Development Of Functional Material Scaffolds for Sensing Applications

by

Madhumati Ramanathan

A dissertation submitted to the Graduate Faculty of Auburn University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

> Auburn, Alabama May 14, 2010

Keywords: Array biosensor, titanium dioxide, silica, immobilization, lysozyme, florisil

Approved by

Aleksandr L. Simonian, Chair, Professor of Materials Engineering Jeffrey W. Fergus, Associate Professor of Materials Engineering ZhongYang Cheng, Associate Professor of Materials Engineering Curtis G. Shannon, Professor of Chemistry

Abstract

Developments in the field of chemical and biological sensors call for sensitive and selective sensing surfaces to provide consistent information about target analytes. Since such sensors are mostly operating in complex multi-component environments, their interaction with interferent can have deleterious effects on their activity. Thus, stable sensing surfaces form the most critical aspect of a biosensor design and their development requires reliable surface morphology and chemistry for allocation of chemical and bio-recognition elements.

Studies in this dissertation were aimed at developing modified titanium dioxide nanoparticles using techniques such as silanization and cross-linking to act as scaffolds for stable immobilization of proteins via direct covalent bonding or encapsulation in silica particles during its bio-inspired lysozyme mediation. Spatially defined presence of protein-reporter agents, on transparent titania nanoparticles coated on glass slides, enabled the development of fluorimetric array biosensors for simultaneous detection of multiple analytes. The different approaches used to develop the functional layers were compared in terms of biosensor sensitivity and stability.

Organophosphorus hydrolase (OPH) was used as the model biosensing enzyme to show the potential application of the above chemistries for the detection of organophosphate (OP) neurotoxins. Reporter pH responsive fluorophores conjugated to enzyme transduced the catalytic hydrolysis of OPs by OPH, into a measurable optical signal. The developed sensor has potential applications for the detection of OP pesticides in environmental samples. As an extension to OP detection, the principle of molecular recognition based on thermodynamic-complex formation of fluoride ions with aluminum(III) octaethylporphyrin contained in a plasticized film with chromoionophore was exploited for the detection of organophosphofluoridates like Diisopropyl Fluorophosphate (structural analogue of Sarin and Soman chemical nerve agents).

Studies also included the activation of magnesium silicate (florisil) particles with reactive chemicals for the development chemical sensors. Modified Nash reagent was utilized in the form of fluoral-P and adsorbed onto florisil micro-particles. These pre-activated particles were attached to the glass slide surface via tape and via dispersion in polydimethyl siloxane (PDMS) matrix. Fluorimetric quantification of photoluminescent product of formaldehyde and fluoral-P activated florisil, namely, 3,5-diacetyl-1,4-dihydrolutidine (DDL) allowed for the detection of formaldehyde.

Acknowledgements

I would like to express my deep sense of gratitude to my mentor, Prof. Aleksandr L. Simonian, for his constant motivation and support during my entire course of study at Auburn. His wisdom, guidance and encouragement have been invaluable in enabling me to develop the confidence and aptitude necessary to realize my goals.

I would like to thank my committee members, Profs. Jeff. Fergus, ZhongYang Cheng and Curtis Shannon for their useful discussions and suggestions in my research. I would also like to thank Dr. Virginia Davis for agreeing to be my external reader. None of this work would be accomplished without the enzyme OPH, generously provided by Prof. James Wild and his team at Texas A&M. I would like to thank Drs. Heather Luckarift, Mark Meyerhoff, Grigory Vertelov and John Lock for their support, discussions and suggestions with various aspects of the work presented in this dissertation.

I would like to thank Maj. Leamon Viveros for introducing to me the basic biochemistry techniques when I first joined the lab. I would additionally like to thank Dr. Joel Golden, for his time to help me become acquainted with Array Biosensor, during my initial stages of study. Ms. Lin Wang (University of Michigan, Ann Arbor) prepared the aluminum octaethyl porphyrin presented in this work.

A special note of appreciation goes to Roy and Alison, for their patience with my requests regarding various aspects of working in the department. I would like to thank our entire research group –Valber, Shankar, Sista, Ainur, Sheetal, Tony, Saroja, Markhabat and Sarah, for their support during my research studies.

I am grateful for being blessed with parents who have always encouraged me to pursue my dreams and have been supportive of my decisions. I would like to thank my Sister, Jeeju and Kaavya, for their support and encouragement throughout my studies and in all aspects of my life. Finally, I would like to thank my husband and parents-in-law, for their love, understanding and support, especially over the past months, while I have been busy with my dissertation writing.

Table of Contents

Abstract ii
Acknowledgements iv
List Of Tables xi
List Of Figures xii
1. Motivation of Research
1.1. Introduction1
1.2. Dissertation organization2
2. Chemical Sensors
2.1. Introduction
2.2. Classification of chemical sensors
2.2.1. Optical transducers7
2.3. Sensing elements9
2.3.1. Ionic recognition10
2.3.2. Molecular recognition11
2.3.3. Bio-molecular recognition13
2.3.3.1. Affinity biosensors
2.3.3.2. Catalytic biosensors15
2.3.4. Bio-Mimetic biosensors

2.4. Integration of sensing and transducing elements: Biosensors
2.4.1. Adsorption17
2.4.2. Covalent binding
2.4.3. Cross-Linking19
2.4.4. Encapsulation and entrapment19
2.5. Nanomaterials for sensing applications
3. Current Art in Sensors for the Detection of Organophosphates
3.1. Introduction
3.2. Toxicity of Organophosphates23
3.3. Need for detection25
3.4. Detection of Organophosphates
3.4.1. Conventional analytical techniques29
3.4.2. Inhibition based biosensors
3.5. Biosensing Element: Direct detection using Organophosphorus Hydrolase33
3.6. Multi-Analyte Sensing Platform: Array Biosensor
4. Array Biosensor Based on Enzyme Kinetics Monitoring By Fluorescence
spectroscopy: Application for Neurotoxins Detection
4.1. Introduction
4.2. Materials and Methods
4.2.1. Chemicals and reagents
4.2.2. Preparation of glass slides
4.2.3. Characterization of titanium dioxide nanoparticles
4.2.4. Sensing principle43

4.2.5. Experimental set-up44
4.3. Results and Discussions46
4.3.1. Characterization of the metal oxide nanoparticles
4.3.2. pH response53
4.3.3. Enzymatic reaction
4.3.4. Specificity of detection
4.3.5. Stability of the immobilized enzyme
4.4. Conclusions60
5. Lysozyme Mediated Formation of Protein-Silica Nanocomposites for Biosensing
Applications
5.1. Introduction
5.2. Materials and Methods64
5.2.1. Chemicals and Reagents
5.2.2. Instrumentation
5.2.3. Preparation of titanium dioxide coated glass slides
5.2.4. Functionalization of pretreated glass slides for lysozyme immobilization65
5.2.5. Formation of OPH encapsulated silica particles on pretreated glass slides67
5.2.6. Microscopic characterization of silica particles
5.2.7. Enzyme assays for OPH activity
5.2.7. OPH activity assays on Array biosensors
5.3. Results and Discussions
5.3.1. Formation of silica particles for OPH encapsulation
5.3.2. Covalent attachment of lysozyme for silica formation75

5.3.3. Encapsulation of fluorescent OPH for studies on array biosensor	77
5.4. Conclusions	80
6. Monitoring of Diisopropyl Fluorophosphate Hydrolysis by Fluoride Selective	
Polymeric Films Using Absorbance Spectroscopy	81
6.1. Introduction	81
6.2. Materials and Methods	85
6.2.1. Chemicals and Reagents	85
6.2.2. Preparation of aluminum octaethyl porphyrin (Al[OEP])	86
6.2.3. Preparation of (Al[OEP])-ETH 7075 cocktail	86
6.2.4. Preparation of slide coated with polymerized film	86
6.2.5. Instrumentation	87
6.2.6. Procedure	88
6.3. Results and Discussions	89
6.4. Conclusions	92
7. Formaldehyde Detection Using Activated Magnesium Silicate Particles	94
7.1. Introduction	94
7.2. Materials and Methods	96
7.2.1. Chemicals and Reagents	96
7.2.2. Florisil treatment with fluoral-P	96
7.2.3. Immobilization of activated florisil on glass slide	97
7.2.4. Instrumentation	98
7.2.5. Sensor characterization	99
7.3. Results and Discussions	100

7.3.1. Formaldehyde detection using activated magnesium silicate particles100
7.3.2. Activation of particles103
7.3.3. Response curve105
7.4. Conclusions110
8. Overall Conclusions111
9. Future work
9.1. Bioinspired mediation of titanium dioxide for coating on glass slides115
9.2. Enzyme stabilization at extreme pH conditions117
References

List of Tables

Table 2.1 Classification of evanescent wave sensors	9
Table 2.2 List of amino acids with ionizable side chains	18
Table 3.1 List of Organophosphates	27
Table 3.2 Some of the conventional analytical techniques to determine OP nerve age	nts
	30

List of Figures

Figure 2.1 Schematic representation of a chemical sensor
Figure 2.2 Classification of chemical sensors based on mode of transduction7
Figure 2.3 Modes of sensing element interaction with analyte10
Figure 2.4 Schematic mechanism of pH optode12
Figure 2.5 Schematic representation of a cross-linker
Figure 3.1 Schematic representation of the general chemical structure of an organophosphorus compound
Figure 3.2 Phosphorylation of AChE in the presence of OP24
Figure 3.3 Annual agricultural use in million pounds for some of the OPs in US26
Figure 3.4 Chemical structures of OP chemical nerve agents
Figure 3.5 Schematic representation of the principle behind inhibition based OP detection
Figure 3.6 Molecular structure of OPH
Figure 4.1 OPH immobilization chemistry on titanium dioxide coated glass slide. R and R ¹ represent neutravidin and OPH/BSA respectively42
Figure 4.2 Schematic representation of paraoxon hydrolysis

Figure 4.7 The changes in net fluorescent intensity of the working and reference/control spots n a particular channel of the glass slide with pH for 10 mM Ches buffer solution..54

Figure 4.11 Bar graph showing the percentage of retained signal value relative to the sensitivity present on day 1 of sensor preparation......60

Figure 5.1 Schematic representation of the chemistry used for lysozyme immobilization66

Figure 5.4 Leaching test: Change in absorbance with time for a paraoxon solution after incubation with OPH encapsulated silica slides using TMOS (\blacksquare) and TEOS (\bullet)......72

Figure 6.3 Absorbance spectra of film on quartz slide treated with various concentrations of DFP hydrolyzed by OPH......90

Figure 7.2 Top: Original Nash reaction. Bottom: Hantzsch Reaction with fluoral p.....101

1. MOTIVATION OF RESEARCH

1.1. Introduction

Employment of suitable chemistries and materials for the development of stable sensing surfaces is critical in a biosensor design as the recognition elements operate in complex environmental matrices that have harmful influence on their stability and sensitivity. In this study, *in-situ* functional material scaffolds for sensing elements using a combination of surface chemistries and morphologies for applications in biosensors and chemical sensors were developed. Particularly, the feasibility of titanium dioxide nanoparticles and magnesium silicate particles as scaffold surfaces with specific potential for signal amplification to enhance sensitivity were studied.

Focus of my earlier studies involved the modification of array biosensor platform (a fluorimetric platform developed by the Naval Research Laboratories (NRL, Washington D.C.) for affinity-based assays), to allow for kinetic studies using enzymes as biorecognition elements. One of the main problems encountered was to implement an appropriate surface chemistry for enzyme immobilization on the planar waveguide of the array platform. Although preliminary results with aminosilane chemistry for OPH immobilization with application for neurotoxin detection were promising, the sensor showed poor reproducibility and relatively low sensitivity. It should be noted here that right choice of material surfaces and its appropriate functionalization for biomolecular immobilization is critical to the performance of biosensors, especially those involving enzyme kinetics, as they will otherwise require constant recalibration before detection, attributed to the loss of enzymatic activity over time, particularly when rebuilt or reused.

The goal of this research is to address the above issues by designing a combination of surface chemistry and morphology that act scaffold materials for stable immobilization with reporter structures, such that the final sensor is sensitive, specific, selective, reusable and portable. Extension of the methodology to pattern the biomolecular recognition elements on the material scaffolds to form an array biosensor has the potential for simultaneous detection of multiple analytes. The broader impacts of this research will include the understanding, development and application of biocompatible nanoparticles as smart material surfaces for biosensors attributed to their multifunctional role of providing a high surface area, chemically stable, transparent wave guiding support for protein immobilization.

Specific use of titanium dioxide nanoparticles, polydimethylsiloxane and magnesium silicate particles (florisil) to act as a scaffold for chemical and biosensing of chemicals like Organophosphate neurotoxins and formaldehyde respectively is studied in this dissertation.

1.2. Dissertation Organization

The current chapter presents the motivation of research in the development of materials and their functionalization for the detection of chemical like Organophosphates and formaldehyde along with an overview of the dissertation. An introduction on concepts of sensing with the techniques used for immobilization of biomolecules on to various surfaces precedes literature review on organophosphates in Chapter 3. Chapter 4

reports on the development of an array biosensor using biocompatible titanium dioxide nanoparticles functionalized with an enzyme for the simultaneous detection of organophosphate neurotoxins. Chapter 5 details the development of an alternative strategy of OPH immobilization via encapsulation in lysozyme mediated silica particles on titanium dioxide coated glass slides. Chapter 6 discusses on the development of a biosensor based on the selective detection of fluoride ions resulting from organophosphofluoridate hydrolysis. Chapter 7 details on the development of chemical sensors based on the activation of magnesium silicate particles for formaldehyde detection. Chapter 8 summarizes the conclusions followed by the future work described in chapter 9. The references are provided after future work.

2. CHEMICAL SENSORS

2.1. Introduction

Birth of chemical sensors and biosensors as alternative analytical tools can be largely attributed to the problems facing the analytical procedures such as the requirement of skilled personnel, sample pretreatment and conditioning; and bulky and expensive instrumentation required to determine the components of a substance termed as an "analyte". According to the International Union of Pure and Applied Chemistry (IUPAC) "A chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal. The chemical information, mentioned above, may originate from a chemical reaction of the analyte or from a physical property of the system investigated" [1]. Similarly, "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" [1, 2]. Hence, chemical and biosensors are comprised of a receptor that interacts with the analyte of interest and a transducer that converts the receptor-analyte interaction into a measurable signal in real time, whose magnitude is proportional to the concentration of specific analyte. Figure 2.1 shows the schematic representation of a chemical/biosensor.



Figure 2.1: Schematic representation of a chemical sensor

The first chemical sensor, from a commercial viewpoint was a glass electrode, developed by Arnold Beckman in 1932 [3]. Nearly three decades later, Clark and Lyons built the first biosensor using glucose oxidase for glucose measurement [4]. Ever since, there has been tremendous progress in the field of chemical sensors and biosensors. With the technological advancements and integration of electronic miniaturization and nano/bio/material science and engineering; and constant need for better sensitivity, portability and ever increasing list of target analytes, the world market is projected to surpass ~ \$ 11.5 billion for chemical sensors [5] by 2010 and ~ \$6.1 billion for biosensors

[6] by 2012. Chemical sensors and biosensors find applications chiefly in areas of health care, control of industrial processes, for environmental monitoring and homeland security. However, medical and environmental markets continue to remain the forefront users of biosensors. Biosensors though treated as a separate field, are generally accepted as a subclass of chemical sensors, in that the receptor is a biomolecular recognition element. Selectivity, sensitivity, accuracy, response times, recovery times and working lifetimes are some of the factors that affect the performance of chemical sensors and biosensors.

2.2. Classification of chemical sensors

Chemical sensors and biosensors are generally classified according to the type of transducer and sensing element employed to measure a particular signal parameter. **Figures 2.2** shows the chemical sensor classification based on the transducer employed for detection. Other classifications include those based on the mode of delivery of the sample analyte as noted by Kissinger as single use continuous and intermittent sensors [7]. Continuous/flow mode results in uninterrupted measurement of analyte while in the intermittent or batch mode, the analyte in introduced in the flow stream of carrier and measurement is done at regular intervals. Though the classification was originally meant for amperometric biosensors, it is also applicable to other transduction modes. Chemical sensors may also be classified according to the fundamental type of chemical recognition process [8]. Every transducer or recognition element has its advantages and disadvantages. For example, optical sensors normally do not require any reference unlike electrochemical transducers, however, electrochemical transducers are easy to miniaturize. Chemical recognition elements have better stability while the biomolecular

receptors have better specificity and selectivity. Hence, the mode of recognition and detection employed is primarily governed by the analyte of interest and the demands of application.



Figure 2.2: Classification of chemical sensors based on mode of transduction

2.2.1. Optical transducers

The sensors developed in this study use optical based transduction methods for detection. Here, a change in an optical property such as absorbance, fluorescence, luminescence and reflection that occurs upon the interaction of sensing layer with analyte forms the basis of detection. Absorption of photon by a molecule occurs when the difference in energy levels of the molecule is equal to the energy of the incident photon. This results in the excitation of the electron from the ground state to an excited state at a specific wavelength. The excited electron may return to the ground state giving out its excess energy in the form of fluorescence or luminescence. Luminescence occurring due to a chemical reaction is termed chemiluminescence and that by a living organism is termed bioluminescence.

Reflectance based measurements involve studying the analyte on an optical surface generally using internal reflectance spectroscopy. Light striking the interface of two medium of refractive index n_1 and n_2 ($n_1 > n_2$) undergoes total internal reflection when the angle of incidence in the denser medium is greater than the critical angle, which is defined by

$$\sin\theta = \frac{n_2}{n_1}$$

Total internal reflection generates an evanescent wave field in the rarer medium that decays exponentially with distance. **Table 2.1** shows the optical sensing methodologies developed using the principle of evanescent waves. Excellent reviews on these methologies have been provided in the following references [9-11].

Method	Description
Attenuated total Reflectance (ATR)	Attenuation of the internally reflected light due to the presence of an absorbing material on the reflecting surface
Total internal reflection fluorescence (TIRF)	Changes in intensity of fluorescence of the fluorophore immobilized on the rarer medium of the interface
Surface plasmon resonance (SPR)	Surface plasmons at the interface of metal and dielectric material interact with evanescent waves producing surface plasmon resonance whose angle of resonance is a function of species on the surface

Table 2.1: Classification of evanescent wave sensors

2.3. Sensing elements

The mode of sensing element and analyte interaction can be ionic, molecular or biological [12]. **Figure 2.3** shows the various modes of sensing elements that can be employed for detection based on the type of interaction. The nature of interaction determines the sensor specificity and selectivity. For example, interactions based on ionic recognition are generally selective, in that the sensing element interacts more selectively to one ion in a matrix sample. Biological interactions are more specific in nature.



Figure 2.3: Modes of sensing element interaction with analyte [12].

2.3.1. Ionic Recognition

Ionic recognition involves the selective detection of ions (guest) by a sensing membrane (host). This mode generally uses ion-selective electrodes [13], modified and screen printed electrodes [14-16]. Ion selective electrodes are mechanically stable, nonporous, water insoluble membrane based devices that allow the permeability of analyte ions to produce a concentration dependent potential or current. Consequently, the signal parameter measured is electrochemical in nature. Selectivity may be enhanced in these electrode systems by introducing polymers either on the surface or matrix of electrode.

2.3.2. Molecular Recognition

Molecular recognition may be used as a principle of detection using chemical recognition agents and using spectroscopic analysis. In the case of chemical recognition elements, the thermodynamic feasibility, molecular size of analyte and rate of reacting species can form the basis of selectivity [12]. If the equilibrium constant (K) of a reaction for a specific analyte is high for one analyte-ligand complex when compared to others, selectivity is realized. This can be represented by the following equation.

$$A + xB \rightarrow AB_x$$

Where,
$$K = \left(\frac{[AB_x]}{[A][B]^x}\right)$$

The signal parameter analyzed is usually optical or electrochemical. For example, a pH responsive indicator, when incorporated in a PVC membrane containing sodium tetraphenyl borate (Na⁺TPB⁻) to maintain electro-neutrality may change its absorbance (red/blue) over a certain pH range that can be measured using a spectrometer [12]. This forms the principle behind the development of many optodes or optical electrodes (**Figure 2.4**). An ideal optical sensor developed using this principle should have a material (ligand) that is sensitive and selective to the analyte, have good photo-stability, not leach in the reagent solution, have quick response times and be reversible in a reasonable time-frame, in its reaction to enable reusability.



Figure 2.4: Schematic mechanism of pH optode [12]

For sensing elements exhibiting same thermodynamic behavior towards analytes "A" and "B", selectivity towards "A" or "B" may be achieved if the kinetics of interaction is different. This is generally the case with enzyme based reactions, which will be detailed in the later sections. Molecular size as the basis of selectivity is achieved for analytes that have different sizes. Here, the host's shape (cup-like or cavity) may play an important role in sieving out the interferent from analyte in a matrix sample for detection.

Molecular recognition based on spectroscopic analysis exploit the principle of internal energy of molecules, which may be expressed as a sum of kinetic, electronic, vibrational, rotational and nuclear energies that are characteristic of particular regions of the electromagnetic spectrum. Methodologies employing this principle are shown in **Figure 2.3**.

2.3.3. Biomolecular recognition

Exploitation of biomolecular elements involved in biochemical processes occurring in living organisms for sensing applications generally provide greater selectivity and specificity in comparison to chemical recognition elements. These chemical sensors are known as biosensors and are regarded as sub-set of chemical sensors or as a separate class of their own. Depending on the type of biochemical interaction between the biomolecular element and the analyte, the sensors might be classified as affinity biosensors or catalytic biosensors. Affinity based biosensors employ antibodies, nucleic acids, receptors or molecularly imprinted polymers for detection purposes while catalytic biosensors employ enzymes, cells/tissues, microorganisms or organelles as recognition elements.

2.3.3.1. Affinity biosensors

Antibodies

Antibodies are proteins and can be developed against almost any antigen (target). Antibody based sensors are also called immunosensors as an organism develops antibodies in response to an attack on their immune system by antigens. The antibodies bind to the antigens and remove them. If "Ab" is antibody and "Ag" is antigen, then their binding can be represented by

$$Ab + Ag = Ab - Ag$$

where, the affinity constant is given by

$$K = \frac{Ab - Ag}{[Ag][Ab]}$$

This value of K is generally around 10^6 [12]. They are characterized by their selectivity, ultra-sensitivity and high affinity for binding with target antigens.

