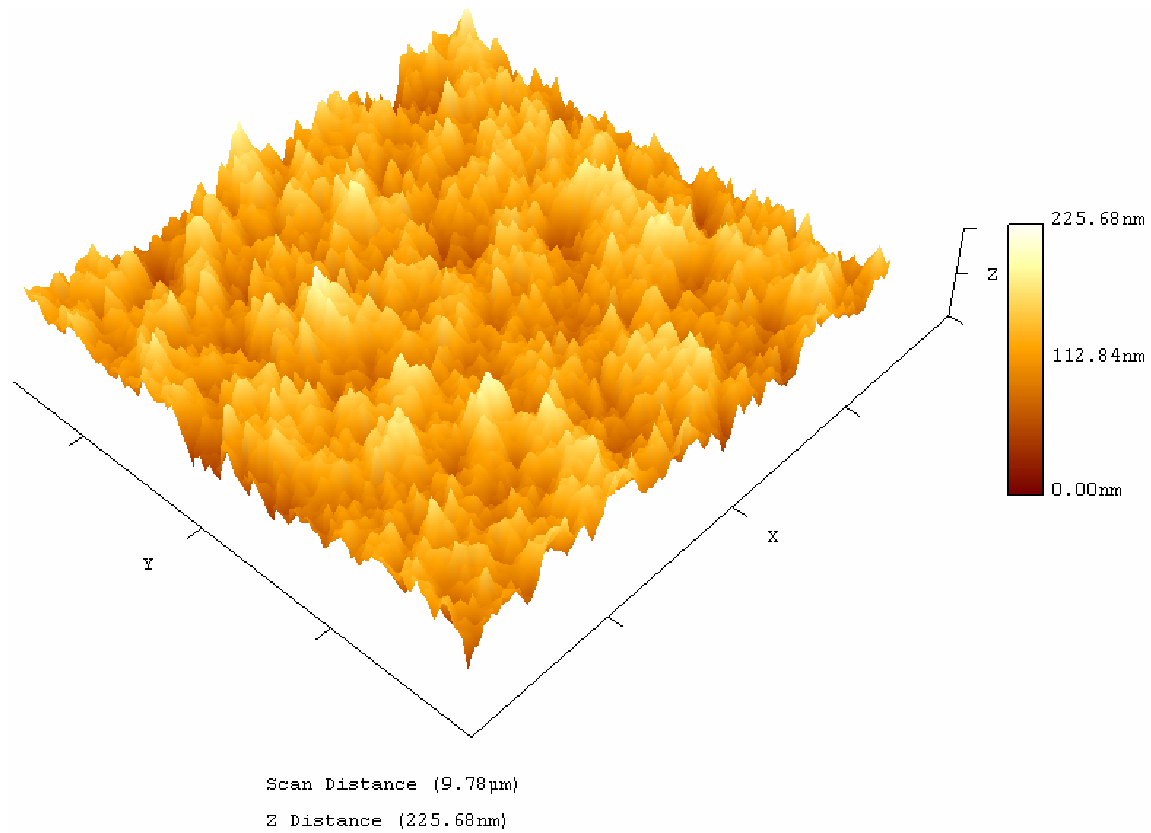
Figure 4.6: AFM image of the glass slide spin coated with Titanium nanoxide paste diluted

with ethanol in the ratio of 1:10 (TiO$_2$: ethanol)

