Organophosphorus compound detection using enzyme encapsulated in peptide nanotubes

Eric B. McDaniel

The University of Toledo

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A Thesis

entitled

Organophosphorus Compound Detection Using Enzyme Encapsulated in Peptide Nanotubes

by

Eric B. McDaniel

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in

Chemical Engineering

Dr. Dong-Shik Kim, Committee Chair

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Dr. Brent Cameron, Committee Member

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College of Graduate Studies

The University of Toledo

December 2013
An Abstract of

Organophosphorus Compound Detection Using Enzyme Encapsulated in Peptide Nanotubes

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Organophosphorus (OP) compounds are commonly used as pesticides as well as nerve agents. These compounds, which are applied in the environment for agricultural and domestic use, must be detected so that residues can be removed to assure safety of humans and the environment. In this research, the goal is to develop a fast, portable, and precise biosensor to detect these OP compounds. To accomplish this, sensors were prepared with enzymes encapsulated in Peptide Nanotubes (PNTs). PNTs were synthesized with diphenylalanine. PNTs can be readily formed by self-assembly of diphenylalanine under mild conditions. Organophosphorus Hydrolase (OPH), an enzyme that has been used to detect OPs, was used in this research as a sensing element to detect malathion, a common OP pesticide. The use of OPH gives a direct method of detecting malathion. Different electrode preparations were used and tested to detect malathion dissolved in water using Cyclic Voltammetry (CV). CV determines current produced by an electro-active species as a response to varying voltage. The increase of a current peak for malathion compared to a blank, on a CV response curve, indicates that there is an electrochemical reaction taking place, presumably due to malathion oxidation facilitated by the OPH enzyme. A linear relationship between the percent increase of peak current versus malathion concentration from 10-100 µM was shown.
Acknowledgements

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<tr>
<td>Ab</td>
<td>Absorbance</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>AChCl</td>
<td>Acetocholine Chloride</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetocholine Esterase</td>
</tr>
<tr>
<td>ATSDR</td>
<td>Agency for Toxic Substances and Disease Registry</td>
</tr>
<tr>
<td>AuSPE</td>
<td>Gold Screen-Printed Electrode</td>
</tr>
<tr>
<td>Ch</td>
<td>Choline</td>
</tr>
<tr>
<td>CNT</td>
<td>Carbon Nanotube</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic Voltammetry</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>HFP</td>
<td>1,1,1,3,3,3-hexafluoro-2-propanol</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>OP</td>
<td>Organophosphorus</td>
</tr>
<tr>
<td>OPH</td>
<td>Organophosphorus Hydrolase</td>
</tr>
<tr>
<td>SPE</td>
<td>Screen-Printed Electrodes</td>
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Chapter 1

Introduction

1.1 Biosensors

In the past few decades, biosensors have been an ever growing area of research that has attracted various scientists from environmental, industrial and medical fields and will continue to expand. Biosensors provide highly selective methods for detecting biological compounds and organisms.

There are various types of sensors available on the market. However, to make fast, potable and precise detections, Screen-Printed Electrodes (SPEs) have often been used. Application of SPEs has been an expanding area of research for the past two decades. Glucose detectors for diabetics were the first commercially available SPEs and their success only strengthened this expansion (Cass et al, 1984). SPEs consist of an auxiliary electrode, reference electrode and working electrode as seen in Figure 1-1, from Pine Research Instrumentation.
SPEs can be mass produced by industry or even laboratory-made through an appropriate screen-printing machine. Depending on the user’s preference, they can be disposed of or re-used after cleaning. For application, SPEs are typically modified by applying various substrates and/or matrices on the working electrode.

Utilizing oxidation and reduction reactions to monitor the current, electrochemical techniques are predominantly used for biosensors. Because of this, “redox enzymes” are commonly used as the substrate or part of the matrix on the working electrode (Gorton, 1995).

1.2 Cyclic Voltammetry

In Electrochemistry, Cyclic Voltammetry (CV) is one of many techniques. CV is a very useful technique that can be used to study a plethora of topics, including modification of electrodes, reaction rates, reaction mechanisms and the concentration of an unknown concentration through calibration curve (Carriedo, 1988).
A CV test involves a scan from positive to negative potential, a reduction sweep, followed by a scan from negative to positive potential, an oxidation sweep (Quiroga, 2013). During the scan, electrochemical reaction/s increases the amount of current at potentials that the electrochemical reaction governs. This can be seen from following the graphs in Figure 1-2 (A and B).