Nucleic Acids

In addition to antibodies, nucleic acids are affinity type biorecognition elements and hence fall under the category of affinity based systems. Their base-pairing characteristics attribute to their selectivity and specificity. DNA (Deoxyribonucleic acid) is the most commonly used nucleic acid for this purpose. Recently, DNA based sensors have been termed "genosensors" [17, 18]. With the advances in genetic engineering, they can be used in combination with other biomolecular elements like enzymes and antibodies, to give rise to a broad range of biosensors for genetic testing, detection of biological agents and diagnosis of human diseases [19-21].

Receptors

Receptors are a special type of protein complexes with carbohydrates/ lipids that interact specifically with biological and chemical substances. The chemical substances are known as messengers, transmitters or ligands. The receptor-ligand interaction triggers a series of biochemical reactions, leading to an amplified physiological response in receptor bearing organism, which can be ion-channel opening, production of second messenger systems or activation of enzymes. They are selective and have affinity for a range of structurally related compounds. For example, phage displayed libraries can be created to contain specific receptors for targets and integrated with transducers to create biosensors [22].

2.3.3.2. Catalytic biosensors

In catalytic biosensors, the analyte, which is in a non-detectible form, on interaction with the biomolecules, undergoes a catalyzed chemical conversion to a form that can be detected by a transducer. The steady state concentration of the species formed, lost or inhibited forms the principle of detection. Catalytic biosensors use enzymes, microbes, sub-cellular organelles, plant tissues or animal tissues as the biorecognition elements.

Enzymes

Enzymes are proteins that are characterized by their catalytic power, specificity and regulation. In biosensors, enzymes react with analytes of interest generally termed as substrates and catalyze reactions to form products that may be detected either directly or indirectly. They are selective in both their interaction and rate of catalytic reaction. The catalytic activity of enzymes is attributed to its ability to reduce the energy of activation required for a reaction to take place. Besides the formation of detectable products, some enzyme-analyte interactions, result in the inhibition of their activity. The inhibition may be competitive or non-competitive, depending on the matrix of substrate. Accordingly, measuring the rate of species formed (product), lost (substrate) or inhibited (enzyme) from an enzyme catalyzed reaction forms the basis of detection. Most of the enzymes that are commonly used in the design of biosensors are oxidoreductases and hydrolases. Enzymes may be used in their wild form or may be purified and engineered to specific targets to improve the efficiency of the biosensor.

Cells & Tissues

Enzymes are generally isolated from cells and tissues and hence, are purified to provide selectivity in detection. However, in the absence of their natural environment, enzymes are known to degrade over time. Instead, cells and tissues containing a variety of enzymes may be utilized directly by their immobilization on transducer surfaces to develop biosensors. These biosensors have a number of advantages, in that; they are less expensive than using purified enzymes. As mentioned above, the enzymes are more stable in their natural environment. The major drawback of such biosensors is the loss of analyte selectivity, especially in complex matrices.

Microbes

Microbial biosensors use microorganisms like bacteria as biorecognition elements. The catalytic biorecognition is based on the biological processes such as consumption of metabolites, production of organic compounds or changes in respiration activity and their detection using appropriate transducers. For example, an ammonia biosensor can be constructed using nitrifying bacteria [23].

2.3.4. Bio-mimetic sensors

They form a separate class of sensors wherein the recognition elements are artificial synthetic systems that mimic the functioning of biological systems. The artificial systems can mimic the function of biocatalyst, affinity receptors or nucleic acids. When considerable chemical modifications of the surface of working electrodes to enable electrocatalytic properties for voltametric and amperometric sensors is produced, artificial biocatalytic sensors are developed. The use of molecularly imprinted polymers [24], wherein the print molecule with functional monomers may be copolymerized to produce a groove in the polymer; can act as site mimics to play the role of affinity based biorecognition materials. In the case of nucleic acids, their synthetic counterparts are known as aptamers. The use of such artificial systems has several advantages. For example, they are easy to manipulate chemically, unlike biological systems, they are more stable and they do not require biological systems for their production, as in the case of antibodies.

2.4. Integration of sensing and transducing elements: Biosensors

The performance of a chemical sensor is largely governed by the integration methodology used for the sensing and transducing elements. In the case of biosensors, biological elements are immobilized onto various insoluble supports like glass, metal oxide particles, polystyrene etc. to stabilize the biomolecules and enable sensor regeneration. This can be achieved using different techniques [25-27] like adsorption, covalent binding, entrapment or encapsulation.

2.4.1. Adsorption

Adsorption is one of the earliest, simplest, fastest and most inexpensive technique used for the immobilization of proteins onto a support. Here, the protein molecules in solution, when in contact with the support, diffuse rapidly from the bulk liquid to the surface of contact, thereby becoming reversibly or irreversibly adsorbed. Adsorption takes place when interactions due to van der Waals forces, hydrophobic interactions, hydrogen bonding or those of ionic nature between the proteins and contact surface are involved. Some of the factors that affect the protein adsorption are pH, temperature, ionic strength and the nature of substrate.

2.4.2. Covalent binding

Nucleophillic attack at the activated functional group of the support material forms the basis of covalent bonding of proteins. Here the ionizable groups of amino acids in their unprotonated forms act as powerful nucleophiles. Among the 20 naturally occurring amino acids, only 7 amino acids have ionizable side chains, making them suitable for conjugation and modification purposes. These are listed in **Table 2.2**.

Amino acid	Reactive group
Arginine	amine
Aspartic acid (aspartate)	carboxylate
Cysteine	sulfhydryl
Glutamic acid (glutamate)	carboxylate
Histidine	imidazole ring
Lysine	amine
Tyrosine	phenolate anion

Table 2.2: List of amino acids with ionizable side chains

The relative order of nucleophilicity in proteins is shown below [27].

$$R-S^- > R-NH_2 > R-COO^- = R-O^-$$

Thus, sulfyhydryls of cysteine are the strongest nucleophiles followed by amines from the lysines, histidines, arginines, the α and N-terminals of a protein. The least potent nucleophiles are the carboxylate groups of glutamic and aspartic acids and phenolate of the tyrosine. Those amino acids, which are not essential for the functionality of a protein (such as catalysis in the case of enzymes and antigen binding in the case of antibodies) can be covalently bonded to support materials using various coupling reactions. Some of the reactions include diazotization, amide (peptide) bond formation, alkylation (arylation), Schiff's base formation, Ugi reaction, amidination reactions, thiol-disulfide reactions, mercury-enzyme interactions and γ -irradiation induced coupling [25].

2.4.3. Cross-linking

Cross-linking techniques use cross-linkers to immobilize proteins onto various surfaces. A cross linker is a molecule with a center spacer arm and reactive groups at either ends. This is shown in **Figure 2.5**. Depending on the type of functional groups present on ends, cross-linkers may be either homo-bifunctional or hetero-bifunctional. These cross-linkers react with the ionizable groups of amino acids to enable their immobilization onto various support materials.



Figure 2.5: Schematic representation of a cross-linker [27]

2.4.4. Encapsulation and entrapment

Encapsulation is a non-covalent technique in which the biomolecules are entrapped in the transducer using membranes such as cellulose acetate (a common dialyzing membrane), polycarbonate materials, polytetrafluoroethylene (Teflon), nafion and other polyurethanes. In the case of entrapment, the biomolecules, generally enzymes are mixed in a polymeric solution, which on gelation entraps the biomolecules, thereby immobilizing them. The type of immobilization technique chosen depends on the type of protein and support used for biosensor development.

2.5. Nanomaterials for sensing applications

Nanomaterials by definition include those objects and systems whose size, in at least one dimension is around 1-100 nm. They have interesting electronic, optical and magnetic properties, which emerge due to the quantum phenomena (confinement) at the nanoscale range. For example, noble metals at the nanoscale exhibit size dependent surface plasmon absorption phenomena in the visible region resulting from the oscillation of electrons at certain frequencies. These surface plasmons when excited by evanescent waves produce a surface sensitive analytical tool known as surface plasmon resonance (SPR) for measuring protein absorption [28] and this methodology has been used to develop biosensors for the detection of biological and chemical agents [10]. In addition to the interesting electronic properties, nanomaterials are characterized by very high specific surface areas, attributed to the inverse dependence of surface to volume ratio over length or radius (S/V α l^{-1} and S/V = 3/R)[29, 30], thereby, making their surface favorable for adsorption of organic materials to lower the interfacial free energies [31]. These interesting properties are often exploited and integrated with the sensing and transducing components to enhance the performance of chemical sensors.

Commonly used nanomaterials for sensing applications include metal oxide nanoparticles (like SiO₂, TiO₂, Al₂O₃, Fe₂O₃), metal nanoparticles (Au, Ag, Pt, Pd), fullerenes (carbon nano wires, nanotubes and fullerenes) and semiconductor nanoparticles (CdS, CdSe, CdTe, ZnSe) etc. Their role in enhancing the performance of

sensors may be direct as in the case of stimuli responsive materials or indirect as in the case of providing a stable support for sensing layer, especially in the case for biosensing elements.
3. CURRENT ART IN SENSORS FOR THE DETECTION OF ORGANOPHOSPHATES

3.1. Introduction

Organophosphates are a large family of phosphorus containing compounds that inhibit the activity of acetyl cholinesterase (AChE), an enzyme that is responsible for the hydrolysis of acetylcholine neurotransmitter [32, 33]. Their chemical structure has two alkyl, aryl, or alkoxyl side chains, a leaving group (which may be O, S, F, C and or N) and a characteristic covalent bond to sulfur or oxygen. **Figure 3.1** shows the schematic representation of an OP compound.

$$\mathbf{R}_{1} \stackrel{\mathbf{O}/\mathbf{S}}{\underset{\mathbf{R}_{2}}{\stackrel{\mathbf{A}}{=}}} \mathbf{X}$$

$$\mathbf{R}_{1} \stackrel{\mathbf{\&}}{\underset{\mathbf{R}_{2}}{\overset{\mathbf{A}}{=}}} \mathbf{X}$$

$$\mathbf{X}: \text{ Leaving group (O, S, F, C and/or N)}$$

Figure 3.1: Schematic representation of the general chemical structure of an organophosphorus compound.

The phosphorus atom is normally attached to four oxygen atoms in organophosphates. However, in the case of phosphonates, there are three P-O bonds and a P-C bond. In the case of phosphinates, there are two P-O bonds and two P-C bonds. The sulfur containing OPs with the $P \rightarrow S$ moiety are called the phosphorothionates. The other

varieties of OPs are mainly derivatives of phosphoric, phosphinic or phosphonic acids [34]. Although these compounds were synthesized as early as the 1800s, their neurotoxic properties were not known until the early 1930s, following which, their application as pesticides became widespread [33]. The more toxic OP compounds are the notorious chemical warfare agents like Sarin, Soman, Vx and Tabun [35].

3.2. Toxicity of OPs

AChE is an enzyme that hydrolyses the central and peripheral neurotransmitter, Acetylcholine (ACh) [33]. Normally, on reacting with ACh, the hydroxyl group of serine at the AChE active site is acetylated, resulting in the release of choline moiety subsequent to which, the AChE activity is recovered within 0.15 milliseconds from hydrolysis of acetylated AChE. However, an OP compound mimics ACh and thus, in their presence, trans-esterification or trans-phophorylation of AChE takes place. The regeneration of a phosphorylated AChE is extremely slow, taking more than 60 hours. **Figure 3.2** represents the schematic of the mechanism of OP inhibition of AChE. In the absence of active AChE, the ACh accumulation occurs, thereby affecting the sensory and motor organs. Uncontrolled convulsions, paralysis and death of the exposed organism follow.

The level of toxicity of an OP compound is dependent on many factors such as its physical and chemical properties to name a few. The degree to which a protein phosphorylation occurs, determines the potency of OP. Proteins generally undergo phosphorylation in serine (OH⁻) since it has an unshared pair of electrons. The central phosphorus present in the OP structure is electrophillic, and hence, the presence of atoms or molecules in the OP side chains that donate electrons, greatly reduce its potency. Consequently, different OPs have different levels of toxicity. Dephosphorylation of

AChE may be facilitated when certain hydroxylamine derivatives such as oximes are used [36]. However, in certain cases, when the enzyme is aged, irreversible binding of OP takes place and reactivation may not occur.



Figure 3.2: Phosphorylation of AChE in the presence of OP (shown in red)

OP may enter the body by multiple routes such as direct adsorption through the skin or by ingesting food contaminated by them, leading to additive toxicity. After they enter the body, they undergo oxidation or hydrolysis by a number of enzymes depending on the type of leaving group present. Some xenobiotic metabolizing enzymes, cytochromes P450 (CYPs) present in liver, play a major role in bio activating the phosphorothionates leading to the formation of an "oxon" or oxygen analog of the parent

OP, while some catalyze reduction reactions [37-39]. These oxons are highly potent and thus oxidation reactions of P=S to P=O bond may attribute to their toxicity [33]. OPs may also covalently bind to other Serine Esterases, namely, Butyryl Cholinesterase (BChE), Carboxyl Esterase (CaE), Neuropathy Target Esterase (NTE) Trypsin, Chymotrypsin and Tyrosine residue of Human Serum Albumin [37, 38, 40-44].

Some organophosphates may result in delayed toxicity due to lipid solubility and fat storage in mammals. Long-term exposures to these compounds in low doses may also result in Organophosphate Induced Delayed Neuro-toxicity (OPIDN) associated with inhibition of the Neuropathy Target Esterase (NTE) [45-47]. The afflictions of the affected human being with a variety of syndromes of central-peripheral, distal, sensory-motor axonopathy are some of the characteristics of OPIDN. An intermediate syndrome may occur sometimes after 24-96 hours of exposure, characterized by acute respiratory paresis, muscular weakness, cranial nerve palsies and depressed tendon reflexes [48].

3.3. Need for Detection

Approximately 50 million tons of OPs are produced for agricultural and nonagricultural purposes in US alone [49]. Graph in **Figure 3.3** shows the annual use of some of the OPs in US. A list of some of the OPs currently in use is provided in **Table 3.1** Although these chemicals are mainly used as pesticides and insecticides; they are toxic to non-target mammals as well. OPs may enter and spread in the environment by a number of processes such as volatilization into air, leaching into the ground soil, runoff from the fields etc. Due their potential afflictions on target and non-target organisms, the Food and Drug Administration (FDA) began employing safe levels of OP/pesticide residue tolerances in food. With the increased awareness of the effects of these pesticides, the United States Environmental Protection Agency (USEPA) in 1973 took over responsibilities in establishing limits for pesticide residues in environment [49]. The rate of introduction of new pesticides in the market decreased from 10-15 per year for every 1800 OPs synthesized during 1955-70 to less than one of every 22000 synthesized per year currently.



Figure 3.3: Annual agricultural use in million pounds for some of the OPs in US [49]

LIST OF OP PESTICIDES			
Acephate	Disulfoton	Oxydemeton-methyl	
Azinphos-methyl	Ethoprop	Phorate	
Bensulide	Fenamiphos	Phosalone	
Chlorethoxyfos	Fenthion	Phosmet	
Chlorpyrifos	Malathion	Phostebupirim	
Chlorpyrifos-methyl	Methamidophos	Pirimiphos-methyl	
Diazinon	Methidathion	Profenofos	
Dichlorvos (DDVP)	Methyl parathion	Terbufos	
Dicrotophos	Mevinphos	Tetrachlorvinphos	
Dimethoate	Naled	Tribufos	

Table 3.1: List of OP pesticides [49]

The analytical procedures for the diagnosis OP exposure may include 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 and phosphonyl-proteins-adducts in plasma [50]. However, these methods have poor specificity since, both OPs and carbamates inhibit the AChE and BChE enzymes. Depending on the severity of exposure, timely administration of atropine sulfate, glycopyrolate or prolidoxime may help reactivate the enzyme [51].

In addition to their use as pesticides, OP compounds form the largest of the notorious group of chemical agents (V and G nerve agents) that are stockpiled in munitions and containers in various locations around the world [52-55]. For example, of the 40,000 metric tones of chemical agents stockpiled in Russia, 80% are nerve agents

(Sarin, Soman and Vx), 2% is mustard and rest is lewsite-mustard mixture [53]. Similarly, 60% of the chemical agents stockpiled in US were Vx and Sarin (GB)[55]. **Figure 3.4** shows the chemical structure of some of these nerve agents. To stall their further proliferation, actions due to the Geneva Convention has led to the process of destruction of these stockpiles and prohibit their development in the future [56, 57].



Sarin

Soman



Tabun



VX

Figure 3.4: Chemical structures of OP chemical nerve agents

Despite the efforts by the USEPA and the world treaties, incidents due to OP compound exposure continue to take place. One such incident is the Mevinphos

poisoning of 26 workers in apple orchard in Washington. (Mevinphos is an OP used to control apple aphids with an oral LD_{50} of 3.7 to 6.1mg/ml in rats) [58]. In 2002, there was an estimated 69,000 child cases of pesticides related poisoning incidents [59]. The OP nerve agents have been exploited for terrorist activities as well [60-62]. These events underscore the need for the development of quick, selective and sensitive sensors for these compounds in the environment to prevent further exposure to non-target organisms and loss of human life.

3.4. Detection of OPs

3.4.1. Conventional analytical techniques

Several methods have been developed for the detection of organophosphates. These may include the use of conventional analytical techniques and chemical sensors. Some of the conventional analytical methods developed for nerve agent detection are listed in **Table 3.2**. In the case of chromatography techniques, they are generally coupled 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. Although most of the above techniques are sensitive, they suffer from disadvantages of conventional analytical techniques, as discussed in the Section 2.1.

Technique	Principle/instruments	OP detected
Acoustic Wave Sensors	Surface acoustic wave using lanthanide compounds	Sarin [a]
Spectrophotometric sensors	Molecular imprinting technique with europium probe	Soman [b]
Chromatographic techniques	Gas chromatography (GC) - Solid phase micro-extraction	Sarin [c]
	GC-Mass spectrometry	Sarin & Soman [d]
Ion Mobility spectrometry	Aspiration Ion mobility	Soman, Vx [e]
ELISA	Using monoclonal antibodies against Soman	Soman [f]

Table 3.2: Some of the conventional analytical techniques to determine OP nerve agents. The references for the above are as follows. a: [63, 64], b: [65, 66], c: [67], d:[68], e: [69] f: [70]

3.4.2. Inhibition based biosensors

Activity of the inhibited cholinesterase due to the presence of OPs against its initial activity forms the basis of detection behind these biosensors. **Figure 3.5** gives the schematic representation of this principle. Several different types of electrochemical, piezoelectric, calorimetric and optical sensors have been developed employing this principle and a number of authors have extensively reviewed this [71-73]. Among these, the electrochemical sensors are the most widely studied and researched method.



Net signal = $I_0 - I_f$

Figure 3.5: Schematic representation of the principle behind inhibiton based OP detection.

Several amperometric [74-78] and potentiometric [79-82] methods have been devised using this principle. Insensitivity to environmental turbidity enables the use of amperometric method for detection of wastewater samples. Potentiometric biosensors are however less sensitive as they are easily influenced by the pH solution conditions and environmental factors.

Conductometric biosensors exploit the principle of changes in ion concentration because of inhibition, for transduction purposes. Thin film planar electrodes immobilized with cholinesterase have been used to detect photo degradation products of methyl parathion using conductometric biosensor [83]. Enzyme inhibition resulting in changes in mass or acoustic wave at the surface of the piezoelectric material was used for building piezoelectric biosensor [84]. They have the advantages of chemical inertness, mass production, low cost and solid construction.

In the case of less invasive and more sensitive optical based transduction methodologies, fiber optic biosensor based acetyl cholinesterase immobilization on Langmuir-Blodgett films by measuring the changes in absorbance of para-nitrophenol [85], cholinesterase immobilization in polyvinylidenefluoride membrane in contact with sol-gel layer incorporated with bromocresol purple [86], AChE conjugated with pH sensitive fluorophore in sol gel network [87], are a few of the many developments. 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 [88].

Selectivity and specificity of biosensors is one of its main features and requirements. In addition to knowing the general wide spectrum of compounds that may affect cholinesterases, it is critical to know the specific compound of the wide spectrum. One of the most disturbing and shocking civilian incidents stressing the importance of selectivity of biosensor was the use of the "unknown knock out gas" by the Russian elite forces to counter the Moscow theatre siege by the Chechen terrorists in October 2002 [89]. Apart from the complications of gas poisoning, its unknown nature made the treatment of the hostages even more difficult. Hundreds of hostages died because of gas poisoning and complications due to mistreatment. One of the chief disadvantages of inhibition based detection principle is that cholinesterases are inhibited by OPs and a wide spectrum of other compounds like carbamates, heavy metal ions etc. In addition, there are restrictions on sensor reusability due to the difficulties involving sensor regeneration. This can be overcome by the employment of sensing elements specific to OP compounds.

3.5. Biosensing Element: Direct detection using Organophosphorus Hydrolase

Although OPs are stable when kept in cool, dark, anhydrous conditions, in the presence of water, heat or light may result in its hydrolysis, oxidation or rearrangement of its structure. The hydrolysis rates may differ for different classes of Organophosphates. The second-generation biosensors use more direct approach by employing enzymes that act as catalysts to hydrolyze and detoxify OPs [90-92]. There are several enzymes like Human Paraoxonase [93], Squid DFPase [94] and Organophosphorus Acid Anhydrolase OPAA-2 [95, 96] that hydrolyze OPs, however, Organophosphorus Hydrolase (OPH, EC 3.1.8.1) is the only enzyme that can catalytically hydrolyze the P-S bond of OP substrates in addition to P-O, P-F and P-CN bonds [97-100].

The current study employs OPH as the bio-sensing element for the detection of OPs. OPH is a remarkably stable metalloenzyme with 2 zinc ions present in its active site. **Figure 3.6** shows the dimeric crystal structure of OPH [101]. Studies have shown that the replacement of zinc ions with cobalt ions can significantly increase its activity. Although OPH has hydrolysing capabilities towards a wide spectrum of OPs, it is highly specific towards those with oxygen as the leaving group. Studies show that OPH

catalyses the hydrolysis of OPs through the nucleophilic attack of the central phosphorus atom.



Figure 3.6: Molecular structure of OPH [101].

The first biosensor using OPH was developed by the group at Texas A&M. In their method, E-coli cells exhibiting OPH activity were cryoimmobilized in polyvinyl alcohol matrix [102]. A pH glass electrode was used to transduce the OP hydrolysis by OPH into a measureable signal. Another immobilization strategy involved the use of solgel matrix for enzyme immobilization for the development of sol-gel modified field effect transistors[103, 104]. Discriminative detection of pesticides like OPs and carbamates was realized with the incorporation of AChE and OPH to develop an amperometric sensor [105, 106]. Immobilization within polyelectrolyte multi-layers on glass beads [107], nanocomposite formation by incorporation of OPH into silicone polymers [108] and use of competitive inhibition of OPH immobilized on planar glass slides [109] are some OPH immobilization strategies developed using optical based spectroscopic methods of detection.