4.3 PH DEPENDENT SPECTRA OF CNF

Carboxynaphthofluorescein, CNF is a pH sensitive fluorophore with an excitation and emission wavelength of 598nm and 668nm respectively at pH 10.0. In order to obtain the pH dependent spectra of CNF, 2ml of 10mM CHES buffer containing 0.1mg/ml CNF was taken in a 2 sided cuvette and emission scans with an excitation wavelength of 598nm were performed on the fluorimeter (Photon Technology International) for different pH ranging from 8.0 to 9.0. As seen from the graph in **Figure 4.7**, the peak intensity, which was obtained at 650nm, was found to decrease with increasing hydrogen ion concentration. As the excitation source used in the array biosensor has a wavelength of 635nm instead of 598nm, it was necessary to investigate the effect of excitation wavelength at 635nm on fluorescent intensity of CNF **(Figure 4.8)**. Studies on the fluorimeter showed more than 50% loss in fluorescent intensity due to excitation at 635nm instead of 598nm (**Figure 4.9**).
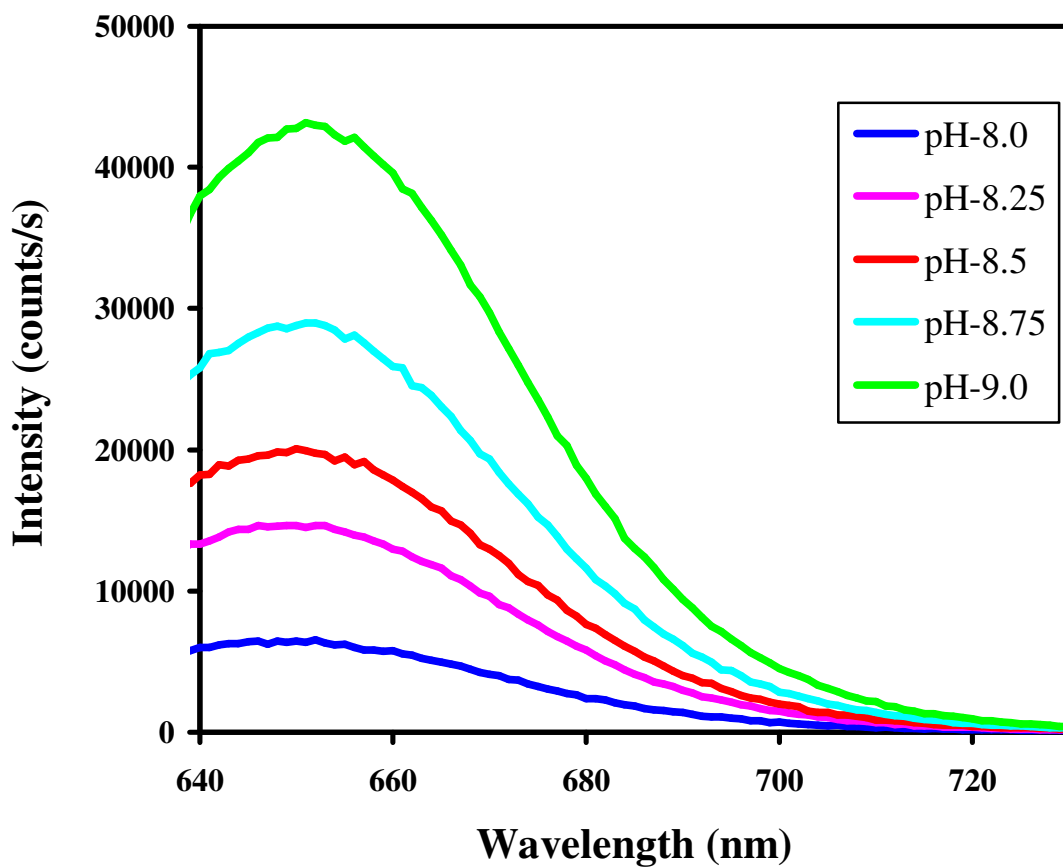
Figure 4.7: pH dependent emission spectra of CNF for an excitation wavelength of 598nm.