![Figure 1-2: Graphs of (A) excitation signal and (B) response signal for Cyclic Voltammetry (Delmar et al, 2013).](image)

The peak at point c, in Figure 1-2B, represents the highest reductive current produced from a generic electrochemical reaction, and point f is the highest oxidation current. Due to addition of a substrate and/or matrix onto the electrode, the peak heights will increase due to the electrochemical reaction. In a series of CV tests on the same solution, however, the peak height will drop as the detection compound gets converted.
1.3 Organophosphorus Compounds

Organophosphorus (OP) compounds are commonly used as pesticides as well as nerve agents. In pest control, OPs are less persistent in the environment as compared to previously used chlorinated hydrocarbons, and thus the use of OP compounds has increased in the past few decades (Racke, 1992). However, OPs are not ideal pesticides due to their toxicity, which can cause death to domestic animals and even humans (Minton et al, 1988).

![Structures and examples of organophosphorus Compounds](Image)

**Figure 1-3: Structures and examples of organophosphorus Compounds (Upadhyay et al, 2009).**

The general structure of OP compounds start with the tetra-substituted phosphorus (V) center (Upadhyay et al, 2009). Oxygen or Sulfur is double-bonded to this as well as
two substituents and a characteristic leaving group, which can be seen from the examples in Figure 1-3. Sarin, Soman, and Tabun are classified as G class nerve agents that were made during World War II by German scientists, though sarin and tabun weren’t used during the conflict. Paraoxon was synthesized by G. Schrader, and had excellent insecticide capabilities, though it is also highly toxic to mammals. Parathion, paraoxon and malathion were later synthesized to lower the toxicity for safer use, with malathion having the least toxicity.

Despite this toxicity, OP pesticides have and are being used in the environment for agricultural and domestic application. It is imperative to have the ability to detect these compounds, in agricultural runoff as well as water used for chemical warfare agent decontamination, to assure safety to humans as well as the environment.

1.4 Enzymes

All living systems control their activity through the use of a great number of enzymes, protein molecules that act as biological catalysts (Ophardt, 2003). Enzymes are highly selective to one or several reactants, called substrates. Enzymes significantly increase the rate of reaction, elsewise the reaction would be too slow and the living system wouldn’t be able to survive. Like inorganic catalysts, enzymes lose activity with time. However enzymes are more biocompatible than most catalysts, and have more potential for biological applications.
In relation with this research, the human nervous system contains acetocholine esterase (AChE), an enzyme that becomes inhibited when regulating the neurotransmitter acetylcholine in the presence of OP compounds as seen in Figure 1-4 (Joshi et al, 2005).

1) \[ \text{AChE} \xrightarrow{\text{Inhibitor}} \text{AChE}_{(\text{inactive})} \]
2) \[ \text{AChCl} \xrightarrow{\text{AChE}} \text{Ch} + \text{HAC} + \text{Cl}^- \]
3) \[ 2\text{Ch} \xrightarrow{} 2\text{Ch}_{(\text{ox})} + 2e^- + 2\text{H}^+ \]

**Figure 1-4: Chemical formulas of (1) inhibition of acetocholine esterase and (2 & 3) acetocholine esterase’s natural reaction.**

The inhibiting compounds, in this reaction scheme, prevents the reaction to produce choline (Ch) and thus electrons. In a CV test, this results in a decrease in the peak height. The change in peak height is related to the degree of inhibition, which can be calibrated to inhibitor concentration, making an indirect detection. In order to obtain the degree of inhibition, it is required to have three CV tests. First is a curve of the electrode in working solution alone, followed by a curve of that solution with the addition of acetylcholine chloride (AChCl), and finally a curve with the inhibiting compound (Roepcke, 2011). The addition of AChCl will increase the peak from the standard working solution, and the presence of an OP compound decreases the peak based on the amount of inhibition.

Horseradish Peroxidase (HRP) has been used for its strong redox potential (Park et al, 2012). HRP, like other peroxidase enzymes, catalyzes the conversion of \( \text{H}_2\text{O}_2 \) to water to consume electrons and reduce electrochemical parameters, such as working
potential and current. Low parameters are desirable for portable detection, because of the decreased device specs requirement.

In this research, instead of using AChE on the electrode to indirectly monitor the concentration of inhibitor, Organophosphorus Hydrolase (OPH) is used for a direct detection. As suggested OPH, instead of being inhibited, directly catalyzes the OP compound hydrolysis reaction, as the name describes. This reaction can be seen in Figure 1-5 (Lihong et al, 2006):

\[
\text{OPs} \xrightarrow{\text{OPH}} \text{organophosphodiester}
\]

Figure 1-5: Enzymatic reaction of Organophosphorus Hydrolase with a general Organophosphorus compound.