Developments in fluorescent-based detection of OPs include the employment of pH responsive fluorophores to transduce the catalytic reaction into a measurable signal. Close proximity between the fluorophore and recognition element enables enhanced monitoring of enzyme-substrate reactions and detection of target OPs. This can be achieved through their conjugation and immobilization on suitable supports. Examples include the encapsulation of OPH-SNAFL [110] and OPH-CNF [111] conjugates in polyethylene glycol and polystyrene waveguides respectively. While these systems provide real-time target analysis apart from being highly sensitive towards different OPs, they suffer from the inability to provide multiple and simultaneous detection of several different analytes.

3.6. Multi analyte sensing platform: Array Biosensor

One of the first multi-analyte sensors employed immunobased assays for biomedical applications [112]. Ever since, there has been significant progress in sandwich-based immunoassay technology for simultaneous detection of multiple analytes. A good number of the methods use the principle of evanescent waves to develop highly surface selective sensors for real-time analysis of biomolecular interactions. Some of the examples include the multi-analyte immunoassays based on surface plasmon resonance [113] and capillary flow systems [114]. Higher sensitivities have been obtained by increasing the optical path length as in the case of use of multiple internal reflection elements like planar waveguides [115-118], planar waveguide interferometers [119-121] and bundled optical fibers [122, 123]. Studies have also been performed to combine optical and electrochemical transduction methods [124]. Here, the planar waveguide is coated with Indium-Tin oxide (higher refractive index, 1.95-2.0) to improve the optical properties for total internal reflection and to electrically regenerate the immuno-sensing surface. There has been significant progress using Array Biosensor developed by the Naval Research Laboratory for simultaneous detection of multiple biothreat agents like Ricin, Cholera Toxin, B. Anthracis, Campylobacter Jejuni, Staphylococcus Enterotoxin B, Ochratoxin and many others [9, 125-132]. Array biosensor will be used in the current study as a fluorimeter to develop an enzyme based sensor for simultaneous detection of multiple OPs.

4. ARRAY BIOSENSOR BASED ON ENZYME KINETICS MONITORING BY FLUORESCENCE SPECTROSCOPY: APPLICATION FOR NEUROTOXINS DETECTION

4.1. Introduction

The aim of the current study was to develop a biosensor array for the detection of OP neurotoxins like paraoxon (P-O), parathion (P-O) and DFP (P-F), using OPH as the bio-recognition element, conjugated with pH-dependent a fluorophore carboxynaphthofluorescein (CNF). The enzyme catalyzes the breakdown of OP neurotoxins and the generation of protons decreases the micro-environmental pH and the measured fluorescence intensity. BSA, that does not show any enzymatic activity towards OP, acts as the scaffold protein for fluorophore immobilization at the reference spots, which allows the realization of a differential mode measurement, by subtracting signals from the "working", and "reference" spots. The main advantage of the differential mode measurement is that, common mode variations such as temperature and bulk pH changes are eliminated while local pH changes due to the enzymatic action at the OPH-loaded spots are amplified.

An array biosensor unit developed at the Naval Research Laboratories (NRL) was adopted as the detection platform and appropriately modified for enzyme-based measurements. A planar multi-mode waveguide was covered with an optically transparent TiO_2 layer to increase the surface area available for immobilization. The nanoparticles modified slides were functionalized with silane to enable covalent immobilization of OPH. Aminosilane (APTS)-gluteraldehyde (Glu) chemistry is one of the most widely utilized glass functionalization techniques for protein immobilization. Earlier studies [133] showed the development of a biosensor for the detection of paraoxon based on covalent immobilization of OPH using APTS-glu chemistry on glass slides coated with titanium dioxide nanoparticles. However, stringent conditions of moisture free environments are required as the use of aminosilane suffers from competing surface reactions and self-polymerization even in the presence of minute quantities of water. Accordingly, the different batches of sensors prepared when checked for paraoxon detection showed poor reproducibility with lower detection limits of 100 µM. Hence, an alternative functionalization technique involving mercaptosilanes with GMBS crosslinker followed by avidin-biotin-NHS was developed for OPH immobilization on titanium dioxide coated glass slides

4.2. Materials and Methods

4.2.1. Chemicals and Reagents

Ti-nanoxide "T" paste was purchased from Solaronix Inc., (Aubonne, SA), absolute ethyl alcohol was purchased from Florida Distillers Co. (Lake Alfred, FL), Sodium/potassium phosphate buffer, dimethyl sulfoxide (DMSO), *N*,*N*,-dimethyl formamide(DMF) and bovine serum albumin (BSA, fract V, cold alcohol precipitated) was purchased from Fisher Scientific, Pittsburgh, PA),. Ches (2-[*N*-cyclohexylamino] ethanesulfonic Acid) was purchased from Alfa Aesar (Ward Hill, MA), toluene

(anhydrous, driSolv,) was purchased from EMD (Gibbstown, NJ), 3-(mercaptopropyl)trimethoxysilane (MPTS, 98%,) and diisopropylfluorophosphate (DFP) was purchased from Sigma Aldrich (St. Louis, MO), *N*-γ-maleimidobutyryloxy succinimide ester (GMBS), neutravidin was purchased from Thermo Fisher Scientific Inc., (Rockford, IL) CNF (5-(and-6-)-carboxynaphthofluorescein-succinimidyl ester, mixed isomers) and biotin-XX, SE ((2-[*N*-cyclohexylamino] ethanesul 6 ((6 ((biotinoyl) amino) hexanoyl) amino) hexanoic acid, succinimidyl ester) was purchased from Molecular Probes (Carlsbad, CA), glass slides were purchased from VWR scientific Inc., (West Chester, PA).

Wild-Type OPH (E.C.3.1.8.1) isolated from recombinant *Escherichia coli* strain was obtained from Texas A&M (Dr. Wild's Lab at TAMU). Paraoxon (diethyl-*p*-nitro phenyl phosphate) and parathion (*O*,*O*-diethyl-*p*-nitro phenyl phosphorothioate) was purchased from Chem. Service (West Chester, PA). DI (de-ionized water, 18.2 M Ω cm) was used throughout and was obtained using a Millipore water purification system.

4.2.2. Preparation of glass slides

In order to remove any organic contaminants, the glass slides were incubated in concentrated hydrochloric acid (12.1N) for 30 min followed by a thorough water rinse. They were further dried under nitrogen atmosphere. The clean surface of the glass slide was coated with Ti-nanoxide paste, to boost the surface area. The composition of the Ti-nanoxide paste obtained from the manufacturer (Solaronix, Switzerland) has $\sim 11\%$ weight of nanocrystalline titanium dioxide anatase particles, water, less than 20% ethanol and polyethylene oxide. The procedure followed for coating the glass slide was "Dr.

Blade squeegee printing" method wherein, the glass slide was strapped on to a flat platform by its sides using an adhesive tape and the Ti-nanoxide paste was spread evenly on the slide with the help of a glass rod. The coated slides were sintered at 450 °C for 30 min, cooled down to room temperature, and cleaned with 1N hydrochloric acid. After cleaning, the slides were thoroughly rinsed in DI water and dried under nitrogen atmosphere. This was followed by silanization with 2% MPTS in dry toluene for 2 h in nitrogen atmosphere. The slides were then thoroughly rinsed in dry toluene. This was followed by incubation in 1 mM GMBS in absolute ethanol (dimethyl sulfoxide was used as the organic solvent to dissolve GMBS). Bonding occurs due to the covalent thioether linkage between the maleimide group of the cross-linker and sulfhydryl group of the silane. Subsequently, the slides were thoroughly rinsed in DI water and incubated in 1 mg/ml of neutravidin (prepared in DI water) at 4 °C for 12 h. This was followed by a thorough water rinse. The neutravidin-coated slides were incubated in freshly prepared 1 mg/ml biotin-XXSE (1 mg biotin in 0.1 ml dimethyl formamide and 10 mM Phosphate buffer of pH 6.5) at room temperature for 1 h. After this, the slides were again rinsed in DI water. Before the immobilization of the enzyme, its activity was checked using the UV spectrophotometer. The extinction coefficient of the product of enzymatic reaction of paraoxon, namely, *para*-nitrophenol was 17,000 at an absorbance of 405 nm. The concentration of bovine serum albumin prepared in 0.1 M phosphate buffer with pH 8.0 was maintained similar to that of OPH. The immobilization procedure employed (by NRL) to pattern the proteins and fluorophores on the glass slide involved the use of the physically isolated patterning (PIP) method. This method was used to generate 1 mm² array of biorecognition elements (antibodies) on the planar waveguide. Here, the

polydimethylysiloxane made physically isolated patterning flow cell, was placed on the surface of the waveguide, and the antibodies were introduced in the channels. The problems faced with the above method for our study were the formation of bubbles that resulted in the non-uniform immobilization of the biomolecules and response variations among different arrays. To avoid these problems; simple, reproducible and long-lasting, multiple-use incubation holders (IH) were designed and engineered using the principle of capillary action and employed as an alternative to the PIP flow chambers [134].

In this study, the principal of capillary action was used as shown in **Figure 4.3** to direct the fluid flow and form rows of proteins and fluorophores on the waveguide surface. This is described in details in the earlier work [133]. The distance of hematocrit tubes from the glass slide surface determined the amount of solution that was needed to be placed on the slide-tube interface. This method of immobilization required $5-10 \,\mu$ l of solution per row of protein/fluorophore array in contrast to the PIP method that required at least 50 µl of solution. The whole incubation holder with the slide was placed in a Petri dish with moistened tissue to maintain humid conditions and prevent evaporation of applied solutions. The activated waveguide surface was incubated for at least 36 h at 4°C with OPH (4 mg/ml, phosphate buffer, pH 8.0) and BSA (4 mg/ml, phosphate buffer, pH 8.0) After protein incubation, the slides were thoroughly rinsed in DI water and air-dried, for the immobilization of fluorophore. The pH sensitive fluorophore used in the study was CNF, functionalized with NHS ester. CNF solution of 1 mg/ml was prepared in 10 mM phosphate buffer, pH 8.0 and DMF was used as the organic solvent to dissolve the CNF. The slides were incubated with CNF for 8 h at 4 °C. After incubation, the slides

were rinsed with DI water and stored in phosphate buffer at 4 °C with cobalt chloride present to maintain the activity of OPH. **Figure 4.1** gives the schematic representation of the slide chemistry.



Figure 4.1: OPH immobilization chemistry on titanium dioxide coated glass slide. R and R¹ represent neutravidin and OPH/BSA respectively.

4.2.3. Characterization of titanium dioxide nanoparticles

Transmission electron microscopy (TEM) was used to study the colloidal particle shape and morphology using Zeiss EM10 transmission electron microscope. The size and shape of the particles coated on the glass slides after heat treatment at 450 °C were studied using JEOL JSM 7000F field emission scanning electron microscope (JEOL USA, Inc., Peabody, MA) at 15-20KV in vacuum mode (< 7.75x 10⁻⁴ Torr). A thin layer of gold was sputtered on the oxide layer to make its surface conductive. The thickness of the coating on glass slide was measured using a profilometer (Tencor Instruments). X-ray diffraction studies were performed using Rigaku DMAX-B vertical diffractometer. The samples were exposed to X-rays (40KV, 40 mA) from a copper target and the intensity of the diffracted X-rays was measured. UV-visible spectroscopy was used to study the transmission characteristics of the coating on glass slide.

4.2.4. Sensing principle

OP neurotoxins undergo catalysed hydrolysis by OPH to generate protons that result in a change in the microenvironmental pH. The presence of a pH responsive fluorophore conjugated to OPH, serves to transduce the pH changes due to hydrolysis with a corresponding change in fluorescent intensity. This forms the basis of detection in the developed array biosensor.



Figure 4.2: Schematic representation of paraoxon hydrolysis

The developed sensor was tested for its sensing characteristics by measuring the pH response, enzyme action and stability. Paraoxon was used as the model OP substrate. The hydrolysis of paraoxon is shown in **Figure 4.3**. For characterization using UV/Vis spectroscopy, the formation of para-nitrophenol was measured at 405 nm for detection purposes.

4.2.5. Experimental Set-up

The slide patterned with biorecognition elements conjugated with fluorophores was rinsed with DI water, air-dried and placed in the flow chamber of array biosensor. The flow chamber is made up of PDMS with six channel wells, each channel having a sample inlet and outlet [134]. As the direction of flow of the samples through the channels is in the direction perpendicular to that of the rows of biomolecules conjugated to fluorophores on the slide, multi-analyte sensing is made possible. A 54 mm, long, hollow, cylindrical aluminum tube with an 8 mm diameter opening was fit 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 (diode laser, 635 nm) was directed to 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 (**Figure D**).



Figure 4.3: Schematic representation of array biosensor development. A: Principle of protein patterning on glass slide. B: Photograph of the incubation holder used for patterning biomolecules with fluorophores. C: CCD image of array of biosensors developed on glass slide. D: Schematic representation of array biosensor.

Q-Capture Suite software (Q-Imaging, USA) was used for taking the images, and the TIFF analysis program developed at NRL, was used for data analysis. The rinsing buffer, 1 mM Ches, pH 8.2 was introduced at the flow rate of 4 ml/min using an Ismatec peristaltic pump, and the fluorescence array pattern was monitored with an exposure time of 2 s. After the background signal level was established, the OP samples prepared in buffer, were allowed to flow for 15 s until it filled the flow system. In order to give the true representation of the catalytic reaction, the maximum slope (change in intensity/time) was calculated for every concentration of the analyte introduced. The above-mentioned monitoring procedure was based on more than 100 experiments performed to optimize the measurement process.

4.3. **Results and discussions**

4.3.1. Characterization of the metal oxide nanoparticles

The titanium dioxide nanoparticles were obtained as a colloidal paste containing 11wt. % of nanocrystalline anatase particles with less than 20% ethanol and some polyethylene oxide. Over months of use, it was realized that these particles often aggregated resulting in cracked, non-adherent films when coated on glass slides. Hence, the sonication of the paste for 30 minutes was performed prior coating step. These particles were characterized using various techniques to ensure the development of sensitive biosensors. **Figure 4.4** shows the transmission electron microscopy image of the titanium dioxide nanoparticles dried over carbon formvar grids. The particles were

distributed between having a spherical and ellipsoidal morphology. Also, the average particle size was found to be 12-13 nm.



Figure 4.4: Transmission electron microscopy image of titanium dioxide particles of the paste within 6 months of being purchased from the manufacturer

To enable good adhesion between the titanium dioxide nanoparticles and the glass slide and to remove the organic solvents present in the paste, a sintering treatment at 450°C was performed after the coated slides were air-dried. **Figure 4.5** shows the X-ray diffraction of the plain and titanium dioxide coated glass slide. The peaks were plotted using JCPDS data (78-2486).



Figure 4.5: X-ray diffraction studies of glass slide coated with titanium dioxide nanoparticles

Peaks show that the nanoparticles are of anatase phase and that the heat treatment at 450°C does not lead to rutile phase transformation. The particle size was calculated using the Scherrer equation.

$$D = \frac{K\lambda}{\beta\cos\theta}$$

where, K is a dimensionless constant ~0.9, λ is 1.541 Å, 20 is the diffraction (Bragg) angle and β is the full width at half maximum (fwhm) of the diffraction peak. The

average crystallite size calculated from (101) peak was found to be 13.3 nm. This is consistent with the TEM approximation of the particle size. Hence, it can be concluded that sintering does not result in coarsening of the particles and this should explain the possible retention of the anatase phase as observed from the XRD peaks. Absence of coarsening is critical from the standpoint that coarsening will decrease the surface area from mechanisms due to increase in particle size and decrease in available pore volume of the film.

The average weight of titanium dioxide nanoparticles coated on glass slide was found to be 7.08 ± 0.7 mg. The thickness of the film measured using a profilometer was around $2.2 \pm 0.01 \,\mu\text{m}$. The surface area and the pore size of the particles as provided by the manufacturer were 120 m²/g and 11 nm respectively. **Figure 4.6** shows the SEM image of plain and the nanoparticles coated on glass slides. The titanium dioxide nanoparticles appear to have a uniform coating with visible pores in-between particles. In addition, the average particle size of what appeared to be aggregated in the film was estimated to be 37 nm.



Figure 4.6: SEM image of plain glass slide (A) and glass slide coated with titanium dioxide nanoparticles (B).

The transparency of glass slides in the visible region of the electromagnetic spectrum along with its low intrinsic fluorescence, chemical inertness and resistance to high temperatures makes it a versatile support for fluorescence-based bioassays. In the case of array biosensor, in addition to being a protein support, the plain microscopic glass slide also acts as a planar waveguide to generate evanescent waves that in turn excites the patterned fluorophores present on the surface and create an array of sensing surfaces for simultaneous detection of multiple analytes. The generation of evanescent waves however requires that the following two conditions be met.

- a) Light is incident from a denser (waveguide) to a thinner medium
- b) Angle of incidence is greater than the critical angle which is defined by

$$\theta_c = \sin^{-1} \left(\frac{n_2}{n_1} \right)$$

where, n_2 is the refractive index of the surrounding solution (analyte, buffer, water etc.) and n_1 is the refractive index of biomolecular support (glass slide, polystyrene etc).

For glass-water interface, the critical angle is 66.5 to 67° . The resulting evanescent field has a penetration depth (D_p)characterized by

$$D_p = \frac{\lambda}{2\pi n_1 \sqrt{\sin^2 \theta - (n_2 / n_1)^2}}$$

Here, λ is the wavelength of incident light and θ is the angle of incidence. In an array biosensor, for an excitation wavelength of 635 nm (diode source), the penetration depth is ~ 760 nm. From the equation, one can modify the parameters such as the wavelength, angle of incidence and refractive indices of materials used to increase or decrease the penetration depth.

The "n" of non-porous titanium dioxide is 2.52. However, since the film prepared in this study has a pore size of 11 nm, the actual value is expected to be much lower. The current array biosensor configuration does not allow for modifications in θ . In order to understand if the titanium dioxide sensing film is the *medium* or the *waveguide*, refractive index of the thin film is of paramount. Since the conventional methods of determining the refractive index were unsuccessful due to the scattering of light by the films, an alternative technique using porosity was explored to determine the refractive index. According to the following equation,

$$Porosity = \frac{Non-solidvolume}{totalvolume(solid+non-solid)}$$

Given that the surface area of the anatase paste is $120 \text{ m}^2/\text{ g}$, the average weight of particles coated on glass slide is $0.007.08 \pm 0.7$ mg, and the average crystal size (d) is 13 nm, assuming that the particles are spherical, the solid volume of anatase on glass can be found by rearranging the following equation.

$$d = \frac{6}{\rho(\text{specificsurfacearea})}$$

where, ρ is the density (mass/volume) of particles on slide. The relationship between porosity and refractive index is given by the following equation [135].

Porosity=
$$\left(1 - \frac{n^2 - 1}{n_d^2 - 1}\right) \times 100(\%)$$

where, n is the refractive index of porous anatase and n_d is the refractive index of nonporous anatase (2.52)[136]. The value of refractive index of the titanium dioxide film coated on glass slide was found to be 1.91 using this method. Ideally, for the developed thin film to act as a waveguide, the angle to incidence should be greater than 44°. The limitations of the array biosensor configuration only allowed for an angle of incidence for glass-water interface, which was ~ 67°. However, possible coupling of light from the slide into the film and its subsequent scattering by the high refractive index particles through the film could act as an excitation source for the patterned fluorophore on film, and thus explain the fluorescence array viewed via CCD camera. Further studies are required to investigate on to the optical properties of the developed film to act as waveguides. However, taking the above results into account, experiments were carried out to investigate on the sensing characteristics of the developed array biosensor.

4.3.2. pH Response

CNF is a pH sensitive fluorophore with an excitation and emission wavelength of 598 nm and 668 nm, respectively at a pH 10.0. As the excitation source used in the array biosensor has a wavelength of 635 nm instead of 598 nm, it was necessary to investigate the effect of excitation wavelength on fluorescent intensity. Studies on the fluorimeter (Photon Technology International) showed more than 50% loss in fluorescent intensity due to excitation at 635 nm instead of 598 nm [133]. **Figure 4.7** shows the pH response of the glass slide with immobilized proteins and fluorophore. The graph depicts that the changes in the intensity of the signal in both working and control channels are positively correlated with the changes in pH. The spot to spot coefficient of variance (CV) was found to be less than 20%.



Figure 4.7: The changes in net fluorescent intensity of the working and reference/control spots in a particular channel of the glass slide with pH for 10 mM Ches buffer solution

4.3.3. Enzymatic Reaction

Figure 4.8 shows the response of the reference and the working spots to the introduction of 0.5 mM paraoxon in 1 mM Ches buffer. As detailed in the graph, the working spot has a huge drop in signal when compared to that of the reference spot. This drop in signal is typical for an enzymatic reaction that occurs due to the catalytic hydrolysis of paraoxon. The drop in intensity identified for the control spot can be attributed to the variations in sample buffer pH. In addition, it can be seen that the

catalytic hydrolysis for this particular concentration of paraoxon is realized during the first 10 s, after sample introduction. Normally, the time taken for the hydrolysis depends on the concentration of paraoxon or target analyte. Thus, for all experiments the maximum slope was calculated for every concentration of paraoxon introduced.



Figure 4.8: Response of reference and working spots to 0.5 mM paraoxon, 1 mM Ches buffer, pH 8.2. Images were taken for every 5 s.

Figure 4.9 shows a concentration-signal relationship for paraoxon by the sensor taking a single pair of working and reference spot into consideration. The presented data was averaged and the standard error was determined for three samples for every concentration of 0.00125 mM, 0.0025 mM, 0.005 mM, 0.01 mM, 0.02 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM and 0.8 mM paraoxon. Each point in the graph is calculated by the subtraction of the signal of the working spot from the control spot according the equation

$$S = -\frac{\Delta I \, w - \Delta I \, ref}{\Delta t}$$

Where,

S = slope, ΔI_w is the change in intensity of the working spot, ΔI_{ref} is the change in intensity of the reference spot and Δt is the change in time.

The graph has the linear region ranging from concentrations of 0.00125 mM to 0.1 mM and the signal saturates at concentrations above 0.35 mM. The inset in **Figure 4.9** shows the linear region of the response curve. Even though changes in intensity for concentrations as low as 0.00125 mM was obtained, it did not meet the statistical criteria of being three times greater than the standard deviation. Thus, the developed sensor was able to detect concentrations as low as 0.0025 mM. In spite of the good sensitivity, only 54% of the potentially available fluorescence is used, due to excitation at 635 nm (laser source wavelength) instead of 598 nm. The system performance should improve significantly by exploiting a light source with 598 nm wavelength.