The concentration of the CNF was 0.1mg/ml in 2ml, 10mM CHES buffer
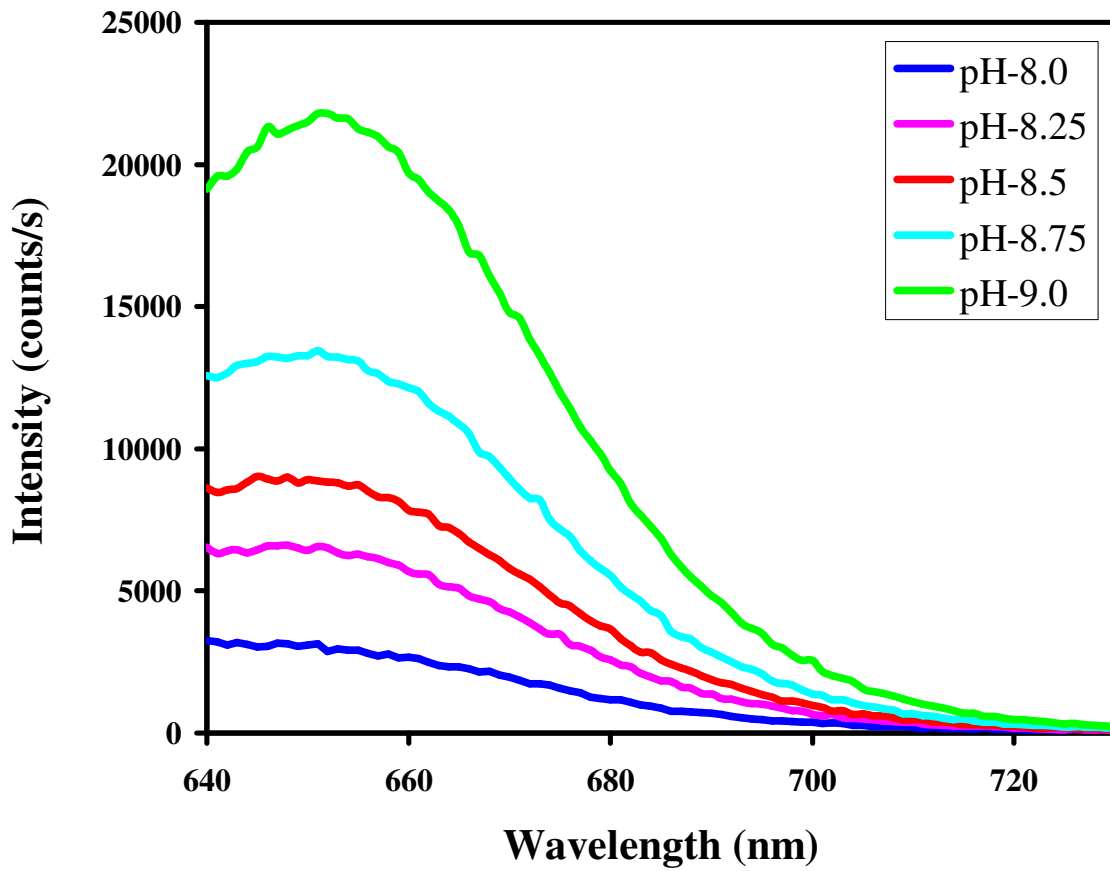
Figure 4.8: pH dependent emission spectra of CNF for an excitation wavelength of 635nm.

The concentration of CNF was 0.1mg/ml in 2ml, 10mM CHES buffer
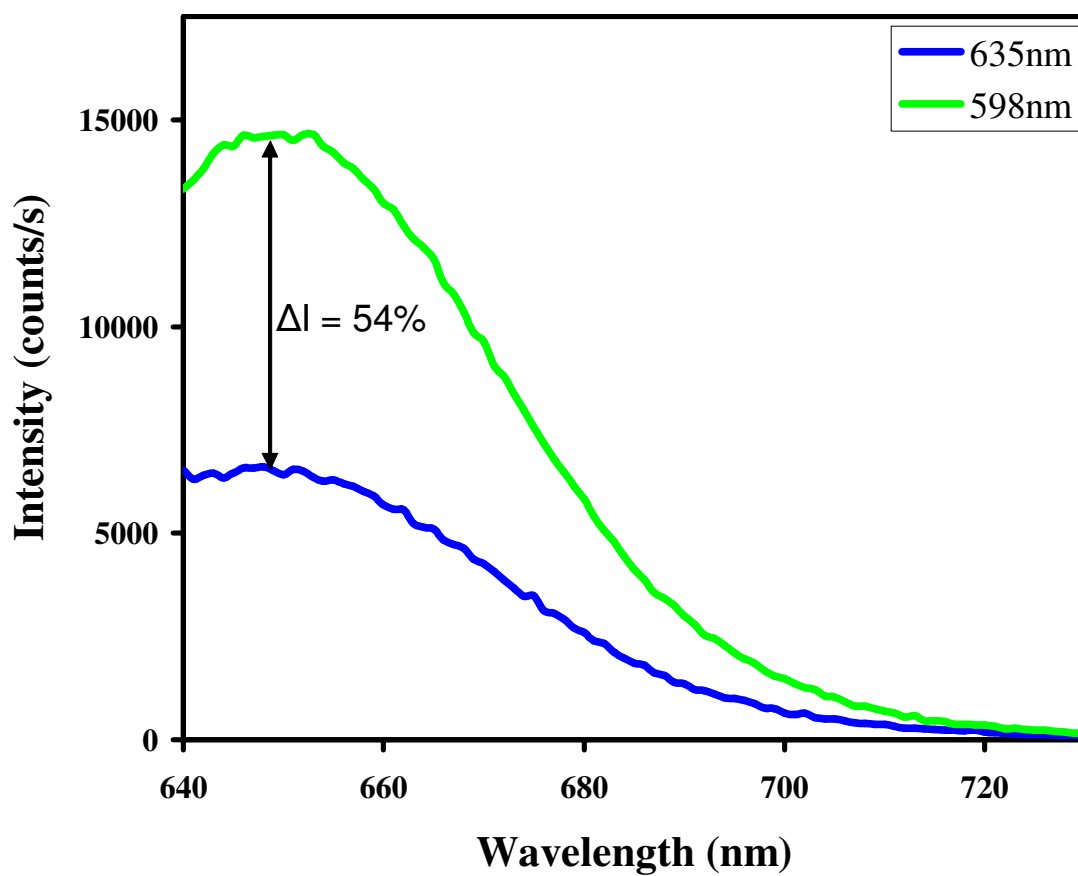
Figure 4.9: Emission wavelength intensities for excitation wavelengths of 598nm and 635nm in 10mM CHES buffer, pH 8.25