Figure 4.9: Response curve of sensor to paraoxon introduction. The standard errors were calculated for three samples introduced for every concentration. The inset shows the linear region of response curve ranging from 2.5 μ M to 0.1 mM paraoxon.

4.3.4. Specificity of Detection

As mentioned at the introduction, there are several enzymes like Human paraoxonases [93], squid DFPase [94] and organophosphorus acid anhydrolase OPAA-2 [95, 96] that hydrolyses OPs, but, OPH (OPH, E.C. 3. 1. 8. 1) is the only enzyme that can catalytically hydrolyze the P-S bond of OP substrates apart from P-O, P-F, P-CN bonds [97-100]. The wild type OPH has different catalytic activities towards the P-O, P-F and P-S bonded OPs
[90, 91] with a turn over number for paraoxon around 6000 s⁻¹, DFP around 75 s⁻¹ and Demeton-S around 4.3 s⁻¹ [137].

The specificity of the biosensor under study was examined using two P-O bond substrates namely paraoxon and parathion, and a P-F bond substrate DFP. It can be seen from **figure 4.10** that the highest response is obtained for paraoxon followed by parathion that has 75 % less sensitivity than paraoxon, with DFP exhibiting the lowest (93% < paraoxon) sensitivity. This is similar to other investigations that have been made on the substrate specificity of OPH-WT with parathion and DFP showing turn over number less than 80% and 97 % respectively, when compared to paraoxon [138]. The lowest concentration the biosensor could detect for DFP and parathion based on the statistical criteria of being three times greater than standard deviation was 10 μ M. By using an array of enzymes with different substrate specificities such as OPH mutants and OPAA, this biosensor should be capable of discriminating different OP agents.



Figure 4.10: Response curves of paraoxon, parathion and DFP for concentrations ranging from 2.5 μM to 0.8 mM prepared in 1 mM Ches, pH 8.2-8.3.

4.3.5. Stability of the immobilized enzyme

The stability of the sensor was investigated by obtaining response curves for the linear region of paraoxon on various days after the waveguide preparation. **Figure 4.11** shows the changes in signal of the sensor relative to day 1 that is considered to be 100%. It can be seen that there is a drastic drop in signal level on the first 18 days after sensor preparation. On the 59th day, only 5% of the initial signal level is present; however, it has been proved enough for 0.01 mM paraoxon detection. The waveguide with patterned

biomolecules and fluorophores prepared was stored in 10 mM phosphate buffer with cobalt chloride at 4 °C.



Figure 4.11: Bar graph showing the percentage of retained signal value relative to the sensitivity present on day 1 of sensor preparation.

4.4. Conclusions

A sensitive and rapid enzyme based array biosensor for OP detection was developed using the innate ability of OPH to catalyze the hydrolysis of OP neurotoxins. Ti-nanoxide coating that was used to boost the surface properties of the waveguide provided good characterization for the immobilization of the biorecognition elements and fluorophores. SEM images of the surface of the coated glass slides showed a uniform coating of Ti-nanoxide. The pH response of reference and working spots on the glass slide were similar allowing the discrimination between the catalytic and non-catalytic based pH changes. The lowest concentration of paraoxon that the biosensor could detect was 0.0025 mM, which is far below the lethal dosage level (0.007 mM in rats and mouse). The specificity of the sensor was validated by the simultaneous detection of paraothion (P-O bond) and DFP (P-F bond) along with paraoxon. The sensor showed lower sensitivity for parathion and DFP, with detection limits of around 0.010 mM for parathion and DFP, respectively than paraoxon. Good storage conditions of the waveguide in 10 mM phosphate buffer with cobalt chloride under refrigerated conditions enabled enough retention of OPH activity (though less than initial 10%) to detect paraoxon concentration after more than 8 weeks.

5. LYSOZYME-MEDIATED FORMATION OF PROTEIN-SILICA NANO-COMPOSITES FOR BIOSENSING APPLICATIONS

5.1. Introduction

The in situ encapsulation of biomolecules directly at a waveguide surface provides a potentially versatile tool with specific application to biosensor development. Various surfaces coated with titania nanoparticles, for example, have found increasing application in fields as diverse as photocatalysis, photovoltaics, photoelectrochromics and sensors due to the high photocatalytic activities of titania coatings [139-142]. Enzymes have been immobilized onto titania-coated waveguides via physical adsorption and covalent immobilization for biosensing applications and these techniques have been shown to enhance protein loading and retention of biomolecular activity [133, 143]. Apart from photocatalytic activity, interesting optical properties of titania nanoparticles, i.e. high refractive index and dielectric constants has enabled their use as waveguides when coated on glass slides and optical fibers[144-146]. Interfacing additional inorganic oxides, such as silica, in layers or core shell configurations is also known to tailor photocatalytic properties and enhance stability [147]. In addition, studies show that specific surface modifications of titania coatings provides appreciable biocompatibility [148].

For biosensor applications, however, complications arise when enzymes are directly introduced into oxide structures due to the extreme conditions required for inorganic oxide synthesis that are unfavorable for retention of activity in biomolecules. Recent research in biomineralization reactions, however, offers an alternative paradigm for the formation of silica nanoparticles under mild conditions that provides stability and retention of activity of the encapsulated biomolecules [149]. Studies on mineralization of silica in biological systems, for example, have led to the isolation of polycationic species that catalyze silica formation including silaffins and silicatein proteins, from marine diatoms and sponges respectively [150-152]. Recently the lysozyme-mediated formation of silica particles has also been demonstrated [153, 154]. Lysozyme as a template for biomineralization is advantageous as it is a ubiquitous protein and commercially available as opposed to specific silicification proteins that are expensive and labor intensive to purify. The resulting bio-nanocomposites of lysozyme with amorphous silica have been shown to retain the proteins native antibacterial activity [155]. Aside from being a benign method for oxide synthesis, the method also provides an effective method for enzyme immobilization that is amenable to encapsulation and stabilization of enzymes at a surface. For example, lysozyme-mediated silica formation was successfully utilized to encapsulate organophosphate hydrolase (OPH) on a gold surface plasmon resonance waveguide [156]. Similarly, a silica-precipitating peptide mediated silica formation and encapsulation of β -Galactosidase *in situ* on functionalized silicon wafers [157]. In both cases, the encapsulated enzymes retained catalytic activity detectable by their respective analytes; namely paraoxon (for OPH) and lactose (for β -Galactosidase) and provides a basis for biosensing applications.

In this study, we extended the versatile mineralization reactions of lysozyme to coat silica particles on waveguides formed on glass slides, pretreated with a titanium dioxide coating. The choice of precursor and immobilization chemistry was studied in order to optimize encapsulation of OPH as a model enzyme. The stabilized OPH composites were patterned to form an array of sensors on the waveguide surface and tested on a commercially available array biosensor to demonstrate the versatility of the enzyme encapsulation methodology for biosensor development.

5.2. Materials and Methods

5.2.1. Chemicals and Reagents

Lysozyme (from hen egg white), tetramethyl orthosilicate (TMOS) and tetraethyl orthosilicate (TEOS) were purchased from Sigma Aldrich (St. Louis, MO). 3mercaptopropyl trimethoxy silane (MPTS) was purchased from Gelest Inc (Morrisville, PA). Potassium phosphate buffer (0.1 N NaOH, 0.1 M KH₂PO₄, pH 8.2), Ches buffer for spectrophotometer assays (0.05 M CHES, 0.05 mM CoCl₂, pH 9.0), Ches buffer for the array biosensor (0.01 M CHES, 0.05 mM CoCl₂, 2.7 mM KCl, 120 mM NaCl, pH 8.5) and phosphate buffered saline (PBS, 10 mM, pH 8.1) were prepared using reagents from standard commercial sources. Hydrochloric acid, acetone, bovine serum albumin (BSA, fract V, cold alcohol precipitated) and dimethyl sulfoxide (DMSO) were all purchased from Fisher Scientific (Pittsburgh, PA). Titanium dioxide nanopaste "T" was purchased from Solaronix Inc, (Aubonne, Switzerland) and plain pre-cleaned glass slides were purchased from VWR Chemicals (West Chester, PA). The solvents, absolute ethanol (Florida Distillers Co.) and anhydrous drisolv toluene were purchased from EMD Biosciences Inc. (Gibbstown, NJ). N- γ -maleimidobutyryloxy succinimide ester (GMBS) was purchased from Pierce (Rockford, IL) and CNF (5-(and-6-)carboxynaphthofluorescein-succinimidyl ester, mixed isomers) was purchased from Invitrogen (Carlsbad, CA). Wild-Type OPH (E.C.3.1.8.1) from recombinant *Escherichia coli* was purified at Texas A&M as described elsewhere[158].

5.2.2. Instrumentation

A standard UV/Vis spectrophotometer (Ultraspec[™] 2100 pro, Amersham Biosciences, Piscataway, NJ) was used for all absorbance-based measurements. An Array Biosensor portable unit from the Naval Research Laboratories (Washington, DC) was used for all fluorescence-based assays with modifications as described previously [159].

5.2.3. Preparation of titanium dioxide coated glass slides

A glass microscope slide forms the basis of the biosensor array and was pretreated with an initial coating of titanium dioxide nanoparticles in a manner described previously [159]. To differentiate between the initial titania paste coating and subsequent modifications, the titania-coated glass slides as prepared above will be referred to simply as 'pretreated glass slides'.

5.2.4. Functionalization of pretreated glass slides for lysozyme immobilization

The pretreated glass slides were chemically functionalized with MPTS and GMBS crosslinker in order to covalently immobilize lysozyme. Pretreated glass slides were treated with 2 % MPTS (in anhydrous toluene) for 45 minutes, and then rinsed in toluene. Silanated slides, after rinsing, were dried and incubated with 10 mM GMBS (prepared in absolute ethanol, diluted from a stock solution prepared in 10 % DMSO v/v) for 45 minutes. GMBS-treated slides were rinsed sequentially in ethanol and water and

incubated with lysozyme (5 mg/ml in 10 mM PBS, pH 8.1) for 5 hours. **Figure 5.1** shows the schematic representation of the chemistry used for lysozyme immobilization. In a control experiment, physical adsorption was used to immobilize lysozyme on pretreated glass slides by incubating with lysozyme (5 mg/ml in 10 mM PBS, pH 8.1) for 5 hours before rinsing with water as before.



Figure 5.1: Schematic representation of the chemistry used for lysozyme immobilization.

5.2.5. Formation of OPH-encapsulated silica particles on pretreated glass slides

Two precursors were investigated for silica formation; in the first method, TMOS (30μ l hydrolyzed with 170\mul of 1mM HCl) was mixed with 820 µl potassium phosphate buffer, (of 0.1 M, pH 8.1) containing 100 µl of OPH (~1.5 mg/ml). A similar solution was prepared using TEOS in place of TMOS. 10µl of the above reaction mixtures were spotted to the pretreated glass slides and incubated for 5 hours at room temperature.

For fluorescence-based assays, immobilization of lysozyme was carried out using the MPTS-GMBS functionalization described in the previous section. OPH and BSA were conjugated with the fluorophore, CNF, using a protocol provided by the manufacturer. A centrifugal filter unit with a 30 kDa molecular weight cut-off was used to separate the conjugated protein from any unreacted dye. BSA was used as a scaffold protein for CNF immobilization as a negative control with no paraoxon-hydrolyzing activity. The methodology for pattering slides with the OPH/BSA-CNF conjugate involved the use of capillary tubes as described in a previous study [159]. **Figure 5.2** shows the lysozyme mediated formation of silica particles on glass slides.

5.2.6. Microscopic characterization of silica particles

Treated slides were sputtered with a thin layer of gold and visualized by Scanning Electron Microscopy (SEM, JEOL, model 840) in order to study and characterize the silica particles formed. No gold coating was required for characterization using optical microscopy (Aetos Technologies Inc, AL).



mediation < 1 day

Figure 5.2: Schematic representation of lysozyme mediated formation of silica particles and its encapsulation of OPH on glass slide coated with titanium dioxide nanoparticles

5.2.7. Enzyme assays for OPH activity

Activity of OPH was determined from the rate of catalytic hydrolysis of paraoxon to produce *p*-nitrophenol (PNP), which has a characteristic absorption peak at 405 nm. The conversion of paraoxon was investigated at a range of paraoxon concentrations after incubation of OPH-immobilized slides for 1 min and measurement of the respective absorbance change at 405 nm.

5.2.8. OPH activity assays on the array biosensor

Array biosensor assays were performed to test the efficiency of immobilization of OPH on the portable fluorimeter platform. Here, a red diode laser (λ =635 nm) directed over the edge of the glass slide undergoes total internal reflection producing evanescent waves that act as an excitation source (and direct measurement) for the labeled biomolecules immobilized onto the slide surface. A CCD camera was used to monitor the resulting fluorescent intensity.

The sensor was characterized for pH variation by passing 10 mM CHES buffer (at a pH ranging from 8.0 to 10.0) at a fixed flow rate of 1.5 ml/min for 30 seconds. The flow was stopped when the images were captured for data analysis. For paraoxon response, different concentrations of paraoxon were prepared in buffer (1 mM CHES, pH 8.4), and flowed through the channels of the sensor to pass through regions containing immobilized OPH and BSA and thereby form an array of sensing spots. Images containing the array of fluorescent spots were taken for exposure times of 3 seconds using Q capture pro software (Q Imaging, Canada). TIFF analyze software developed at NRL was used to quantify the fluorescent intensity of the captured images.

5.3. **Results and Discussions**

5.3.1. Formation of silica particles for OPH encapsulation

Lysozyme catalyzes the formation of silica from its precursor solutions. Initially, the formation of silica particles and their ability to encapsulate OPH was investigated to ensure that the lysozyme-mediated formation of particles was not hindered when directed onto a pretreated surface. Lysozyme immobilization was achieved by physical adsorption and silica particles were formed in situ. OPH became encapsulated as the silica particles formed. The efficacy of the procedure was evaluated by monitoring the catalytic activity of encapsulated OPH for the hydrolysis of paraoxon. The graph in Figure 5.3 shows the absorbance of PNP, obtained as a result of paraoxon hydrolysis by OPH encapsulated within silica composites formed directly on the pretreated glass slides. The response of OPH activity was comparable and the encapsulation efficiency showed little variation between the two silicate precursors investigated (TEOS and TMOS). Quantification of the amount of immobilized lysozyme template was determined by measuring the concentrations of lysozyme in solution before and after immobilization and in the subsequent wash steps. Under the reaction conditions described, approximately 30% of the initial lysozyme was immobilized on the pretreated glass slide by adsorption alone. Interestingly, similar studies performed for OPH adsorption on the pretreated glass slides resulted in no retention of OPH and is attributed to poor electrostatic and/or hydrophobic interactions with the pretreated glass slide.



Figure 5.3: Hydrolysis of paraoxon to para-nitrophenol by OPH encapsulated in silica using TMOS (●) and TEOS (▼) precursors

For silica encapsulation of OPH, TMOS as silicate precursor appeared to result in a silica matrix with increased sensitivity at low concentrations compared to TEOS but the standard error of measurements was more variable with TMOS as the precursor. It was considered that variability in the measurements may be due to OPH leaching from the silica particle matrix during analysis. In order to test this observation, the change in absorbance of the reaction solution was monitored after the functionalized slides had been removed from the reaction solution. After the removal of slides that contained OPH in silica prepared from TMOS, the hydrolysis of paraoxon continued, as evidenced by an increase in absorbance at 405 nm, indicating that OPH had leached from the surface of this preparation (**Figure 5.4**). In contrast, silica formation from TEOS produced a stable composite that prevented OPH from leaching over time.



Figure 5.4: Leaching test: Change in absorbance with time for a paraoxon solution after incubation with OPH encapsulated silica slides using TMOS (•) and TEOS (•)

The activity and retention of the encapsulated OPH may depend upon a number of factors, one of them being the morphology of particles formed during encapsulation. SEM analysis was used in order to characterize the matrix morphology and the effect of precursor on the resulting composites. The initial pretreatment of glass slides with T-paste (titanium dioxide coating) results in a homogeneous coverage of nanoparticles that

can be clearly differentiated from a non-coated glass slide. As such, encapsulated OPH was visualized for both pretreated and untreated glass slides. Figure 5.5 shows that silica particles formed using TEOS appear pronounced and distinct with a wide particle size distribution (100-500 nm). This can be seen more clearly on plain glass slides than for pretreated glass slides due to the presence of the titania background coating. In the case of TMOS, the distribution of silica particles is more homogenous and uniform and produces a gel-like coating of silica particles. Optical images of the silica particles encapsulating OPH confirm that the TMOS precursor forms silica particles that are small and homogeneous with an even distribution of encapsulated enzyme, while the silica particles formed with the TEOS precursor again appear larger and possibly aggregated. This difference in particle morphology between the two silicate precursors requires further investigation as porosity of the matrix may contribute significantly to substrate interaction with the encapsulated enzyme and influence the stability of the matrix against leaching. In addition, preliminary observations suggest that the rate of silicification differs for the two precursors when catalyzed by lysozyme with silica formation from TEOS being a significantly more rapid process compared to TMOS (data not shown). The small particle distribution for TMOS-silica creates a large surface area that could explain the enhanced leaching of OPH as was observed in **Figure 5.4**. Thus, despite the heterogeneity of TEOS-based silica particles, the structure provides optimal retention for encapsulating OPH under the given experimental conditions. As such, for further experimental studies, TEOS was used as the precursor for silica formation.



Figure 5.5: SEM and optical images of silica particles using TEOS and TMOS with schematics. SEM images of Plain (a) and Pretreated (b) slides, SEM images of silica particles formed using TMOS precursor on plain (c) and pretreated (d) slides, SEM images of silica particles formed using TEOS precursor on plain (e) and pretreated (f) respectively. Optical microscopic images of OPH encapsulated silica particles formed in solution mediated by lysozyme using TEOS (g) and TMOS (h) precursors respectively.

5.3.2. Covalent attachment of lysozyme for silica formation

Preliminary studies confirmed that physical adsorption of lysozyme to pretreated glass slides did not hinder the formation of silica particles and OPH was successfully encapsulated in the resulting inorganic matrix. However, adsorption strategies of immobilization suffer from potential problems of protein leaching due to physiological changes in pH and ionic strength. For long term operation of a biosensor, the stability of the matrix and corresponding retention of enzyme activity (of the sensing component) is paramount. In the study described herein, the possibility of lysozyme leaching from the preformed structure could in turn destabilize the encapsulated OPH immobilized on the surface. An alternative is to anchor the template lysozyme in place using covalent attachment chemistries to potentially enhance the stability and reusability of the final biosensor.

Covalent immobilization by MPTS modification allowed subsequent orientation of GMBS to preferentially bind the lysine residues of lysozyme. MPTS/GMBSfunctionalized glass slides provided a platform for silica formation indicating that the orientation of lysozyme using covalent attachment did not significantly hinder the silicification reaction of lysozyme (**Figure 5.6**). The protein immobilization efficiency indicated that ~90% of lysozyme was retained by MPTS/GMBS chemistry providing a significant increase to the loading capacity over physical adsorption alone (~30%). The efficiency of enzyme immobilization in silica was determined by measuring the activity of the encapsulated OPH. MPTS/GMBS-functionalized slides demonstrated linear detection rates (slope: 0.1945) corresponding to catalytic activity with increasing concentrations of paraoxon in the range 0.024 mM to 0.495 mM. The detection limit for the encapsulated OPH was approximately 0.05 mM paraoxon.



Figure 5.6: Activity of OPH encapsulated in silica particles with covalently immobilized lysozyme

Protein immobilization in which the immobilization matrix is covalently anchored to the pretreated glass slide should theoretically improve the long-term stability and hence applicability for bio-analytical devices. In addition, the use of a 3-dimensional matrix of silica significantly increases the surface area for protein loading[143, 159]. In order to test the stability of the silica-encapsulated OPH, the retention of OPH activity was investigated following controlled changes in physiological conditions. High salt concentrations, for example, can be detrimental to protein stability in immobilized systems. The presence of 0.5 M sodium chloride in buffer solutions, for example, results in complete desorption of cytochrome C (a cationic protein with an isoelectric point (pI) similar to lysozyme, pI=10 and 10.5 respectively) [160]. Pretreated glass slides were functionalized with MPTS/GMBS and lysozyme as described above and used to mediate the formation of silica containing OPH. The resulting functionalized slides were then treated with 0.5 M NaCl and retention of OPH activity was determined. No loss of OPH activity was observed following the high salt treatment, indicating that the silica particles provided stable encapsulation of OPH and offer protection against environmental changes. In contrast, a control slide in which OPH was attached in the absence of lysozyme for silica mediation resulted in 33% loss in activity. The possibility of OPH to mediate silica formation that could explain the presence of initial activity requires further studies.

5.3.3. Encapsulation of fluorescent OPH for studies on array biosensor

Array biosensor, developed at the Naval Research Laboratories was used to demonstrate the enzyme encapsulation described herein on a portable system. To directly integrate with the NRL array biosensor, the reporter for detection activity was fluorescent-based and detectable using fluorescent dyes that excite at 635nm and emit in the 665-680 nm range [161]. OPH was conjugated with the pH-dependent fluorophore, CNF, resulting in the utilization of only 50 % of the actual fluorescent intensity of CNF due to the variations in available excitation wavelength (635 nm) from that required for CNF (598 nm) [133, 159]. The catalytic hydrolysis of organophosphates by OPH generates protons which directly influences the fluorescence intensity of CNF.

Mercaptosilane chemistry was used for lysozyme immobilization in order to pattern biomolecules for the biosensor array. MPTS/GMBS chemistry allowed for direct comparison with previous studies [159] that involved covalent immobilization of OPH. The pretreated glass slides were patterned with immobilized lysozyme and incubated with a mixture of dye-conjugated OPH and silicate precursor solution. The lysozymemediated silica formation encapsulates the fluorescently-labeled OPH directly at the sensor surface in a series of well-defined 'spots'. Control spots were similarly derived using fluorescently-labeled BSA which allowed for direct variations in physiological conditions and environmental pH changes to be evaluated in order to eliminate external variations. Figure 5.7 A shows the patterned slide with 2 rows of control (BSA) spots and 4 rows of working (OPH) spots. The presence of the fluorescent spots indicated that the planar waveguide properties were not affected by a layer of silica at the surface. Figure 5.7 B shows the pH response of OPH conjugated to the pH sensitive fluorophore, CNF, encapsulated and patterned on the pretreated glass slide. BSA-CNF provides a control to differentiate between any changes in pH that may occur from hydrolysis of paraoxon and those due to sample-buffer variations. The graph shows a good correlation in pH response between the BSA and OPH spots, confirming that BSA was encapsulated in silica and was a suitable control for our assay (Figure 5.7 B).

Figure 5.7 C shows the paraoxon response, determined by calculating the slope for changes in fluorescent intensity over a period of time for specific concentrations of paraoxon. Though concentrations as low as 7μ M paraoxon could be detected, the statistical detection limit was ~35 μ M. To determine the stability of the encapsulated enzymes, the measurements were repeated after 12 and 66 days of sensor preparation. The sensor retained more than 60% of its original activity even after 2 months of storage. By comparison, covalent attachment of OPH by MPTS/GMBS chemistry (to a similarly pretreated glass slide) lost ~80% of activity within 18 days and 95% of OPH enzyme activity over a period of 59 days[159].



Figure 5.7: Response of pretreated glass slides with patterned encapsulation of OPH-CNF on array biosensor. A) Array of spots formed on glass slides B) pH response of working (OPH-W) and control (BSA-R) spots C) Paraoxon (prepared in 1 mM Ches, pH 8.4) response of sensors over a period of nearly 2 months

5.4. Conclusions

In this study, the utility of lysozyme to template silica nanoparticle formation on glass slides that have been precoated with a titanium dioxide paste was demonstrated. OPH was introduced during the synthesis procedure along with the ceramic precursors and the encapsulated OPH retained catalytic activity towards paraoxon. Under the given experimental conditions, TEOS as a precursor for silica formation was superior in encapsulating OPH than TMOS. Covalent attachment of lysozyme prior to silicification resulted in the formation of a stable silica matrix for OPH encapsulation with reproducible linear detection rates for paraoxon. Extension of the methodology to pattern silica and encapsulate OPH that was conjugated with a fluorescent indicator enabled the development of a spectro-fluorimetric array biosensor with OPH. OPH activity was retained within the resulting sensor array for over 60 days demonstrating a significant improvement in stability over previously reported configurations [159]. In addition, encapsulation in silica using this method is rapid with preparation times of a few hours, compared to the several days for covalent immobilization of OPH as reported previously. OPH encapsulation was demonstrated herein as a model enzyme system, but the encapsulation methodology is applicable to a wide variety of biomolecules, providing the potential for multiple enzyme immobilizations and simultaneous analysis of multiple analytes [157].

6. MONITORING OF DIISOPROPYL FLUOROPHOSPHATE HYDROLYSIS BY FLUORIDE-SELECTIVE POLYMERIC FILMS USING ABSORBANCE SPECTROSCOPY

6.1. Introduction

Organophosphonofluoridates (OPFs) belong to a subclass of organophosphates (OPs) with anti-cholinesterase properties [33]. The presence of fluorine attached to the central phosphorus atom differentiates OPFs from other OPs with O, S, or CN as the leaving groups following chemical hydrolysis. Among the most notorious chemical warfare agents, Sarin and Soman both belong to this phosphonofluoridate subclass, known as GB and GD agents, respectively. Consequently, significant effort is being directed into the research and development of relatively simple sensors for their detection in the environment. Some of the conventional OFP detection systems include chromatographic techniques interfaced with mass spectrometers [162], surface acoustic wave sensors based on changes in properties of a piezoelectric crystal upon the adsorption of specific analyte [163] and molecular imprinting techniques in combination with spectrometers using a europium probe to measure the hydrolysis products of Sarin [66]. As such, these techniques offer good sensitivity; however, most of these sophisticated technologies have limited portability, and their complex operation requires skilled personnel.

In lieu of these approaches, the employment of enzyme-based biosensing systems may be a less expensive alternative with better operational abilities for field detection purposes. In previous studies, the inhibition of acetyl-cholinesterase was used as a means to detect and quantify neurotoxic OPs providing good sensitivity [164]. However, their limits in application included poor selectivity and limited reusability attributed to the nearly irreversible AChE enzyme inhibition. A more specific biorecognition element such as organophosphorus hydrolase (OPH, E.C. 3.1.8.1) that catalyses the hydrolysis of various OPs with dramatically different kinetic parameters has the potential to discriminate certain OPs against other cholinesterase inhibitors [97, 99, 165]. For example, the K cat values at 7.0 pH and 25 °C of OPH from Pseudomonas diminuta that hydrolyzes a variety of OPs such as paraoxon (P-O), DFP (P-F), Soman (P-F) and Sarin (P-F) was found to be 2070 s⁻¹, 41 s⁻¹, 4.8 s⁻¹ and 56 s⁻¹ respectively [97]. Extensive studies using site-directed mutagenesis have shown tailored catalytic activities towards substrates having different bonds [166]. There is a related enzyme, organophosphorus acid anhydrolase (OPAA, EC 3.1.8.2) that is specific towards OFP (type G) [95] hydrolysis, which has the potential to selectively detect OPFs over other OPs.

The biosensors based on OPH or OPAA reported to date, which are specific for OFPs, chiefly rely on the generic detection of protons [111, 133, 159, 167] and/or the specific detection of fluoride ions [168] generated upon hydrolysis of the target species. Proton detection as a means for OFP quantification suffers from several disadvantages. First, pH-based detection systems are largely influenced by the buffering capacity of the target analyte sample. High buffering capacity of the sample may prevent the ability to monitor changes in proton activity, and poorly buffered samples may render the system

unstable. Hence, extremely good referencing techniques are crucial in order to avoid false positive signals, and they are difficult to sustain. Secondly, all OPs, regardless of their leaving groups or their application as pesticides or chemical warfare agents (CWAs), produce hydrogen ions upon hydrolysis, thereby creating challenging selectivity issues. Addressing the selectivity requirement of an OP biosensor is critical to differentiate the presence of a pesticide such as parathion or acephatae from a chemical nerve agent such as Sarin or Soman. This discrimination is critical in order for the local emergency planning committees (LEPC) to act appropriately for remediation and prevent public panic. Thirdly, the reuse of enzyme-based sensors over extended periods is limited by the loss of their enzymatic activity requiring constant recalibration (and even during) detection analysis. These issues may be addressed by developing a system that combines the advantages of immobilized enzymatic hydrolysis and selective fluoride ion sensing devices. One such development involves the use of a commercially available pF ionselective electrode coupled to OPH immobilized onto a silica gel in a batch mode set-up [167]. The system however suffers from poor sensitivity.

Recent studies of fluoride detection systems based on polymeric films have demonstrated that thin films prepared with Al(III) porphyrins in conjunction with a lipophillic pH indicator such as dibromofluorescein can be employed to create a highly sensitive and reusable fluoride ion sensor based on the coextraction principle [169]. In this preliminary study, we show that it is possible to develop a stable, enzyme-affiliated OPF sensing system by utilizing such a fluoride ion-selective optical film to transduce the OPF hydrolysis by OPH into a measureable signal. The interfacing of an Al[OEP]-ETH 7075 ion-selective membrane with an enzyme-based measurement greatly widens the window of dynamic range of detection. The principle of OFP detection in the current study is shown in **Figure 6.1**.



Figure 6.1: Schematic representation of the principle of DFP hydrolysis by OPH and subsequent detection by optical sensing film.

Diisopropylfluorophosphate (DFP), is a structural analogue of the class-G chemical warfare agents and is used as the model target analyte in these studies. Here, DFP undergoes catalytic hydrolysis in the presence of OPH to give equimolar concentration of fluoride ions. The generated products are exposed to fluoride sensing polymer film coated on quartz slide resulting in the coextraction of fluoride ions and protons from solution phase by the aluminum porphyrin and chromoionophore (ETH 7075), respectively, into the film. This coextraction results in the protonation of ETH 7075 which is measured as a change (decrease) in absorbance at $\lambda_{\mu\alpha\xi}$ of the depronated form of the chomoionophore The entire set-up of the sensing film with analyte solution was contained in a standard 1 cm cuvette (**Figure 6.2**).

6.2. Materials and Methods

6.2.1. Chemicals and reagents

Octaethyl porphyrin (OEP) was purchased from Frontier Scientific (Logan, UT) and aluminum chloride (AlCl₃) was purchased from Strem Chemicals, Inc. (Newburyport, MA). Diisopropyl fluorophosphates (DFP), 4',5'-dibromofluorescein octadecyl ester (ETH 7075), 2-nitrophenyloctylether (o-NPOE), poly(vinyl chloride), sodium fluoride, benzonitrile and tetrahydrofuran was purchased from Sigma Aldrich (St. Louis, MO). L-Glycine, phosphoric acid and quartz slides were purchased from Fisher Scientific (Pittsburgh, PA). Ches (N-cyclohexyl-2-aminoethanesulfonic acid) was purchased from Alfa Aesar (Ward Hill, MA). Wild-type OPH (E.C. 3.1.8.1) from recombinant *Escherichia coli* was purified at Texas A&M as described elsewhere [158]. De-Ionized water (18.2 M Ω cm) was used from Millipore water purification system for the preparation of buffer solutions.

6.2.2. Preparation of Aluminum octaethyl porphyrin (ALOEP)

The Al[OEP] was synthesized by refluxing 0.5 mmol OEP and 2 mmol aluminum chloride in 25 ml benzonitrile overnight under nitrogen atmosphere. Following the evaporation of benzonitrile, the crude product was dissolved in methanol and precipitated in 3 M hydrochloric acid solution. The collected precipitate was further washed with 3 M hydrochloric acid. Purification of the crude product was achieved by flash chromatography on silica using dichloromethane as the solvent. The final product was characterized by mass spectrometry yielding a desired molecular weight peak at m/z = 591.3 and using UV/Visible spectrometry.

6.2.3. Preparation of Al[OEP]-ETH 7075 cocktail

The ALOEP-ETH 7075 optical fluoride sensing film was prepared using the procedures reported by Badr and Meyerhoff [170]. The most effective formulation as discussed by Badr et. al. resulted from the mixture of 2.14 mg of Al[OEP], 2.08 mg ETH-7075, 30 mg of 2 nitrophenyloctylether, and 15 mg of poly(vinyl chloride) with 1 ml of tetrahydrofuran. Complete dissolution of the reagents and mixing was ensured (Al[OEP]-ETH 7075) using sonication. The cocktail was then sealed in a glass vial and stored at 4°C until use. Cocktails were used within 3 days of their preparation.

6.2.4. Preparation of slide coated with polymerized film

Quartz slide was cleaned using acetone prior coating with polymeric films. Doctor blade method was used to coat 20 μ l of Al[OEP]-ETH cocktail on each of the cleaned slides. The slides were with the optical sensing film were allowed to dry, and then stored in dark until use.

6.2.5. Instrumentation

UV/vis spectrophotometer (UltraspecTM 2100 pro, Amersham Biosciences, Piscataway, NJ) was used for all absorbance measurements. The schematic of the experimental set-up comprising of a cuvette with sensing film is shown in **Figure 6.2**. The slide coated with the polymer film is placed in a standard 1 cm cuvette containing the analyte solution. A slot with inside groves made on the cuvette cap was used to hold the slide in a position such that it was perpendicular to the light path. The arrangement was sturdy enough to prevent the movement of slide during handling and experimentation. The whole system was in turn placed in the standard cuvette holder of the spectrophotometer for measurement purposes.



Figure 6.2: Schematic of the experimental set-up of the sensing film in cuvette

6.2.6. Procedure

For the detection of DFP, various concentrations of DFP were formulated in CHES buffer, pH 8.5, and were completely hydrolyzed by treatment with 10 μ l of ~ 7 µg/ml of OPH for 2 min. The reaction solutions were then further diluted in glycine phosphate buffer (2.0 M, pH 3.0) such that the final concentrations of DFP in contact with Al[OEP]-ETH 7075 doped film were in the range of 0.1 μ M, 1 μ M, 10 μ M, 25 μ M, 50 μ M, 75 μ M and 100 μ M. The absorbance spectra of the polymer films in contact with the hydrolyzed/diluted DFP solutions for 10 min were recorded on a UV-vis spectrophotometer (Ultaspec 2100pro, Amersham Biosciences). The absorbance peaks corresponding to the chromoionophore's deprotonated state were monitored for changes and plotted against the various DFP concentrations. This two step procedure of hydrolysis and detection was similar to the studies performed for glucose sensing using the Al[OEP]-ETH 7075 polymer films [171]. To reference against possible nonenzymatic hydrolysis of DFP over the same assay time, ALOEP-ETH 7075 films were also exposed to samples that were not hydrolyzed by OPH. The percentage changes in the absorbance (%A) were calculated as follows:

$$\% A = \left[\left(\frac{A_o - A_x}{A_o} \right)_{DFP-OPH} - \left(\frac{A_o - A_x}{A_o} \right)_{DFP} \right] \times 100$$

where, A_o is the absorbance of the polymer film coated slide in glycine phosphate buffer solution and A_x is the absorbance of the slide when treated with "x" M DFP.

6.3. Results and discussions

The use of Al[OEP]-ETH 7075 polymeric film on quartz slide to detect fluoride ions has been discussed in detail by Badr et. al [170]. Studies have shown its application for glucose sensing by coupling enzyme-based reactions with these polymeric films on microtitre plate wells [171]. Since OPH is rendered inactive due to the protonation of its active site [172] at pH 3.0, the hydrolysis and detection of DFP is carried out in two steps under different pH conditions similar to the procedure followed by Robboh et. al for glucose detection [171].

Although OPH activity highest substrate specificity has its with phosphotriesterase (P-O) bonds, its hydrolyzing capability towards OFPs is well established [106, 111, 159, 167]. As shown in **Figure 6.1**, complete hydrolysis of DFP results in the production of equimolar concentration of fluoride ions. Figure 6.3 shows the absorbance spectrum of the polymeric films treated with various concentrations of DFP. From the figure, the absorbance peak corresponding to the de-protonated form of the pH chromophore was found to be 538 nm. It should be noted that changes in the 380-400 nm range, correspond to spectral changes of the Al[OEP] species due to dimerization of the Al[OEP] species upon interaction with the extracted fluoride ions [170]. This spectral region can also be utilized for analytical purposes, although there is potential for more interferences in this region when examining real-world samples. Figure 6.4 shows the percentage change in absorbance at 538 nm as calculated using equation 1 for various concentrations of DFP tested. A single optical polymer film was used to test the various

concentrations of DFP samples hydrolyzed by OPH for a single experimental data set.



Figure 6.3: Absorbance spectra of film on quartz slide treated with various concentrations of DFP hydrolyzed by OPH.

Another fluoride sensing film was used as a control for DFP samples with similar concentrations that were not hydrolyzed by OPH. The error bars of mean values were calculated for 3 sets of experiments using three different fluoride sensing films. The response was nearly linear in the range of DFP concentrations (100 nM – 100 μ M), over which the sensing system was tested (**Figure 6.4**). Based on the response of sensor to DFP samples not treated with OPH (considered blank), it was found that concentrations

as little as 0.1 μ M were detectable, which are much lower than those obtained using earlier DFP biosensor studies with pH FETs (20-25 μ M DFP) [168] and optical waveguides (1-10 μ M DFP) [111, 159]. This level of DFP detection is below the oral lethal dosage limits in humans (~21 μ M) for DFP and dermal lethal dosage limits for GB (0.7 μ M) and GD (0.5 μ M) nerve agents. The sensor is however restricted by the slow response times governed by the fluoride detection step.



Figure 6.4: Response curve of the % change in absorbance at 538 nm for various concentrations of hydrolyzed DFP referenced against non-hydrolyzed DFP. The data was fit for linear regression (y = 0.4086 x + 6.465, $R^2 = 0.966$). The standard error was calculated for n=3 (3 different pairs of reference and working sensors).

It should be noted that in the current configuration, the enzymatic reaction requires a high pH to be efficient (pH 8.5) while the sensing chemistry of the polymer film requires low pH (pH 3.0). Hence, optimal analytical capabilities are only achieved when the reaction and detection process is carried out in two separate steps. However, it may be possible to ultimately overcome this limitation by locally changing pH values near the sensing film using electrochemical methods to generate protons [173] or by encapsulating the enzyme in silica matrix with appropriate surfactants to protect it against extreme acid conditions [174].

6.4. Conclusions

The utility of enzyme-based, catalytic hydrolysis of organophosphosphate neurotoxins that release fluoride ions in conjunction with an amplifying fluoride optical sensing film is demonstrated here as a potential approach to overcome the current shortcomings for the development of OFP-specific sensing systems. The combined sensing strategy (catalytic DFP hydrolysis monitoring by fluoride selective polymeric films) uses simple absorbance spectroscopy to enable the detection of sub-micromolar quantities of analyte, a significant improvement over earlier OFP detection systems. The introduction of chromoionophore for monitoring absorbance changes at ~ 538 nm for the presence of fluoride ions enables the potential use of less expensive optical substrates such as commercially available glass slides and those coated with metal oxides as opposed to the expensive quartz slides, as their absorption bands influence on the pH chromophore absorbance measurements become insignificant. This system can be readily adapted to portable spectrophotometers such as the commercially available Ocean Optics USB 2000 spectrometer (Denedin, FL). Further enhancements in sensitivity may be introduced by increasing the path length of light by using the thin fluoride sensing films in a wave-guide configuration [175]. The only limitation at present for integration with OPH/OPAA immobilized biosensors is the two-step reaction conditions required for hydrolysis and detection. When overcome, the sensing approach described here has the potential for creating highly selective and simple detection system capable of screening for the presence of a wide spectrum of OPs.
7. FORMALDEHYDE DETECTION USING ACTIVATED MAGNESIUM SILICATE PARTICLES

7.1. Introduction

Formaldehyde is one of most widely found aldehydes that may be introduced in the atmosphere via primary emissions from natural vegetation and industrial plants [176-179]. The more sophisticated pathways of formaldehyde introduction may involve photochemical degradation of organic compounds such as methyl-tert-butyl ether (MTBE) [177, 180] and pollutants from mobile and stationary sources [181]. Formaldehyde being a possible health hazard is a matter of concern. Although its carcinogenic properties are controversial and questionable, the International Agency for Research on Cancer (IARC) identifies the chemical as a probable Class 2A carcinogen [182]. Some of the possible health effects of formaldehyde exposure may include eye and upper respiratory irritation, allergic skin reactions, drowsiness, headache and nausea [183]. In spite of its simple chemical structure, its toxicity and widespread applications have led to extensive studies to control and detect its presence in the environment. More recently, health issues related to the presence of formaldehyde and its release from trailers used for temporary housing following the Iowa Floods in 2008 [184] and hurricanes Katrina and Rita in 2005 [185-187] have demanded the attention and investigation by organizations like FEMA.

Importance to the research and development of formaldehyde detection methods has grown over years for reasons in addition to its toxicity. For example, quantification of formaldehyde when present as an end product is also used to detect carbohydrates (to study cigarette smoke [188] and acyl-gylcerides [188, 189]. Chromatographic methods are the most commonly used techniques for the detection of formaldehyde [190-196]. These methods typically require preliminary derivation of formaldehyde using hydrazines, such as 2,4-dinitrophenylhydrazine (DNP) before analysis. Despite their sensitivity (with detection limits up to 3 nM), these methods lack selectivity.

An attractive alternative is the approach introduced by Nash [197]- the condensation of ammonia and 2,4-pentane-dione with formaldehyde, which gives a fluorescent compound 3,5-diacetyl-1,4-dihydrolutidine (DDL). Subsequent modification of Nash's original approach has enhanced the sensitivity of formaldehyde detection. Among the most significant was the introduction of fluoral-P reagent, which is a mixture of pre-reacted ammonia and 2,4-pentanedione [198]. Fluoral-P, when utilized in a flow-injection mode in conjunction with high performance liquid chromatography analysis of the products, enabled sub-micromolar detection limits [199-201]. A more recent development involved the use of a solid support coupled with a flow-injection technique [202] wherein fluoral-P was absorbed onto C-18 beads and then placed onto the column for detection. Application of artificial neural networks for quantification of formaldehyde to optimize the dynamic range of detection has also improved analytical capabilities [203].

The current study builds on Nash method to develop a simple and robust formaldehyde sensor. Magnesium silicate (florisil) micro-particles were functionalized with Nash reagent in the form of Fluoral-P. The pretreated particles were attached to the glass slide surface and exposed to various concentrations of formaldehyde. The intensity of fluorescence was monitored using a fluorimeter. The experimental conditions for particle attachment were optimized for maximum sensitivity.

7.2. Methods and Materials

7.2.1. Chemical reagents

Formaldehyde solution (37% W/W), ammonium acetate and acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ); Florisil particles 60-100/PR mesh size ($280m^2/g$ surface area), Silica gel 200-425 mesh size ($480m^2/g$ surface area) and aluminum oxide 150 mesh size ($155m^2/g$ surface area) were purchased from Sigma Aldrich (St Louis, MO); 2,4-pentane-dione was purchased from Acros Organics (NJ, USA), De-Ionized (DI, 18.2 M Ω .cm) water was obtained from Millipore water purification system and polydimethyl siloxane (PDMS) was purchased from Hisco (Atlanta, GA). 12.1 mM formaldehyde stock solution was prepared in DI water, from which further dilutions were made for assays. The sample solutions were tested within 24 hours of their preparation. Ammonium acetate was vacuum dried prior use. All chemicals were used as received unless otherwise mentioned.

7.2.2. Florisil treatment with fluoral-P

The florisil particles were treated with fluoral-P using the procedure described below that was modified from the original Nash method. Fluoral P was prepared by weighing 15.4 g of ammonium acetate and mixing it with 300 μ L of acetic acid, 200 μ l of

2,4-pentane-dione and 4.5 ml of DI water. The resulting mixture was slightly heated in a microwave for ~ 10 s to dissolve the chemicals and form a solution.

For treatment of the particles, to 1.1 g of florisil, 2 ml of the above fluoral P stock solution and 8 ml DI water was added and mixed thoroughly. After 20 minutes of the treatment, the solution was filtered using a Buchner funnel. The florisil particles were airdried for about 2 minutes, stored in containers and sealed with paraffin film. This was essential to preserve the reactivity of particles since it was inevitable to have formaldehyde emissions from cabinets present in the lab. We will refer to such prepared fluoral-P treated particles as "activated florisil".