4.4 AMINO-SILANE FUNCTIONALIZED WAVE GUIDE

4.4.1 pH response

An experiment to determine the pH responsiveness of the amino-silane functionalized glass slides immobilized with proteins and fluorophore was performed by injecting 25mM CHES buffer for pH ranging from 7.2 to 8.6. **Figure 4.11** shows that the response of both the reference and the working spots (highlighted in **Figure 4.10**) are positively correlated to changes in pH. Also, we can see that the intensity response to pH above 8.3 is not as much as seen for pH range of 7.9 to 8.3. Based on the results obtained for other spots (8 reference and 8 working), it was found that the coefficient of variance was less than 30%.
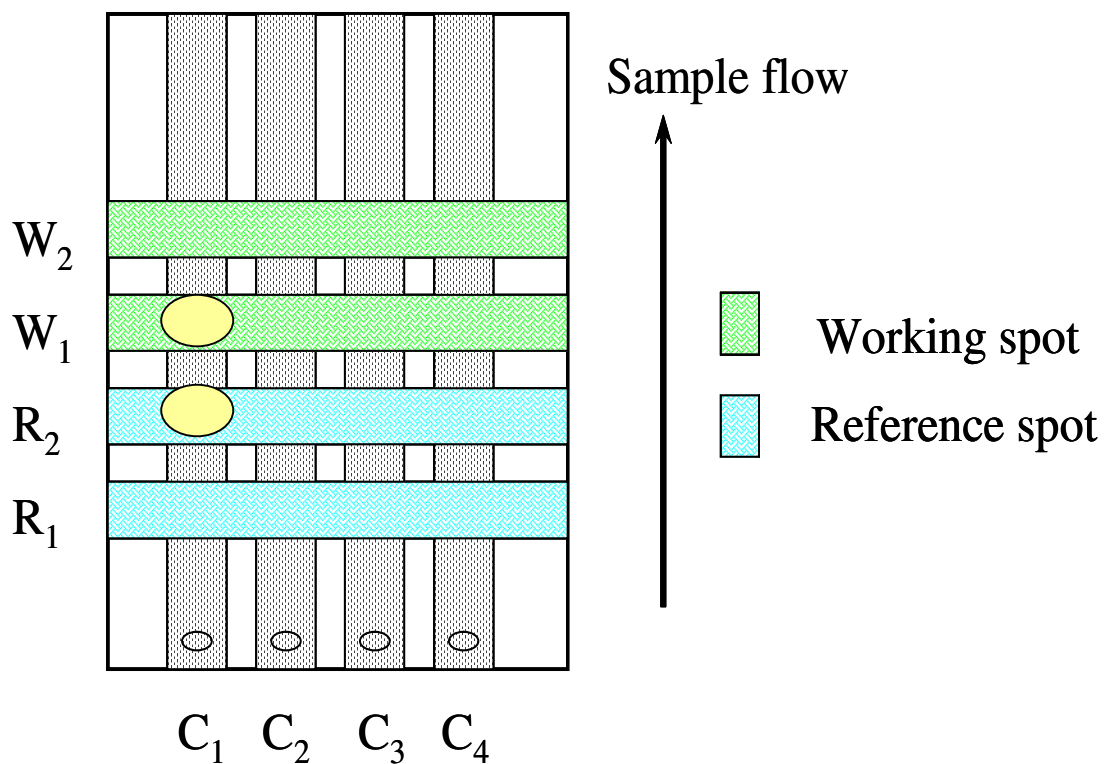
Figure 4.10: Schematic of the glass slide with the working and the reference spots. The spots that are highlighted were considered for data analysis in **Figure 4.11**
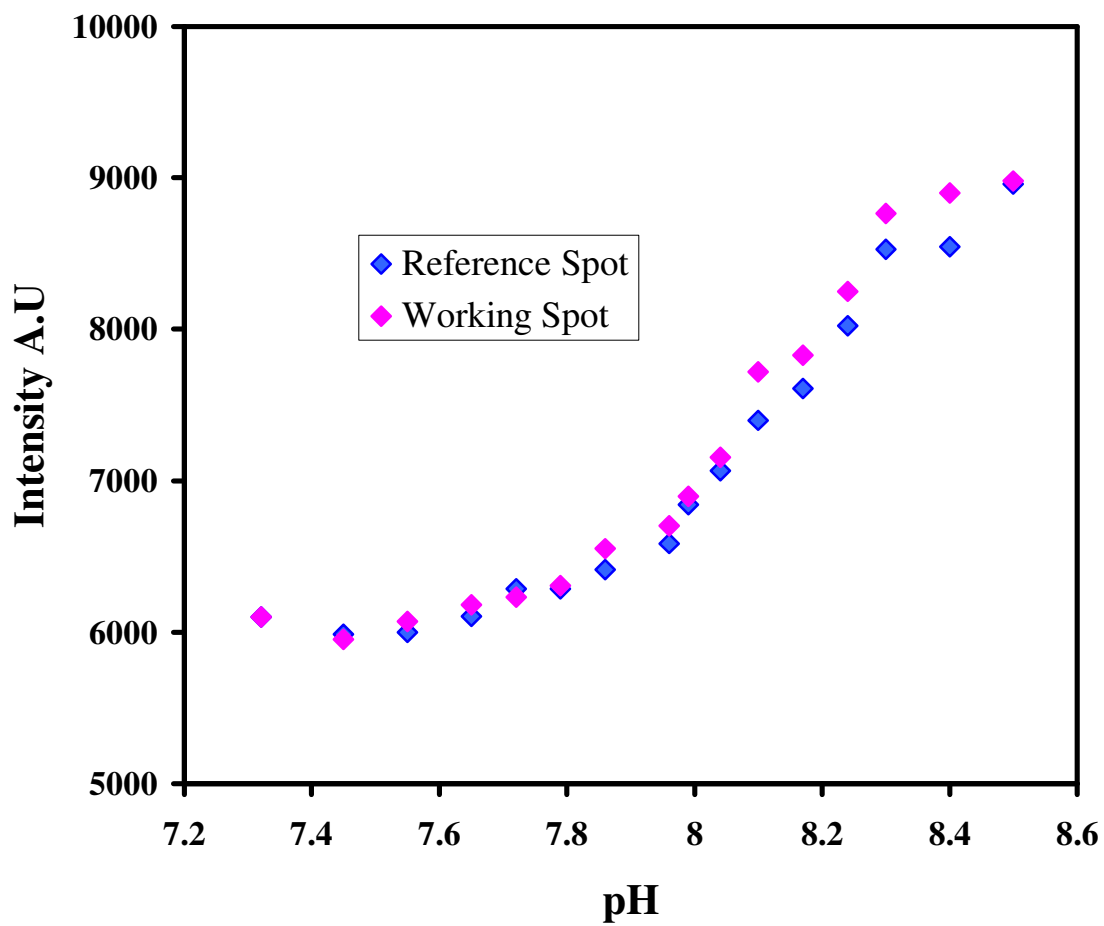
Figure 4.11: The changes in Net fluorescent intensities of the working and reference spots in a particular channel of the glass slide with increasing pH for 25mM CHES Buffer