7.2.3. Immobilization of activated florisil on glass slide

The glass slides were cut (2.5 x 1.75 cm) using a diamond tip cutter to fit onto the slide holder of the fluorimeter. Two different techniques were used to attach the florisil particles on glass slides. The first technique involved the use of a double-sided adhesive tape (Permanent, Scotch 3M). A 5 mm hole drilled in plexiglass was used as a template to control and maintain the position, quantity and area of particles covering the slide. In the second method, PDMS was used as a matrix scaffold to enable robust attachment of activated florisil on glass slides. PDMS was prepared using the standard procedure in which parts A and B were weighed in the ratio of 10:1 and mixed thoroughly. The resulting air bubbles were degassed with a vacuum pump. The precut glass slides were plasma cleaned and spin coated with the above PDMS at 500 rpm for 2 minutes. The activated florisil was sprinkled onto the PMDS-coated surface and the slide was cured at 100°C for 15 minutes. The slides with attached florisil were then cooled to room temperature and stored in sealed containers until use.

7.2.4. Instrumentation

Figure 7.1 shows the schematic of the experimental setup. The fluorescence measurements were collected with a Photon Technology International fluorimeter. The glass slide was placed at 45° to the detector and light source (**Figure 7.1 A**). The area of particles attached to the glass slide (Φ 5mm) was within the area exposed to the detector (approximately 10mm x 8mm) to prevent the DDL formation outside the window of detection (**Figure 7.1 A&B**).

In the case of gas phase detection, the florisil area of coverage was equal to the area of window (approximately 10mm x 8mm) that allowed the incoming and emitting light travelling from source and to the detector respectively. The glass slide with these particles attached via tape was held with the activated florisil facing the inside walls of the 4-sided methacrylate cuvette on which another window was made to allow the interaction between the particles and gaseous HCHO present inside the cuvette (**Figure 7.1 C**).



Figure 7.1: Schematic representation of the instrumental set-up. A: Fluorimeter with the glass slide placed at an angle of 45° with respect to the incoming light source and outgoing fluorescence to the detector. B: Sensor involving cut glass slide with activated florisil particles attached using a double-sided adhesive tape or PDMS. The diameter of the area enclosing particles was 5 mm. C: Schematic of the gas phase sensor with a window made on the cuvette to place the slide with activated florisil particles attached via tape facing the solution inside cuvette.

7.2.5. Sensor Characterization

Formaldehyde detection was carried out by placing 10 μ L of formaldehyde solution on the florisil particles attached to the glass slide via tape/PDMS. Evaporation of HCHO was prevented by covering the glass slide with a watch glass. This was also done

to avoid any cross contamination of HCHO that may be released from the cabinets and other secondary sources present in the laboratory. After 15 minutes of reaction, emission scans were performed in the range of 450 nm to 530 nm with an excitation wavelength of 410 nm. To account for false positive signals, water was used as a reference. For detection of formaldehyde in air, various formulations of aqueous formaldehyde was placed in a closed cuvette with florisil particles positioned in the headspace above the samples (Figure 1C). Time based scans were obtained for excitation and emission wavelengths of 410 nm and 482 nm, respectively.

7.3. Results and discussions

7.3.1. Formaldehyde detection using activated magnesium silicate particles

Though introduced a few decades ago, the Nash approach for formaldehyde detection remains among the most sensitive and selective methods available. The underlying mechanism depends on the reaction in the presence of ammonia and acidic media that progresses rapidly with formaldehyde, but has increasingly negligible rates of reaction for higher-order ketones. For example, the same reaction with acetaldehyde is about 1000 times slower [197]. In addition, considerable fluorescence is expected to result from the product, 3,5-diacetyl-1,4-dihydrolutidine (DDL), only when formaldehyde is involved in the reaction. Therefore, fluorimetric detection is advantageous compared to absorbance spectroscopy. DDL has an emission peak around 510 nm when excited at 410 nm [197]. Extending the conjugated chain length of the reaction intermediates could shift this emission into the red or infrared regions.



Figure 7.2: Top: Original Nash reaction. Bottom: Hantzsch Reaction with fluoral p

The Nash method is based on the well known Hantzsch pyridine synthesis (**Figure 7.2**). With the introduction of fluoral-P [198], which is a pretreated mixture of ammonium acetate, 2,4-pentane-dione and acetic acid, the reaction conditions are more convenient with only HCHO remaining to form fluorescence product. As an imine, fluoral P is unstable in acidic media and decomposes to 2,4-pentane dione and ammonia. Hence, they are normally stored under refrigerated conditions, once prepared.

To demonstrate the retention of fluoral-P reactivity of the activated florisil particles towards HCHO, the prepared particles were tested visually for fluorescence when treated with intermediate concentrations of formaldehyde (**Figure 7.3**) by shining UV light (ActicureTM, Quebec, Canada) onto them. **Figure 7.3** (d) shows the fluorescent nature of the DDL product upon exposure to UV light for activated particles. Fluorescence was not observed for particles that were not activated with fluoral-P when treated with formaldehyde (3 c). These results demonstrate the presence of fluoral-P on florisil surface to produce and detect DDL. To further optimize the solid support that would best retain the fluoral-P reactivity, we also chose alumina and silica in addition to florisil particles for use as potential adsorbents by treating them with fluoral-P for activation. The activated florisil particles produced the best response in terms of fluorescence intensity resulting from DDL formation (data not shown) and were used for all future experimental work.



Reference: No HCHO

Figure 7.3: Fluorescent and colorimetric response of fluoral-P treated florisil particles. "a" and "b" correspond to untreated and activated florisil particles under visible light respectively, "c" and "d" correspond to untreated and activated florisil particles respectively, when treated with formaldehyde under UV light (350 nm). The figure on the right shows the calorimetric changes in activated florisil particles when treated with different concentrations of formaldehyde solutions.

7.3.2. Activation of florisil particles

The objective behind treating the florisil particles with fluoral-P was to provide a high surface area support that could retain the fluoral-P reactivity towards formaldehyde. In addition, this treatment would preserve the fluoral-P activity on solid support. **Figure 7.4** shows the time based scans for DDL resulting from 12.1 mM HCHO reaction with

fluoral-P solution before and after it is used to treat florisil particles for an excitation and emission wavelength of 410 and 490 nm, respectively. The reaction of HCHO with residual fluoral-P from the florisil treatment produced fluorescence with an intensity about one order of magnitude less than fresh fluoral-P, which indicated that the majority of fluoral-P was successfully adsorbed on to florisil to form "activated" particles.



Figure 7.4: Effect of florisil treatment with fluoral P. Time based intensities intensity of DDL product obtained on reaction between HCHO and fluoral-P before its treatment with florisil and the reaction between HCHO and filterant of fluoral-P solution obtained after its treatment with florisil particles. The inset shows the reduced scale of intensity for the treated fluoral-P solution (FP activity after florisil treatment.

7.3.3. Response Curve

Though the described method of activating florisil particles showed a large step towards simplicity for formaldehyde detection, it still requires a robust setup for sensor development. A method was devised to use glass slides as test substrates, distribute activated florisil onto the surface, and then add analyte solution. The resulting fluorescence appeared on the treated side, which was monitored. We exploited two techniques to attach pretreated florisil particles on glass slides. The first method involved the use of a double-sided adhesive tape. Optical noise was one of the problems encountered while using a tape to immobilize the florisil particles on glass slides. Another problem encountered with the use of tape was loss of particle adhesion upon the addition of HCHO. To address these problems, polydimethyl siloxane (PDMS) was used as a matrix to support the florisil particles on the glass slide. PDMS is optically transparent in the visible range and inert to most chemicals.

The sensitivity of the activated particles on the glass slide was characterized by exposing them to increasingly dilute concentrations of HCHO solutions. Water was used as a control to account for fluorescence resulting from unknown HCHO that may be present in the environment. In the earlier section, it was stated that the emission peak was around 490nm. However, it was also found that lower concentrations of HCHO resulted in the shift of emission peaks to lower wavelengths with an average maximum at 485 nm for the micro molar concentration range (**Figure 7.5**). Thus, the response curve was plotted for the % change in fluorescence intensity of DDL product at 485 nm for varying concentrations of formaldehyde.

Percentagechange in Intensity=
$$\left(\frac{Id-Iw}{Iw}\right) \times 100$$

where, I_d is the intensity of DDL from reaction with HCHO solution and I_w is the intensity for control (water).



Figure 7.5: Influence of formaldehyde concentration on the fluorescence emission wavelength. The inset graph shows the variation of peak emission wavelength and intensity as a function of formaldehyde concentration.

Figure 7.6 shows the response curve obtained for the different concentrations of HCHO when treated with activated florisil particles attached to glass slides using an adhesive tape and PDMS. Although the response curves for tape and PDMS supports show similar trends, the lowest concentration that could be detected was about 5μ M for PDMS and 2.5 μ M for adhesive tape immobilization. The range for linear response for formaldehyde was between 2 μ M and 50 μ M HCHO for both florisil supports. The lower sensitivity with the use of PDMS matrix may be attributed to decreased wetting of the PDMS surface by the aqueous HCHO solution due to the hydrophobic nature of PDMS. In addition, the exposed surface of the activated particles may have been reduced through the use of PDMS did not affect the trend of HCHO response that was obtained using tape. It should also be noted that the activity of fluoral-P in PDMS was retained despite the thermal cure during sample preparation.



Figure 7.6: Response curve obtained for the different concentrations of formaldehyde when treated with activated fluoral-P when immobilized on glass slide using an adhesive tape and PDMS. Inset graph shows the linear range of response for either florisil support with linear regression plots. For tape: y= 0.9894 x + 6.8908, R = 0.983, for PDMS y= 0.8520 x + 20.8076, R = 0.9643.

Vapor detection of HCHO was tested by exposing the fluoral P activated fluorisil particles to the headspace of a sample cell loaded with varying concentrations of HCHO in water. Here, the activated particles were attached to the glass slides using the adhesive tape configuration only. The resulting fluorescence response curve is shown in **Figure 7.7.** The slope was calculated over a time interval of 200 s and subtracted from the

response obtained for water. As seen in the graph, the time required to attain the maximum fluorescence intensity decreased from approximately 8 minutes (<1 mM HCHO) to less than 3 minutes with increasing formaldehyde concentrations. Concentrations above 1 mM HCHO took less than 5 minutes to attain saturation intensity. It was found that for concentrations above 1mM HCHO, DDL formation took place within the first 5 minutes while nearly 10 minutes were required for HCHO concentrations under 1 mM.



Figure 7.7: Variation in the response time to achieve maximum fluorescent intensity (**•**) and slope (•) of DDL fluorescence for different concentrations of formaldehyde for gas phase detection. The inset shows the linear range of response for formaldehyde of concentrations from 0.025 mM to 1 mM. (y=0.1108x + 9.1625, R = 0.9934).

7.4. Conclusions

In this study, a simple, yet robust fluorescence detection method for formaldehyde by utilizing magnesium silicate (florisil) micro particles activated with modified Nash reagent in the form of fluoral-P was developed. The presence of a solid support confined the reaction environment to these particles within the window of detector, thereby increasing the system sensitivity. The sample volume requirement of the developed sensor is within 10 microliters, which is a significant improvement over earlier detection systems [204] that require several hundreds of microliters. The robust characteristic of the sensor with the introduction of PDMS for the attachment of activated florisil to glass slides parallels the ease of litmus paper testing for acid/base analysis. The resemblance is stronger considering that the DDL product produces visible changes in color in addition to fluorescence observed under UV light. This method is compatible with on-site field analysis of trailer homes using a portable fluorimeter and a small-format light source and emission filters.

8. OVERALL CONCLUSIONS

Integration of sensing and transducing components using appropriate surface chemistries and morphologies are critical to the detection of analytes with good sensitivity, stability, selectivity and quick response times for applications in chemical and biological sensors.

The studies presented in this dissertation investigate the feasibility of titanium dioxide nanoparticles and magnesium silicate particles as potential surfaces conducive for sensing elements with the specific aim to enhance sensitivity via signal amplification of reporter molecules that traduce target-recognition element interactions. To achieve this, a planar waveguide coated with titanium dioxide nanoparticles was used as a biomaterial scaffold to develop an enzyme based evanescent array biosensor. Characterization experiments showed the presence of ~ 13 nm anatase phase crystalline particles in the film of thickness ~ 2.2 μ m. Theoretical calculations for estimations on its refractive index showed the presence of a relatively low refractive index anatase film of ~ 1.9 as compared to 2.52 for a pore free anatase film. To make the surface amenable for covalent immobilization of proteins for biosensor development, modifications were made using mercaptosilane and cross-linkers. An enzyme organophosphorus hydrolase (OPH), conjugated with a pH-sensitive fluorophore, carboxynaphthofluorescein (CNF) was used as a biosensing element and immobilized on the titanium dioxide surface for the detection

of organophosphate neurotoxins. Bovine serum albumin (BSA) was used as a nonenzymatic scaffold protein for CNF attachment at the reference spots to allow for the discrimination of pH changes due to enzymatic and non-enzymatic reactions. An array biosensor unit developed at the Naval Research Laboratories (NRL) was adopted as the detection platform and appropriately modified for enzyme-based measurements. The developed sensor enabled the detection of $\sim 2.5 \,\mu\text{M}$ paraoxon, and $\sim 10 \,\mu\text{M}$ DFP and parathion, respectively. Very short response time of 30 s was achieved with a total analysis time of less than 2 min. When operated at room temperature and stored at 4 °C, the waveguide retained reasonable activity for greater than 45 days. However, there was a significant drop in sensitivity of signal within 3 weeks of sensor preparation. Desorption of some portion of adsorbed biomolecules from porous substrates and loss of enzymatic activity over time due to its covalent immobilization are some of the possible reasons behind this loss in signal. The experiments conducted to measure the presence of OPH in the storage buffer to check for desorption over a period of 2 months did not result in measureable concentrations of OPH. Hence, the loss in enzymatic activity was attributed to drop in sensitivity for covalently immobilized enzyme on titanium dioxide glass slides.

In light of the above obervations, an alternative strategy of protein immobilization via its encapsulation in silica particles was studied. This involved rapid enzyme immobilization directly on a waveguide surface by encapsulation in a silica matrix. OPH was again used as a model enzyme to demonstrate the utility of lysozyme-mediated silica formation for enzyme stabilization. Silica morphology and the efficiency of OPH encapsulation were directly influenced by the precursor choice used in silica formation. Covalent attachment of the lysozyme template directly to the waveguide surface provided a stable basis for silica formation and significantly increased the surface area for OPH encapsulation. OPH conjugated to a pH-responsive fluorophore was encapsulated in silica and patterned to a waveguide surface to demonstrate the immobilization strategy for the development of an organophosphate array biodetector. Silica-encapsulated OPH retained its catalytic activity for nearly 60 days with a detection limit of paraoxon of \sim 35 µM. The encapsulation technique provides a potentially versatile tool with specific application to biosensor development.

From the two methodologies developed to immobilize enzyme on glass slides coated with commercially obtained titanium dioxide nanoparticles, it was concluded that the covalent immobilization technique required longer sensor preparation times (~ 3 days) and provided better sensitivity for paraoxon detection while the encapsulation technique of immobilization required ~ 10 hours for sensor preparation and provided relatively superior OPH stability. As an extension to OP detection, a novel system for the detection and quantification of organofluorophosphonates (OFP) was developed by using an optical sensing polymeric membrane to detect the fluoride ions produced upon OFP Diisopropyl fluorophosphate (DFP), a structural analogue of Type G hydrolysis. Chemical Warfare Agents such as Sarin (GB) and Soman (GD) nerve agents, was used as the surrogate target analyte. An optical sensing fluoride-ion-selective polymeric film was formulated from plasticized PVC containing aluminum(III) octaethylporphyrin and ETH 7075 chromoionophore (ALOEP-ETH 7075). Selected formulations were used to detect the fluoride ions produced by the catalytic hydrolysis of DFP by the enzyme organophosphate hydrolase (OPH, EC 3.1.8.1). The changes in absorbance that corresponded to the deprotonated state of chromoionophore within the film resulted from

simultaneous co-extraction of fluoride and protons, as DFP hydrolysis occurred in the solution phase in contact with the film. The developed sensing system demonstrated excellent sensitivity for concentrations as low as $0.1 \mu M$ DFP.

Finally, the feasibility of magnesium silicate particles as scaffolds for chemical sensing elements was tested for the development of chemical sensors. To test this, a sensing strategy for formaldehyde (HCHO) detection in liquid and vapor samples was designed. Preliminary results showed that the sensors, in addition to being sensitive and flexible sensor, was extremely simple to build. Modified Nash reagent is utilized in the form of fluoral-P and is absorbed onto magnesium silicate (florisil) micro-particles. These pre-activated particles were attached to the glass slide surface via tape and via dispersion in polydimethyl siloxane (PDMS) matrix. Fluorimetric quantification of a photoluminescent product of formaldehyde and fluoral-P activated florisil, namely, 3,5diacetyl-1,4-dihydrolutidine (DDL) allows detection of aqueous HCHO for concentrations as low as 2 µM. The only stipulation with its implementation on portable devices for on-site detection was the availability of appropriate excitation sources for DDL product to emit fluorescence.

9. FUTURE WORK

9.1. Bioinspired mediation of titanium dioxide for coating on glass slides

Studies have shown the capability of lsyzome to mediate silica and titanium dioxide nanoparticles under room temperature conditions [205]. The titanium dioxide nanoparticles used as a scaffold material for enzyme immobilization detailed in chapters 4 and 5, was synthesized via hydrothermal method (obtained from the manufacturer). Silica encapsulation of proteins conjugated to reporter fluorophores during its mediation by lysozyme on glass slide surfaces coated with titanium dioxide nanoparticles was studied in chapter 5. As an extension to this study and to develop an alternative technique for titanium dioxide deposition, glass slide that has immobilized lysozyme, can be treated with titanium dioxide precursor such as Titanium (IV) bis(ammonium lactate) dihydroxide (TiBALDH) solution. This method has the potential for direct encapsulation of enzymes on glass slide surfaces with titanium dioxide nanoparticles.

Figure 9.1 shows the preliminary results on the deposition of titanium dioxide nanoparticles on glass slide via lysozyme mediation in alternative layers. 4 layers and 8 layers of deposition showed the presence of titanium dioxide covering the entire glass slide surface. Two different concentrations of lysozyme (10 mg/ml and 100 mg/ml) were used for comparison purposes. The layers appear to be porous for both the concentrations used. However, films prepared with 10 mg/ml lysozyme appeared produce a film of

particles similar in size to the titanium dioxide used in the earlier studies. Further studies are required to control and determine the surface area, porosity and refractive index of the film to enable its wave guiding properties. Since the lysozyme is expected to get encapsulated during the mediation process, the developed films also have potential antibacterial applications due to the presence of lysozyme on their surface.



Figure 9.1: Titanium dioxide deposition on glass slides via lysozyme mediation.

8L-100: 8 alternative layers of lysozyme (100 mg/ml) and titanium dioxide precursor. 8L-10: 8 alternative layers of lysozyme (10 mg/ml) and titanium dioxide precursor. 4L-100: 4 alternative layers of lysozyme (100 mg/ml) and titanium dioxide precursor. 4L-10: 4 alternative layers of lysozyme (10 mg/ml) and titanium dioxide precursor.

9.2. Enzyme stabilization in extreme pH conditions

Enzymes are rendered inactive at extreme conditions of pH. For example, a basic enzyme like OPH, which has an isoelectric point of ~8.3, does not hydrolyze OPs at pH of 3.0, attributable to its protonation. These biomolecules are specifically inclined to undergo denaturing when present in solution. Immobilization of enzymes such as their encapsulation in sol gel matrices are known to make them stable attributed to the ceramic cage possibly preventing "unfolding-refolding" motions thereby preserving enzyme activity. Studies by Frenkel-Mullerad et. al. have shown that alkaline phosphatase, that is active in a pH range of 9-10, can maintain its activity at pH as low as 0.9, when encapsulated in silica gel with an anionic surfactant [174]. According to Frenkel-Mullerad et.al., the pH inside the cage with enzyme containing a reservoir of ~ 100 water molecules has only a few protons (implying greater pH) in comparison to the external reservoir containing many water molecules (implying lower pH). The higher internal pH is thus sufficient to maintain the enzyme activity.

Chapter 6 in this dissertation shows the development of DFP sensor using a fluoride ion selective membrane that operates at pH 3.0. Here, the hydroxyl ions interfere with fluoride ions to complex with aluminum porphyrin membrane when the system is operated at high pH and OPH in inactived at low pH due to its protonation. Hence, a two step reaction system of DFP hydrolysis at high pH following which, the solutions were diluted to reduce the pH for fluoride ion detection at pH 3.0, was designed. To circumvent this two step reaction, OPH can be encapsulated within silica particles synthesized using sol-gel process as described by Frenked- Mullerad et.al. for alkaline

phosphotase, to preserve its activity at pH 3.0. Figure shows the modified experimental set-up with preliminary results for DFP detection carried out under single pot reaction conditions.



Figure 9.2: Top figure shows the schematic of the experimental set-up for single pot reaction. The graph shows the response of the polymer film to different concentrations of DFP.

By introducing biosensing enzymes in the titanium dioxide precursor solution during its mediation via lysozyme as was explained in section 9.1, along with appropriate surfactant during encapsulation as explained in section 9.2; the potential application of titanium dioxide nanoparticles to protect enzymes at extreme pH may be realized. Further, the influence of nanoparticle size to modulate the microenvironmental pH may be studied. With this, the possibility of titanium dioxide nanoparticles to act as smart passive materials that enhance the functional properties of sensing layer by providing a biocompatible, high surface area, waveguiding support with the capability for biomolecular stabilization conditions may be realized. at extreme

REFERENCES

- [1] A. Hulanicki,S. Geab andF. Ingman, Chemical Sensors: Definitions and classifications, Pure & App.chem., 63, (1991) 1247-1250.
- [2] Biosenosor, IUPAC: Glossary for chemists of terms used in biotechnology, 64, (1992) 148.
- [3] M. S. Frant, Historical perspective. History of the early commercialization of ionselective electrodes, Analyst, 119, (1994) 2293 - 2301.
- [4] L. C. Clark Jr. and C. Lyons, Electrode systems for continuous monitoring in cardiovascular surgery, Ann. N.Y. Acad. Sci., 102, (1962) 29-45.
- [5] Sensors Market to Reach \$61 Billion by 2010. 2007 [cited 2009 20 November 2009]; Sensors: A Global Strategic Business Report]. Available from: <u>http://www.electronics.ca/presscenter/articles/649/1/Sensors-Market-to-Reach-61-Billion-by-2010/Page1.html</u>.
- [6] Market for Biosensors to Reach \$6.1 Billion by 2012, According to New Report by Global Industry Analysts, Inc. 2008 [cited 2009 1 November 2009]; Available from: http://www.azonano.com/news.asp?newsID=8571.
- [7] P. T. Kissinger, Biosensors a perspective, Biosens.Bioelectron., 20, (2005) 2512-2516.
- [8] U. E. Spichiger-Keller, *Chemical Sensors and Biosensors for Medical and Biological Applications* Wiley-VCH: 1998, p 413.
- [9] C. R. Taitt, G. P. Anderson and F. S. Ligler, Evanescent wave fluorescence biosensors, Biosens. Bioelectron., 20, (2005) 2470-2487.