4. 4. 2 Enzymatic action

To show the enzymatic action of the OPH immobilized on amino-silane functionalized glass slide, 0.5mM paraoxon (Paraoxon) was injected on the channel having the working and the reference spots. The highlighted spots in **Figure 4.10** were considered to study the enzymatic response. It can be seen from the graph in **Figure 4.12** that the working spot shows a much greater drop in intensity on the Paraoxon injection than the reference spot. This drop in signal may be attributed to the hydrogen ion production as a result of catalytic OP hydrolysis. Also, from the graph, it can be concluded that it takes approximately 30-35s for the realization of the first phase of catalytic hydrolysis after sample introduction. Thus, for all experiments the % change in intensity was calculated for the first 30s after sample introduction. The drop in intensity for reference channel may be attributed to the pH variations between buffer and paraoxon sample.
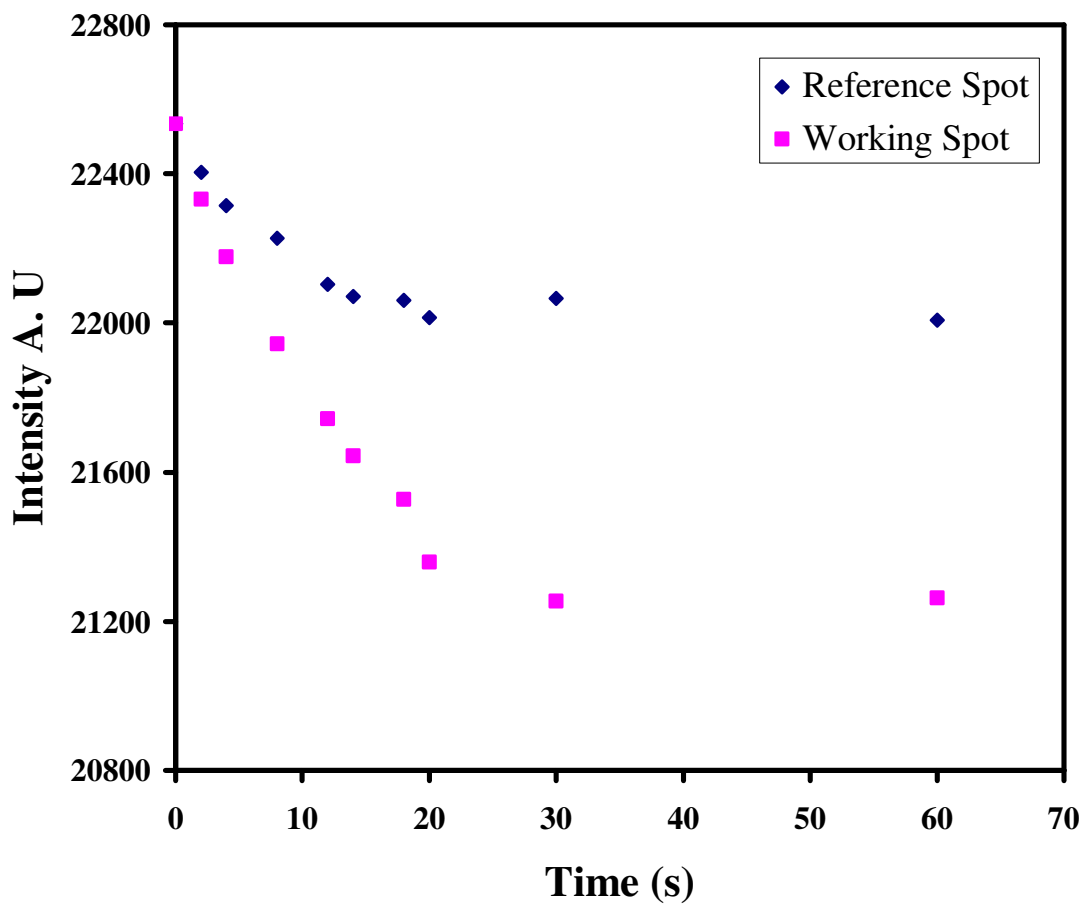
Figure 4.12: Response of the reference and the working spots to 0.5mM Paraoxon prepared in 1mM CHES, 8.2 pH.

4. 4. 3 Response curve

  The **Figure 4.13** shows a concentration-signal relationship for paraoxon by the sensor taking a single pair of working and reference spots into consideration. The differences in percentage change in intensity between the reference and working spots after 30s of sample introduction gave the actual changes in intensities due to enzymatic hydrolysis. **Figure 4.10** shows the spots that were considered for response curve analysis. From the graph, it can be seen that although response was obtained for concentrations as low as 3µM, the lowest concentration of paraoxon that could be detected was 6µM taking the signal to noise ratio into account.
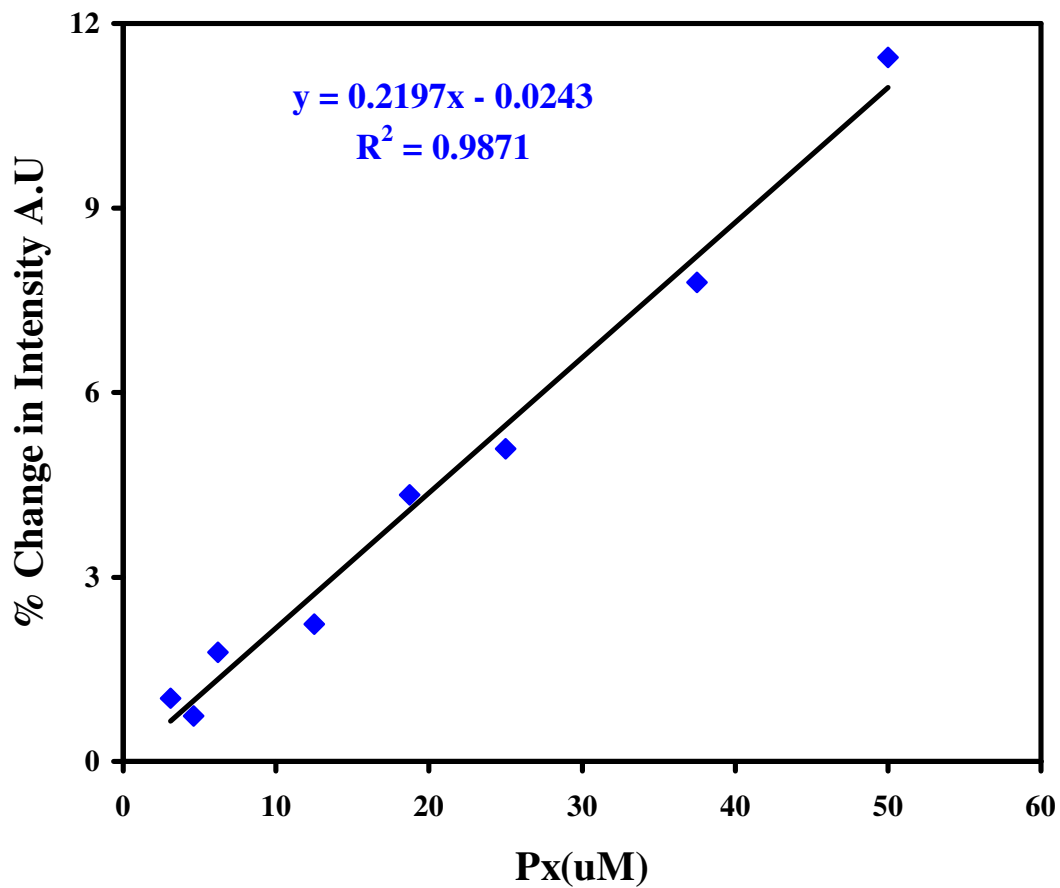
Figure 4.13: Response curve showing the linear region for Paraoxon concentrations of 3μM, 4μM, 6μM, 12μM, 18μM, 25μM, 37μM and 50μM in 1mM CHES with pH of 8.2-8.3.