- [10] X. D. Hoa, A. G. Kirk and M. Tabrizian, Towards integrated and sensitive surface plasmon resonance biosensors: A review of recent progress, Biosens. Bioelectron., 23, (2007) 151-160.
- [11] A. Arzamastsev, N. Sadchikova and A. Titova, Current state of IR spectroscopy applied to pharmaceutical analysis, Pharm. Chem. J., 42, (2008) 466.
- [12] B. E. Eggins, in: R.B. Eggins, Editor[^]Editors*Chemical Sensors and Biosensors*, *Analytical Techniques in the Sciences*. 2007.
- [13] S. S. Kaun and G. G. Guilbault, *Ion Selective electrodes and bisensors based on ISEs* Oxford University Press.: 1987, p.
- [14] O. D. Renedo, M. A. Alonso-Lomillo and M. J. A. Martínez, Recent developments in the field of screen-printed electrodes and their related applications, Talanta, 73, (2007) 202-219.
- [15] I. Švancara, A. Walcarius, K. Kalcher and K. Vytřas, Carbon paste electrodes in the new millennium, Cent. Eur. J. Chem., 7, (2009) 598-656.
- [16] E. Jubete, O. A. Loaiza, E. Ochoteco, J. A. Pomposo, H. Grande and J. Rodríguez, Nanotechnology: A Tool for Improved Performance on Electrochemical Screen-Printed (Bio)Sensors, Journal of Sensors, 2009, (2009) 1-13.
- [17] A. Bonanni, M. Esplandiu, M. Pividori, S. Alegret and M. del Valle, Impedimetric genosensors for the detection of DNA hybridization, Anal. Bioanal.Chem., 385, (2006) 1195-1201.
- [18] M. T. Castañeda, A. Merkoçi, M. Pumera and S. Alegret, Electrochemical genosensors for biomedical applications based on gold nanoparticles, Biosens. Bioelectron., 22, (2007) 1961-1967.
- [19] G. Liu,Y. Wan,V. Gau,J. Zhang,L. Wang,S. Song andC. Fan, An Enzyme-Based E-DNA Sensor for Sequence-Specific Detection of Femtomolar DNA Targets, J. Am. Chem. Soc., 130, (2008) 6820-6825.
- [20] PichonAnne, Electrochemical DNA sensors: Turn on the signal, (2008).

- [21] K. Hashimoto, K. Ito and Y. Ishimori, Microfabricated disposable DNA sensor for detection of hepatitis B virus DNA, Sens. Actuators, B, 46, (1998) 220.
- [22] V. A. Petrenko and V. J. Vodyanoy, Phage display for detection of biological threat agents, J. Microbiol. Methods, 53, (2003) 253.
- [23] R. Cui,W.-J. Chung andD. Jahng, A rapid and simple respirometric biosensor with immobilized cells of Nitrosomonas europaea for detecting inhibitors of ammonia oxidation, Biosens. Bioelectron., 20, (2005) 1788-1795.
- [24] K. Yano and I. Karube, Molecularly imprinted polymers for biosensor applications, TrAC, Trends Anal. Chem., 18, (1999) 199.
- [25] R. F. Taylor, *Protein Immobilization: Fundementals and Applications*. Vol. 14 Marcel Dekker, Inc: 1991, p 377.
- [26] T. Cass and F. S. Ligler, *Immobilized Biomolecules in Analysis: A Practical Approach* Oxford University Press: 1998, p.
- [27] G. T. Hermanson, *Bioconjugate Techniques*. Second, Elsevier: 2008, p 1202.
- [28] R. B. M. Schasfoort and A. J. Tudos, *Handbook of Surface Plasmon Resonance* The Royal Society of Chemistry: 2008, p.
- [29] C. Burda,X. Chen,R. Narayanan andM. A. El-Sayed, Chemistry and Properties of Nanocrystals of Different Shapes, Chem. Rev., 105, (2005) 1025-1102.
- [30] J. C. Love, L. A. Estroff, J. K. Kriebel, R. G. Nuzzo and G. M. Whitesides, Self-Assembled Monolayers of Thiolates on Metals as a Form of Nanotechnology, Chem. Rev., 105, (2005) 1103-1169.
- [31] A. W. Adamson, *Physical chemistry of surfaces* Wiley-Interscience: 1982, p.
- [32] L. G. Sultatos, Mammalian toxicology of organophosphorus pesticides, J. Toxicol. Environ. Health, 43, (1994) 271-289.

- [33] H. W. Chambers, J. S. Boone, R. L. Carr and J. E. Chambers, *Chemistry of Organophoshorus Insecticides* Academic Press: 2001, p.
- [34] R. C. Gupta, in: R.C. Gupta, Editor^EditorsClassification and Uses of Organophosphates and Carbamates, Toxicology of Organophosphate & Carbamate Compounds. Academic Press, 2006. pp. 5-24.
- [35] S. M. Somani, *Chemical Warfare Agents* Academic Press, Inc.: 1992, p 443.
- [36] L. G. Costa, Current issues in organophosphate toxicology, Clinica Chimica Acta, 366, (2006) 1-13.
- [37] M. K. Johnson and P. Glynn, Neuropathy target esterase (NTE) and organophosphorus-induced delayed polyneuropathy (OPIDP): recent advances, Toxicol. Lett., 82-83, (1995) 459-463.
- [38] R. M. Black, J. M. Harrison and R. W. Read, The interaction of sarin and soman with plasma proteins: the identification of a novel phosphonylation site, Arch. Toxicol., 73, (1999) 123-126.
- [39] E. Hodgson, In vitro human phase I metabolism of xenobiotics I: pesticides and related compounds used in agriculture and public health, May 2003, J. Biochem. Mol. Toxicol., 17, (2003) 201-206.
- [40] A. J. J. Ooms and C. V. Dijk, The reaction of organophosphorus compounds with hydrolytic enzymes—III: The inhibition of chymotrypsin and trypsin, Biochem. Pharmacol., 15, (1966) 1361-1377.
- [41] H. L. Boter and A. J. Ooms, Stereospecificity of hydrolytic enzymes in their reaction with optically active organophosphorus compounds—II The inhibition of aliesterase, acetylesterase, chymotrypsin and trypsin by S-alkyl p-nitrophenyl methylphosphonothiolates, Biochem. Pharmacol., 16, (1967) 1563-1569.
- [42] D. J. Ecobichon and A. M. Comeau, Pseudocholinesterases of mammalian plasma: Physicochemical properties and organophosphate inhibition in eleven species, Toxicol. Appl. Pharmacol., 24, (1973) 92-100.

- [43] M. K. Johnson, Organophosphorus esters causing delayed neurotoxic effects: mechanism of action and structure activity studies, Arch. Toxicol., 34, (1975) 259-288.
- [44] F. Fonnum,S. H. Sterri,P. Aas andH. Johnsen, Carboxylesterases, importance for detoxification of organophosphorus anticholinesterases and trichothecenes, Fundam. Appl. Toxicol., 5, (1985) S29-S38.
- [45] J. J. Gordon, R. H. Inns, M. K. Johnson, L. Levence, M. P. Maidment, D. G. Upshall, G. H. Cooper and R. L. Rickard, The delayed neuropathic effects of nerve agents and some other organophosphorus compounds, Arch. Toxicol., 52, (1983).
- [46] M. Lotti, A. Moretto, R. Zoppellari, R. Dainese, N. Rizzuto and G. Barusco, Inhibition of lymphocytic neuropathy target esterase predicts the development of organophosphate-induced delayed polyneuropathy, Arch. Toxicol., 59, (1986) 176-179.
- [47] K. A. Funk, C. H. Liu, R. J. Higgins and B. W. Wilson, Avian Embryonic Brain Reaggregate Culture System II. NTE Activity Discriminates between Effects of a Single Neuropathic or Nonneuropathic Organophosphorus Compound Exposure, Toxicol. Appl. Pharmacol., 124, (1994) 159-163.
- [48] N. Senannayake and L. Karalliedde, Neurotoxic effects of organophosphate insecticides: an intermediate syndrome, N. Engl. J. Med., 316, (1987) 716-763.
- [49] USEPA: Pesticide Reregistration Status for Organophosphates. 2009, United States Environmental Protection Agency.
- [50] F. Worek, M. Koller, H. Thiermann and L. Szinicz, Diagnostic aspects of organophosphate poisoning, Toxicology, 214, (2005) 182-189.
- [51] S.W. Wiener and R.S. Hoffman, Nerve Agents: A Comprehensive Review, Journal of intensive care medicine, 19, (2004) 22-37.
- [52] Potentail exosure to sarin from teh demolitions at Khamisiyah, Iraq on March 10, 1991 [cited 2009 28 November]; Available from: http://www.gulflink.osd.mil/dugway/low_lv_chem.htm.

- [53] *Chemical weapons*. Weapons of mass destruction [cited 2009 20 November]; Available from: <u>http://www.fas.org/nuke/guide/russia/cbw/cw.htm</u>.
- [54] C. Raab. *Iraq sarin stockpiles found*. 2006 [cited 2009 28 November]; Available from: http://www.news.com.au/story/0,10117,19552050-23109,00.html.
- [55] Chemical Weapons. Weapons of Mass destruction 2005 28 April 2005 [cited 2009 28 November]; Available from: <u>http://www.globalsecurity.org/wmd/systems/cw.htm</u>.
- [56] R. Pfirter. *The Chemical Weapons Ban: Facts and Figures*. Organization for the prohibition of chemical weapons [cited 2009 28 November].
- [57] Arms control Association. Fact Sheets: The Chemical Weapons Convention at a Glance. 2003 [cited 2006 6 February]; Available from: http://www.armscontrol.org/factsheets/cwcunderstanding.asp.
- [58] Occupational Pesticide Poisoning in Apple Orchards -- Washington, 1993. 1993
 [cited 2006 7 February]; Available from: http://www.cdc.gov/mmwr/preview/mmwrhtml/00023208.htm.
- [59] *Pesticides and Child Safety*. [cited 2006 7 February]; Available from: <u>http://www.epa.gov/pesticides/factsheets/childsaf.htm</u>.
- [60] Sarin gas attack on the Tokyo subway. 1995 [cited 2009 28 November]; Available from: <u>http://en.wikipedia.org/wiki/Sarin_gas_attack_on_the_Tokyo_subway</u>.
- [61] *Halabja poison gas attack*. [cited 2009 28 November]; Available from: <u>http://en.wikipedia.org/wiki/Halabja_poison_gas_attack</u>.
- [62] *Bomb said to hold deadly sarin gas explodes in Iraq.* MSNBC 2007 [cited 2009 28 November]; Available from: http://www.msnbc.msn.com/id/4997808/.
- [63] M. S. Nieuwenhuizen and J. L. N. Harteveld, Development of a surface acoustic wave gas sensor for organophosphorus nerve agents employing lanthanide compounds as the chemical interface, Talanta, 41, (1994) 461-472.

- [64] M. S. Nieuwenhuizen and J. L. N. Harteveld, Studies on a surface acoustic wave (SAW) dosimeter sensor for organophosphorous nerve agents, Sens. Actuators, B, 40, (1997) 167-173.
- [65] A. L. Jenkins, O. M. Uy and G. M. Murray, Polymer-Based Lanthanide Luminescent Sensor for Detection of the Hydrolysis Product of the Nerve Agent Soman in Water, Anal. Chem., 71, (1998) 373-378.
- [66] A. L. Jenkins and S. Y. Bae, Molecularly imprinted polymers for chemical agent detection in multiple water matrices, Analytica Chimica Acta, 542, (2005) 32.
- [67] S. D. Harvey, D. A. Nelson, B. W. Wright and J. W. Grate, Selective stationary phase for solid-phase microextraction analysis of sarin (GB), J. Chromatogr., A, 954, (2002) 217-225.
- [68] D. Richardson and J. Caruso, Derivatization of organophosphorus nerve agent degradation products for gas chromatography with ICPMS and TOF-MS detection, Anal. Bioanal. Chem., 388, (2007) 809-823.
- [69] K. Tuovinen,H. Paakkanen andO. Hänninen, Determination of soman and VX degradation products by an aspiration ion mobility spectrometry, Anal. Chim. Acta, 440, (2001) 151-159.
- [70] M. H. Erhard, R. Kühlmann, L. Szinicz and U. Lösch, Detection of the organophosphorus nerve agent soman by an ELISA using monoclonal antibodies, Archives of Toxicology, 64, (1990) 580-585.
- [71] B. Kuswandi, R. Andres and R. Narayanaswamy, Optical fibre biosensors based on immobilised enzymes., Analyst, 126, (2001) 1469-1491.
- [72] S. L. Sole´,A. Merkoc,I andS. Alegret, Determination of Toxic Substances Based on Enzyme Inhibition. Part I. Electrochemical Biosensors for the Determination of Pesticides Using Batch ProceduresChemistry, Crit. Rev. Anal. Chem., 33, (2003) 33-89.
- [73] B. Kuswandi and M. Mascini, Enzyme Inhibition Based Biosensors for Environmental Monitoring, Curr. Enzyme Inhib., 1, (2005) 207-221.

- [74] G. Palleschi, M. Bernabei, C. Cremisini and M. Mascini, Determination of organophosphorus insecticides with a choline electrochemical biosensor, Sens. Actuators, B, 7, (1992) 513-517.
- [75] P. Skladal andM. Mascini, Sensitive detection of pesticides using amperometric sensors based on cobalt phthalocyanine-modified composite electrodes and immobilized cholinesterases, Biosens. Bioelectron., 7, (1992) 335-343.
- [76] T. T. Bachmann and R. D. Schmid, A disposable multielectrode biosensor for rapid simultaneous detection of the insecticides paraoxon and carbofuran at high resolution, Anal. Chim. Acta, 401, (1999) 95-103.
- [77] T. T. Bachmann,B. Leca,F. Vilatte,J.-L. Marty,D. Fournier andR. D. Schmid, Improved multianalyte detection of organophosphates and carbamates with disposable multielectrode biosensors using recombinant mutants of Drosophila acetylcholinesterase and artificial neural networks, Biosens. Bioelectron., 15, (2000) 193-201.
- [78] W. Zhao, P.-Y. Ge, J.-J. Xu and H.-Y. Chen, Selective Detection of Hypertoxic Organophosphates Pesticides via PDMS Composite based Acetylcholinesterase-Inhibition Biosensor, Environ. Sci. Technol., 43, (2009) 6724-6729.
- [79] A. M. N. Hendji, N. Jaffrezic-Renault, C. Martelet, P. Clechet, A. A. Shlu'ga, V. I. Strikha, L. I. Netchiporuk, A. P. Soldatkin and W. B. Wlodarski, Sensitive detection of pesticides using a differential ISFET-based system with immobilized cholinesterases, Anal. Chim. Acta, 281, (1993) 3-11.
- [80] K. Wan,J. M. Chovelon and N. Jaffrezic-Renault, Enzyme-octadecylamine Langmuir-Blodgett membranes for ENFET biosensors, Talanta, 52, (2000) 663-670.
- [81] T. Montesinos, S. Perez-Munguia, F. Valdez and J.-L. Marty, Disposable cholinesterase biosensor for the detection of pesticides in water-miscible organic solvents, Anal. Chim. Acta, 431, (2001) 231-237.
- [82] H.-S. Lee, Y. Ah Kim, Y. Ae Cho and Y. Tae Lee, Oxidation of organophosphorus pesticides for the sensitive detection by a cholinesterase-based biosensor, Chemosphere, 46, (2002) 571-576.

- [83] S. V. Dzyadevych, A. P. Soldatkin and J.-M. Chovelon, Assessment of the toxicity of methyl parathion and its photodegradation products in water samples using conductometric enzyme biosensors, Anal. Chim. Acta, 459, (2002) 33-41.
- [84] J. M. Abad, F. Pariente, L. Hernandez, H. D. Abruna and E. Lorenzo, Determination of Organophosphorus and Carbamate Pesticides Using a Piezoelectric Biosensor, Anal. Chem., 70, (1998) 2848-2855.
- [85] J.-W. Choi, Y.-K. Kim, I.-H. Lee, J. Min and W. H. Lee, Optical organophosphorus biosensor consisting of acetylcholinesterase/viologen hetero Langmuir-Blodgett film, Biosens. Bioelectron., 16, (2001) 937-943.
- [86] V. G. Andreou and Y. D. Clonis, A portable fiber-optic pesticide biosensor based on immobilized cholinesterase and sol-gel entrapped bromcresol purple for infield use, Biosens. Bioelectron., 17, (2002) 61-69.
- [87] R.-A. Doong andH.-C. Tsai, Immobilization and characterization of sol-gelencapsulated acetylcholinesterase fiber-optic biosensor, Anal. Chim. Acta, 434, (2001) 239-246.
- [88] B. J. White, J. Andrew Legako and H. James Harmon, Spectrophotometric detection of cholinesterase inhibitors with an integrated acetyl-/butyrylcholinesterase surface, Sens. Actuators, B, 89, (2003) 107-111.
- [89] *Gas 'killed Moscow hostages*. 2002 [cited 2009 20 November]; Available from: http://news.bbc.co.uk/2/hi/europe/2365383.stm.
- [90] W. J. Donarski, D. P. Dumas, D. P. Heitmeyer, V. E. Lewis and F. M. Raushel, Structure-Activity Relationships in the Hydrolysis of Substrates by the Phosphotriesterase from Pseudomonas diminuta, Biochemistry, 28, (1989) 4650-4655.
- [91] D. P. Dumas, J. R. Wild and F. M. Raushel, Diisopropylfluorophosphate hydrolysis by a phosphotriesterase from Pseudomonas diminuta., Biotechnol. appl. biochem., 11, (1989) 235-243.
- [92] Y. Ashani, N. Rothschild, Y. Segall, D. Levanon and L. Raveh, Prophylaxis against organophsphate poisoning by an enzyme hydrolysing organophosphorus compounds in mice, Life Sci., 49, (1991) 367-374.

- [93] C. Hassett,R. J. Richter,R. Humbert,C. Chapline,J. W. Crabb,C. J. Omiecinski andC. E. Furlong, Characterization of cDNA clones encoding rabbit and human serum paraoxonase: The mature protein retains its signal sequence, Biochemistry, 30, (1991) 10141-10149.
- [94] F. C. Hoskin and A. H. Roush, Hydrolysis of nerve gas by squid-type diisopropyl phosphorofluoridate hydrolyzing enzyme on agarose resin., Science, 215, (1982) 1255-1257.
- [95] J. J. Defrank, W. T. Beaudry, T.-C. Cheng, S. P. Harvey, A. N. Stroup and L. L. Szafraniec, Screening of halophilic bacteria and Alteromonas species for organophosphorus hydrolyzing enzyme activity, Chem.-Biol. Interact., 87, (1993) 141-148.
- [96] T. C. Cheng,S. P. Harvey andG. L. Chen, Cloning and expression of a gene encoding a bacterial enzyme for decontamination of organophosphorus nerve agents and nucleotide sequence of the enzyme, Appl. Environ. Microbiol., 62, (1996) 1636-1641.
- [97] D. P. Dumas, H. D. Durst, W. G. Landis, F. M. Raushel and J. R. Wild, Inactivation of organophosphorus nerve agents by the phosphotriesterase from Pseudomonas diminuta, Arch. Biochem. Biophys., 277, (1990) 155-159.
- [98] M. Y. Chae, J. F. Postula and F. M. Raushel, Stereospcific enzymatic hydrolysis of phosphorus-sulfur bonds in chiral organophosphate triesters, Bioorg. Med. Chem. Lett., 4, (1994) 1473-1478.
- [99] K. Lai, N. J. Stolowich and J. R. Wild, Characterization of P-S Bond Hydrolysis in Organophosphorothioate Pesticides by Organophosphorus Hydrolase, Arch. Biochem. Biophys., 318, (1995) 59-64.
- [100] J. E. Kolakowski, J. J. Defrank, S. P. Harvey, L. L. Szafraniec, W. T. Beaudry, K. Lai and J. R. Wild, Enzymatic hydrolysis of the chemical warfare agent VX and its neurotoxic analogues by organophosphorus hydrolase. Biocatalysis and Biotransformation, 15, (1997) 297-312.
- [101] M. M. Benning, H. Shim, F. M. Raushel and H. M. Holden. *High resolution X-ray structures of different metal-substituted forms of phosphotriesterase from Pseudomonas diminuta*. 2001 [cited 2009 20 November]; Available from: <u>http://www.rcsb.org/pdb/explore/jmol.do?structureId=1HZY&bionumber=1</u>.
- [102] E. I. Rainina, E. N. Efremenco, S. D. Varfolomeyev, A. L. Simonian and J. R. Wild, The development of a new biosensor based on recombinant E. coli for the direct detection of organophosphorus neurotoxins, Biosensors and Bioelectronics, 11, (1996) 991-1000.
- [103] A. K. Singh,A. W. Flounders,J. V. Volponi,C. S. Ashley,K. Wally andJ. S. Schoeniger, Development of sensors for direct detection of organophosphates. Part I: immobilization, characterization and stabilization of acetylcholinesterase and organophosphate hydrolase on silica supports, Biosensors and Bioelectronics, 14, (1999) 703-713.
- [104] A. W. Flounders, A. K. Singh, J. V. Volponi, S. C. Carichner, K. Wally, A. S. Simonian, J. R. Wild and J. S. Schoeniger, Development of sensors for direct detection of organophosphates.: Part II: sol-gel modified field effect transistor with immobilized organophosphate hydrolase, Biosensors and Bioelectronics, 14, (1999) 715-722.
- [105] A. L. Simonian, E. I. Rainina and J. R. Wild, A new approach for discriminative detection of organophosphate neurotoxins in the presence of other cholinesterase inhibitors, Anal. Lett., 30, (1997) 2453–2468.
- [106] A. L. Simonian, E. N. Efremenko and J. R. Wild, Discriminative detection of neurotoxins in multi-component samples, Anal. Chim. Acta, 444, (2001) 179-186.
- [107] Y. Lee, I. Stanish, V. Rastogi, T.-c. Cheng and A. Singh, Sustained Enzyme Activity of Organophosphorus Hydrolase in Polymer Encased Multilayer Assemblies, Langmuir, 19, (2003) 1330-1336.
- [108] G. Iqbal andB. Antonio, Degradation of organophosphorous nerve agents by enzyme-polymer nanocomposites: Efficient biocatalytic materials for personal protection and large-scale detoxification, Biotechnology and Bioengineering, 70, (2000) 400-410.
- [109] B. J. White and H. J. Harmon, Optical solid-state detection of organophosphates using organophosphorus hydrolase, Biosensors and Bioelectronics, 20, (2005) 1977-1983.