4. 4. 4 Stability of the Sensor

The stability of the proteins immobilized on the glass slide was determined by obtaining the response curve for different days after the sensor preparation. **Figure 4.14** shows the response curves for Day 1, Day 13 and Day 23 after the sensor preparation. It can be seen from the graph that even after 23 days of sensor preparation, the OPH on the sensor was still active enough to be able to detect the presence of Paraoxon. The lowest detectable concentration of paraoxon however reduced from 6μM to 10μM.
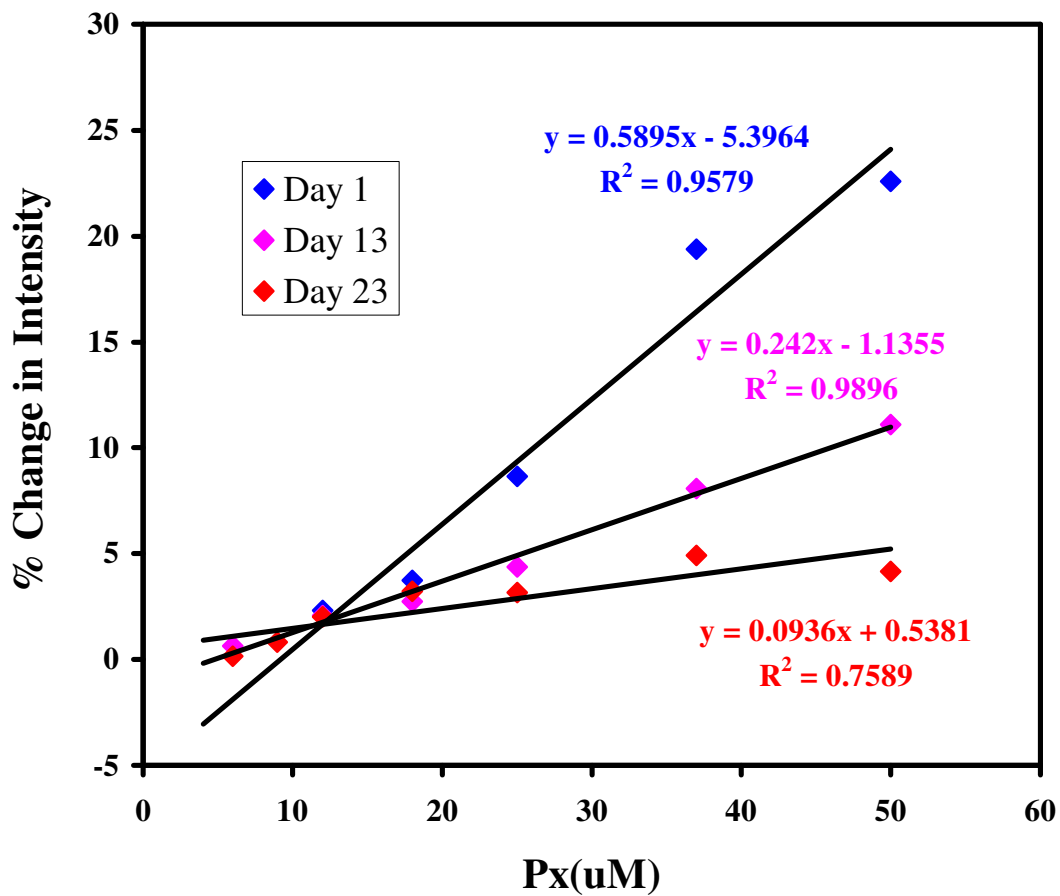
Figure 4.14: Response curves for day 1, day 13 and day 23 after sensor preparation. The paraoxon concentrations used were 3μM, 6μM, 12μM, 18μM, 25μM, 37μM and 50μM.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 CONCLUSIONS:

An optical array biosensor installation was developed for enzyme kinetics monitoring by fluorescence spectroscopy. The Array biosensor detection platform developed at Naval Research Laboratories was significantly modified to suit the needs of enzyme-based detection. New incubation holders were developed using plexiglass as a replacement for the physically isolated patterning method using the PDMS which was easy and reusable for patterning enzymes and fluorophores on the glass slide surface. Ti-Nanoxide coating used to boost the surface properties of the glass slide physically and optically provided good characterization for the immobilization of the Biorecognition elements and fluorophores. SEM and AFM enabled the surface characterization studies.

The response of CNF conjugated to BSA and OPH to changes in micro-environmental pH were similar. Hence, BSA acted as a good scaffold protein for CNF and functioned as a reference in order to discriminate between the catalytic and non-catalytic based pH changes. The lowest detectable concentration of paraoxon was 6μM which is below the lethal dosage level (7μM/kg in rats and mouse). Good storage conditions of the sensor in 10mM Phosphate buffer with cobalt chloride under

refrigerated conditions enabled enough retention of OPH activity (less than initial 10%) to detect paraoxon concentration as low as 10μM after 30 days of preparation.

5.2 FUTURE WORK:

This work can be extended for the detection of different OPs having P-S and P-F bonds using the OPH mutants with tailored substrate specificity, developed at Texas A&M University and the OPAA enzyme having only P-F bond activity. By immobilizing another enzyme, AChE along with the other mutants of OPH, discriminative detection of other pesticides from OPs that inhibit AChE but are not hydrolyzed by OPH can be achieved. Detection of OPs in environmental samples and studies on the effect of interferents like heavy metal ions on the detection of OPs can be performed. Also, it is possible to combine the above sensing strategy with the already developed NRL detection methods for other bio-threat agents, thereby enabling both chemical and biological detection simultaneously. Reducing the sample volume requirements by using micro-sized flow channels, replacement of the CCD camera with compact photodiode array and use of LED with tunable wavelength instead of laser as the excitation source of fluorophore can enable the miniaturization of the whole system.

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