- [110] R. J. Russell, M. V. Pishko, A. L. Simonian and J. R. Wild, Poly(ethylene glycol) Hydrogel-Encapsulated Fluorophoreâ[^]Enzyme Conjugates for Direct Detection of Organophosphorus Neurotoxins, Analytical Chemistry, 71, (1999) 4909-4912.
- [111] L. Viveros, S. Paliwal, D. McCrae, J. Wild and A. Simonian, A fluorescence-based biosensor for the detection of organophosphate pesticides and chemical warfare agents, Sensors and Actuators B: Chemical, 115, (2006) 150-157.
- [112] R. Ekins, F. Chu and E. Biggart, Fluorescence spectroscopy and its application to a new generation of high sensitivity, multi-microspot, multianalyte, immunoassay, Clinica Chimica Acta, 194, (1990) 91-114.
- [113] C. E. H. Berger, T. A. M. Beumer, R. P. H. Kooyman and J. Greve, Surface Plasmon Resonance Multisensing, Analytical Chemistry, 70, (1998) 703-706.
- [114] U. Narang, P. R. Gauger, A. W. Kusterbeck and F. S. Ligler, Multianalyte Detection Using a Capillary-Based Flow Immunosensor, Analytical Biochemistry, 255, (1998) 13-19.
- [115] Y. Zhou, J. V. Magill, R. M. De La Rue, P. J. R. Laybourn and W. Cushley, Evanescent fluorescnece immunoassays performed with a disposable ionexchanged patterned waveguide, Sens. Actuators, B, 11, (1993) 245-250.
- [116] A. Brecht, A. Klotz, C. Barzen, G. Gauglitz, R. D. Harris, G. R. Quigley, J. S. Wilkinson, P. Sztajnbok, R. Abuknesha, J. Gascón, A. Oubiña and D. Barceló, Optical immunoprobe development for multiresidue monitoring in water, Analytica Chimica Acta, 362, (1998) 69-79.
- [117] F.S. Ligler, D.W. Conrad, J.P. Golden, M.J. Feldstein, B.D. Maccraith, S.D. Balderson, J. Czarnaski and C. A. Rowe, Array biosensor for multi-analyte sensing, Proceedings of SPIE-The International Society for Optical Engineering, 3258, (1998) 50-55.
- [118] R. M. Wadkins, J. P. Golden, L. M. Pritsiolas and F. S. Ligler, Detection of multiple toxic agents using a planar array immunosensor, Biosensors and Bioelectronics, 13, (1998) 407-415.
- [119] A. Klotz, A. Brecht and G. Gauglitz, Channel waveguide mode beat interferometer, Sensors and Actuators B: Chemical, 39, (1997) 310-315.

- [120] W. Lukosz, C. Stamm, H. R. Moser, R. Ryf and J. Dübendorfer, Difference interferometer with new phase-measurement method as integrated-optical refractometer, humidity sensor and biosensor, Sensors and Actuators B: Chemical, 39, (1997) 316-323.
- [121] B. H. Schneider, J. G. Edwards and N. F. Hartman, Hartman interferometer: versatile integrated optic sensor for label-free, real-time quantification of nucleic acids, proteins, and pathogens, Clin Chem, 43, (1997) 1757-1763.
- [122] B. G. Healey, L. Li and D. R. Walt, Multianalyte biosensors on optical imaging bundles, Biosensors and Bioelectronics, 12, (1997) 521-529.
- [123] K. L. Michael, L. C. Taylor, S. L. Schultz and D. R. Walt, Randomly Ordered Addressable High-Density Optical Sensor Arrays, Anal. Chem., 70, (1998) 1242-1248.
- [124] A. N. Asanov, W. W. Wilson and P. B. Oldham, Regenerable Biosensor Platform: A Total Internal Reflection Fluorescence Cell with Electrochemical Control, Anal. Chem., 70, (1998) 1156-1163.
- [125] C. A. Rowe-Taitt, J. W. Hazzard, K. E. Hoffman, J. J. Cras, J. P. Golden and F. S. Ligler, Simultaneous detection of six biohazardous agents using a planar waveguide array biosensor, Biosensors and Bioelectronics, 15, (2000) 579-589.
- [126] C. A. Rowe-Taitt, J. J. Cras, C. H. Patterson, J. P. Golden and F. S. Ligler, A Ganglioside-Based Assay for Cholera Toxin Using an Array Biosensor, Analytical Biochemistry, 281, (2000) 123-133.
- [127] C. A. Rowe-Taitt, J. P. Golden, M. J. Feldstein, J. J. Cras, K. E. Hoffman and F. S. Ligler, Array biosensor for detection of biohazards, Biosensors and Bioelectronics, 14, (2000) 785-794.
- [128] M. M. Ngundi,L. C. Shriver-Lake,M. H. Moore,M. E. Lassman,F. S. Ligler andC. R. Taitt, Array Biosensor for Detection of Ochratoxin A in Cereals and Beverages, Analytical Chemistry, 77, (2004) 148-154.
- [129] M. M. Ngundi, C. R. Taitt and F. S. Ligler, Simultaneous determination of kinetic parameters for the binding of cholera toxin to immobilized sialic acid and

monoclonal antibody using an array biosensor, Biosensors and Bioelectronics, 22, (2006) 124-130.

- [130] M. M. Ngundi, C. R. Taitt, S. A. McMurry, D. Kahne and F. S. Ligler, Detection of bacterial toxins with monosaccharide arrays, Biosensors and Bioelectronics, 21, (2006) 1195-1201.
- [131] K. E. Sapsford, M. M. Ngundi, M. H. Moore, M. E. Lassman, L. C. Shriver-Lake, C. R. Taitt and F. S. Ligler, Rapid detection of foodborne contaminants using an Array Biosensor, Sensors and Actuators B: Chemical, 113, (2006) 599-607.
- [132] K. E. Sapsford, C. M. Soto, A. S. Blum, A. Chatterji, T. Lin, J. E. Johnson, F. S. Ligler and B. R. Ratna, A cowpea mosaic virus nanoscaffold for multiplexed antibody conjugation: Application as an immunoassay tracer, Biosensors and Bioelectronics, 21, (2006) 1668-1673.
- [133] M. Ramanathan, *Array Biosensor for the Detection of Organophosphates*. 2006, Auburn University. p. 113.
- [134] M. Feldstein, J. Golden, C. Rowe, B. MacCraith and F. Ligler, Array Biosensor: Optical and Fluidics Systems, Biomed. Microdevices, 1, (1999) 139-153.
- [135] B. E. Yoldas and D. P. Partlow, Formation of broad band antireflective coatings on fused silica for high power laser applications, Thin Solid Films, 129, (1985) 1-14.
- [136] W.D. Kingery, H.K. Bowen and D.R. Uhlmann, *Introduction to Ceramics*.second ed, Wiley, New York: 1976, p.
- [137] G. A. Omburo, J. M. Kuo, L. S. Mullins and F. M. Raushel, Characterization of the zinc binding site of bacterial phosphotriesterase, J. Biol. Chem., 267, (1992) 13278-13283.
- [138] E. N. Efremenko and V. S. Sergeeva, Organophosphate hydrolase an enzyme catalyzing degradation of phosphorus-containing toxins and pesticides, Russ. Chem. Bull., Inter. ed., 50, (2001) 1826-1832.

- [139] P. Fracois, F. Suzanne, J. P. Roland and A.G. Brian, Flexible Solid-State Photoelectrochromic Windows, J. Electrochem. Soc., 146, (1999) 4324-4326.
- [140] M. Gratzel, Photoelectrochemical cells, Nature, 414, (2001) 338-344.
- [141] A. Fujishima, T. N. Rao and D. A. Tryk, Titanium dioxide photocatalysis, J. Photochem. Photobiol., C, 1, (2000) 1-21.
- [142] K. D. Benkstein and S. Semancik, Mesoporous nanoparticle TiO2 thin films for conductometric gas sensing on microhotplate platforms, Sens. Actuators, B, 113, (2006) 445-453.
- [143] E. Topoglidis, A. E. G. Cass, G. Gilardi, S. Sadeghi, N. Beaumont and J. R. Durrant, Protein Adsorption on Nanocrystalline TiO2 Films: An Immobilization Strategy for Bioanalytical Devices, Anal. Chem., 70, (1998) 5111-5113.
- [144] B. Mohammed, M. Jacques, L. Liren and S. Jean. 1992
- [145] H. P. Kao,N. Yang andJ. S. Schoeniger, Enhancement of evanescent fluorescence from fiber-optic sensors by thin-film sol-gel coatings, J. Opt. Soc. Am. A., 15, (1998) 2163-2171.
- [146] R. Mechiakh,F. Meriche,R. Kremer,R. Bensaha,B. Boudine andA. Boudrioua, TiO2 thin films prepared by sol-gel method for waveguiding applications: Correlation between the structural and optical properties, Opt. Mater., 30, (2007) 645-651.
- [147] P. Viravathana and D. W. M. Marr, Optical Trapping of Titania/Silica Core-Shell Colloidal Particles, J. Colloid Interface Sci., 221, (2000) 301-307.
- [148] D. S. Kommireddy, A. A. Patel, T. G. Shutava, D. K. Mills and Y. M. Lvov, Layerby-Layer Assembly of TiO2 Nanoparticles for Stable Hydrophilic Biocompatible Coatings, J. Nanosci. Nanotechnol., 7, (2005) 1081-1087.
- [149] L. Betancor and H. R. Luckarift, Bioinspired enzyme encapsulation for biocatalysis, Trends Biotechnol., 26, (2008) 566-572.

- [150] K. Shimizu,J. Cha,G. D. Stucky andD. E. Morse, Silicatein α: Cathepsin L-like protein in sponge biosilica, Proc. Natl. Acad. Sci. U. S. A., 95, (1998) 6234-6238.
- [151] N. Kröger, R. Deutzmann and M. Sumper, Polycationic peptides from diatom biosilica that direct silica nanosphere formation, Small, 286, (1999) 1129-1132.
- [152] N. Kroger, R. Deutzmann and M. Sumper, Silica-precipitating Peptides from Diatoms, J. Biol. Chem., 276, (2001) 26066-26070.
- [153] T. Coradin, A. Coupé and J. Livage, Interactions of bovine serum albumin and lysozyme with sodium silicate solutions, Colloids Surf., B, 29, (2003) 189-196.
- [154] R. L. Heather, B. D. Matthew, H. S. Kenneth and C. S. Jim, Rapid, Room-Temperature Synthesis of Antibacterial Bionanocomposites of Lysozyme with Amorphous Silica or Titania 13, Small, 2, (2006) 640-643.
- [155] H. R. Luckarift, M. B. Dickerson, K. H. Sandhage and J. C. Spain, Rapid, Room-Temperature Synthesis of Antibacterial Bionanocomposites of Lysozyme with Amorphous Silica or Titania13, Small, 2, (2006) 640-643.
- [156] H. R. Luckarift, S. Balasubramanian, S. Paliwal, G. R. Johnson and A. L. Simonian, Enzyme-encapsulated silica monolayers for rapid functionalization of a gold surface, Colloids Surf., B, 58, (2007) 28-33.
- [157] L. Betancor, H. R. Luckarift, J. H. Seo, B. Oliver and J. C. Spain, Three-dimensional immobilization of beta-galactosidase on a silicon surface, Biotechnol. Bioeng., 99, (2008) 261-267.
- [158] J. K. Grimsley, J. M. Scholtz, C. N. Pace and J. R. Wild, Organophosphorus Hydrolase Is a Remarkably Stable Enzyme That Unfolds through a Homodimeric Intermediateâ€, Biochemistry, 36, (1997) 14366-14374.
- [159] M. Ramanathan and A. L. Simonian, Array biosensor based on enzyme kinetics monitoring by fluorescence spectroscopy: Application for neurotoxins detection, Biosens. Bioelectron., 22, (2007) 3001-3007.

- [160] E. Topoglidis, C. J. Campbell, A. E. G. Cass and J. R. Durrant, Factors that Affect Protein Adsorption on Nanostructured Titania Films. A Novel Spectroelectrochemical Application to Sensing, Langmuir, 17, (2001) 7899-7906.
- [161] F.S. Ligler,K.E. Sapsford,J.P. Golden,L.C. Shriver-Lake,C.R. Taitt,M.A. Dyer,S. Barone andC.J. Myatt, The Array Biosensor: Portable, Automated Systems, Anal. Sci., 23, (2006) 5-10.
- [162] C. Aguilar,F. Borull andR. M. Marce, Identification of pesticides by liquid chromatography-particle beam mass spectrometry using electron ionization and chemical ionization, J. Chromatogr., A, 805, (1998) 127-135.
- [163] G. R. Asbury, W. U. Ching, W. F. Siems and H. H. Hill, Separation and identification of some chemical warfare degradation products using electrospray high resolution ion mobility spectrometry with mass selected detection, Anal. Chim. Acta, 404, (2000) 273-283.
- [164] J. Kulys and E. J. D'costa, Printed amperometric sensor based on TCNQ and cholinesterase., Biosens. Bioelectron., 6, (1991) 109-115.
- [165] B. D. Di Sioudi, C. E. Miller, K. Lai, J. K. Grimsley and J. R. Wild, Rational design of organophosphorus hydrolase for altered substrate specificities, Chem.-Biol. Interact., 119-120, (1999) 211-223.
- [166] J. K. Grimsley, B. Calamini, J. R. Wild and A. D. Mesecar, Structural and mutational studies of organophosphorus hydrolase reveal a cryptic and functional allosteric-binding site, Arch. Biochem. Biophys., 442, (2005) 169-179.
- [167] A. L. Simonian, B. D. diSioudi and J. R. Wild, An enzyme based biosensor for the direct determination of diisopropyl fluorophosphate, Anal. Chim. Acta, 389, (1999) 189-196.
- [168] A. L. Simonian, J. K. Grimsley, A. W. Flounders, J. S. Schoeniger, T.-C. Cheng, J. J. DeFrank and J. R. Wild, Enzyme-based biosensor for the direct detection of fluorine-containing organophosphates, Analytica Chimica Acta, 442, (2001) 15-23.
- [169] I. H. A. Badr and M. E. Meyerhoff, Highly Selective Optical Fluoride Ion Sensor with Submicromolar Detection Limit Based on Aluminum(III)

Octaethylporphyrin in Thin Polymeric Film, J. Am. Chem. Soc., 127, (2005) 5318-5319.

- [170] I. H. A. Badr and M. E. Meyerhoff, Fluoride-Selective Optical Sensor Based on Aluminum(III)â[^]Octaethylporphyrin in Thin Polymeric Film: Further Characterization and Practical Application, Anal. Chem., 77, (2005) 6719-6728.
- [171] H. S. M. Abd-Rabboh and M. E. Meyerhoff, Determination of glucose using a coupled-enzymatic reaction with new fluoride selective optical sensing polymeric film coated in microtiter plate wells, Talanta, 72, (2007) 1129-1133.
- [172] S. D. Aubert, Y. Li and F. M. Raushel, Mechanism for the hydrolysis of Organophosphates by the bacterial Phosphotriesterase, Biochemistry, 43, (2004) 5707-5715.
- [173] E. Greenbaum, M. S. Humayun, C. A. Sanders, D. Close, H. M. O'Neill and B. R. Evans, Metabolic Prosthesis for Oxygenation of Ischemic Tissue, IEEE T. Bio-Med Eng, 56, (2009) 528-531.
- [174] H. Frenkel-Mullerad andD. Avnir, Solâ''Gel Materials as Efficient Enzyme Protectors: Preserving the Activity of Phosphatases under Extreme pH Conditions, J. Am. Chem. Soc., 127, (2005) 8077-8081.
- [175] Y. Kang and M. E. Meyerhoff, Rapid response optical ion/gas sensors using dimer-monomer metalloporphyrin equilibrium in ultrathin polymeric films coated on waveguides, Anal. Chim. Acta., 565, (2006) 1-9.
- [176] P. Carlier, H. Hannachi and G. Mouvier, The chemistry of carbonyl compounds in the atmosphere--A review, Atmos. Environ. (1967), 20, (1986) 2079-2099.
- [177] L. G. Anderson, J. A. Lanning, R. Barrell, J. Miyagishima, R. H. Jones and P. Wolfe, Sources and sinks of formaldehyde and acetaldehyde: An analysis of Denver's ambient concentration data, Atmos. Environ., 30, (1996) 2113-2123.
- [178] G. D. Smith, L. T. Molina and M. J. Molina, Measurement of Radical Quantum Yields from Formaldehyde Photolysis between 269 and 339 nm, J. Phys. Chem. A, 106, (2002) 1233-1240.

- [179] M. Possanzini, V. D. Palo and A. Cecinato, Sources and photodecomposition of formaldehyde and acetaldehyde in Rome ambient air, Atmos. Environ., 36, (2002) 3195-3201.
- [180] L. G. Anderson, J. A. Lanning, R. Barrell, J. Miyagishima, R. H. Jones and P. Wolfe, Sources and sinks of formaldehyde and acetaldehyde: An analysis of Denver's ambient concentration data, Atmospheric Environment, 30, (1996) 2113.
- [181] K. C. Gupta, A. G. Ulsamer and P. W. Preuss, Formaldehyde in indoor air: Sources and toxicity, Environ. Int., 8, (1982) 349-358.
- [182] IARC, Monographs on the evaluation of the carcinogenic risks to humans, Formaldehyde, 2-Butoxyethanol and 1-tert-Butoxy-2-Propanol. Vol. 88, Lyon, France: 2004, p 483.
- [183] J. C. Harris, B. H. Rumack and F. D. Aldrich, Toxicology of urea, formaldehyde and polyurethane foam insulation, JAMA, 245, (1981) 243-246.
- [184] S. Gravelle, *FEMA mobile home count continues to drop*, in *The Gazette*. 2009: Cedar Rapids.
- [185] M. Brunker. *Are FEMA trailers 'toxic tin cans'*? 2006 25 July 2006 [cited 2008; Available from: <u>http://risingfromruin.msnbc.com/2006/07/are_fema_traile.html</u>.
- [186] M. Kunzelman, *Suit Filed Over FEMA Trailer Toxins*, in *The Washington Post*. 2007, The Associated Press: New Orleans.
- [187] J. Palank. FEMA Faulted on Response to Risks in Trailers. The New York Times 2007 July 20 2007 [cited 2007 16 November 2007]; Available from: <u>http://www.nytimes.com/2007/07/20/washington/20fema.html</u>.
- [188] R. R. Baker, The generation of formaldehyde in cigarettes--Overview and recent experiments, Food Chem. Toxicol., 44, (2006) 1799-1822.
- [189] S. Honda, Postcolumn derivatization for chromatographic analysis of carbohydrates, J. Chromatogr., A, 720, (1996) 183-199.

- [190] I. G. Casella and M. Contursi, Determination of aliphatic aldehydes by liquid chromatography with pulsed amperometric detection, J. Chromatogr., A, 1063, (2005) 129-135.
- [191] J.-f. Liu, J.-f. Peng, Y.-g. Chi and G.-b. Jiang, Determination of formaldehyde in shiitake mushroom by ionic liquid-based liquid-phase microextraction coupled with liquid chromatography, Talanta, 65, (2005) 705-709.
- [192] S. Kim andH.-J. Kim, Comparison of standard methods and gas chromatography method in determination of formaldehyde emission from MDF bonded with formaldehyde-based resins, Bioresour. Technol., 96, (2005) 1457-1464.
- [193] M.-A. del Barrio, J. Hu, P. Zhou and N. Cauchon, Simultaneous determination of formic acid and formaldehyde in pharmaceutical excipients using headspace GC/MS, J. Pharm. Biomed. Anal., 41, (2006) 738-743.
- [194] B. Hanoune, T. LeBris, L. Allou, C. Marchand and S. Le Calve, Formaldehyde measurements in libraries: Comparison between infrared diode laser spectroscopy and a DNPH-derivatization method, Atmos. Environ., 40, (2006) 5768-5775.
- [195] X. H. H. Huang, H. S. S. Ip and J. Z. Yu, Determination of trace amounts of formaldehyde in acetone, Anal. Chim. Acta, 604, (2007) 134-138.
- [196] M. T. Oliva-Teles, P. Paiga, C. M. Delerue-Matos and M. C. M. Alvim-Ferraz, Evaluation of Formaldehyde in Foundry Waste Sands Using Liquid Chromatography, Anal. Lett., 42, (2009) 492-504.
- [197] T. Nash, The Calorimetric Estimation of Formaldehyde by Means of teh Hantzsch Reaction, Biochem. J, 55, (1953) 416-421.
- [198] B. J. Compton and W. C. Purdy, Fluoral-P, a member of a selective family of reagents for aldehydes, Anal. Chim. Acta, 119, (1980) 349-357.
- [199] S. Dong and P. K. Dasgupta, Fast fluorometric flow injection analysis of formaldehyde in atmospheric water, Environ. Sci. Technol., 21, (1987) 581-588.

- [200] H. Tsuchiya,S. Ohtani,K. Yamada,M. Akagiri andN. Takagi, Determination of Formaldehyde in Reagents and Beverages Using Flow Injection, Analyst, 119, (1994) 1413-1416.
- [201] S. B. Jones, C. M. Terry, T. E. Lister and D. C. Johnson, Determination of Submicromolar Concentrations of Formaldehyde by Liquid Chromatography, Anal. Chem., 71, (1999) 4030-4033.
- [202] H. L. C. Pinheiro, M. V. de Andrade, P. A. de Paula Pereira and J. B. de Andrade, Spectrofluorimetric determination of formaldehyde in air after collection onto silica cartridges coated with Fluoral P, Microchem. J., 78, (2004) 15-20.
- [203] H. C. Loh, K. W. Chong and M. Ahmad, Quantitative Analysis of Formaldehyde Using UV-VIS Spectrophotometer Pattern Recognition and Artificial Neural Networks, Anal. Lett., 40, (2007) 281-293.
- [204] L. S. G. Teixeira, E. S. Leao, A. F. Dantas, H. L. C. Pinheiro, A. C. S. Costa and J. B. de Andrade, Determination of formaldehyde in Brazilian alcohol fuels by flowinjection solid phase spectrophotometry, Talanta, 64, (2004) 711.
- [205] H. R. Luckarift, M. B. Dickerson, K. H. Sandhage and J. C. Spain, Rapid, roomtemperature synthesis of antibacterial bionanocomposites of lysozyme with amorphous silica or titania, Small, 2, (2006) 640-643.