With the direct reaction with the OP compound, there are only two CV curves required. The first is the curve of the electrode in working solution, followed by the solution with OP compound. A calibration curve can be made based on the difference in peak height of the two curves for various OP concentrations.
1.5 Peptide Nanotubes

There has been great interest and progress in the development of nanomaterials including simple as well as complex structures that show superior electronic, magnetic and mechanical properties as compared with their larger counterparts (Tarabout et al, 2011). Due to their small size and various shapes, these materials are known for their increased catalytic behavior in many reaction based systems. Common formations of these materials include nanoparticles, nanocrystals, nanotubes and nanowires.

Nanomaterials have been used as electrodes or as components in a matrix that is applied on the surface of electrodes. Nanowires are commonly used as microelectrodes, which provide highly sensitive real-time detections for proteins and micro-organisms (Engel et al, 2010). However, due to size and shape, there are limits to modifications done to nanowires. Nanoparticles have been used as substrates that are placed on working electrodes as a paste, most commonly as carbon paste (Svancara et al, 2009). Nanotubes are used as components of a matrix that is applied on the working electrode surface.

There are a few types of nanotubes used for sensors, carbon and peptide nanotubes. Carbon Nanotubes (CNTs) are tubes with nanometer diameters and have advantageous electronic, thermal and structural properties (Hirlekar et al, 2009). However the processes to make CNTs in a large scale are limited due to the energy requirement and, for some processes, yield is also an issue (Rafique et al, 2011).

Just as CNTs, Peptide Nanotubes (PNTs) are nanotubular structures that offer a variety of applications. In contrast to CNTs, PNTs are self-assembled and relatively easy
to make. Peptides are comprised of a short polymer of amino acids linked through peptide bonds (Park et al, 2010, 2011A and 2012). Unlike proteins, however, the thermal and chemical stability of PNTs are remarkable. Due to the numerous types of amino acids, there are a great variety of structures, sizes and functionality possible with PNTs, providing a plethora of applications.

Figure 1-6: Examples of Peptide Nanotubes (Reches et al, 2006).

PNTs are primarily used for biosensors as a medium in which to encapsulate enzymes. The encapsulation of enzymes helps retain enzyme activity and stability for longer times, thus increasing the shelf-life of a prepared electrode (Park et al, 2010, 2011A and 2012). This retention of activity is due to the shielding from the environment as well as the ability of peptides to form covalent bonds with organic molecules and organometals.
1.6 Organophosphorus Pesticide Detections

Like all pesticides, the use of OP pesticides requires monitoring due to the impact of these compounds on the environment and human health. The Environmental Protection Agency (EPA) sets regulations or recommendations for toxic substances that are expressed in air, water, soil and/or foods. For these OP pesticides, EPA has set regulations on the drinking water and residue on foods. Drinking water regulations can be seen in the following Table 1.1, obtained from ATSDR toxicology profiles of the compounds.

Table 1.1: OP Pesticide Regulations in Drinking Water

<table>
<thead>
<tr>
<th>OP Pesticide</th>
<th>Regulation (mg/L)</th>
<th>Regulation (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malathion</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Methyl Parathion</td>
<td>0.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Diazinon*</td>
<td>0.02</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*Diazinon for up to 10 day exposure

The use of different enzymes has been explored for detecting OP compounds. One general approach is the utilization of the enzyme AChE as previously discussed where OP compounds are known to inhibit this enzyme's function in the nervous system (Marrs, 1993). OPH, with its direct interaction with OP compounds has gained attention. Due to the complex process to synthesize, there are limitations on the ability to mass
produce the enzyme and thus OPH is obtained through special microbiology laboratories (Prieto-Simón et al, 2006).

The development and use of different types of electrodes always has been an integral part of electrochemistry. Each type of electrode as well as the material it is made of has its advantages and disadvantages. For example, SPEs can be used in portable applications and are disposable, like the glucose sensors for diabetics. On the other hand, the cost per test is often more expensive as well as the amount of tests, and stationary equipment have the possibility of simultaneous testing (Chin et al, 2013).

The material at which the working electrode is made of is also varied to meet different needs. Gold has high electron conductivity and is relatively inert as compared to other materials. Self-Assembled Monolayer formation has been explored, where adsorption of thiols, sulfides and disulfides on gold surfaces have been observed to promote electron transfer to the surface (Chidsey et al, 1990). Glassy Carbon and graphite are commonly used as working electrodes as a less expensive alternative to gold, while retaining desirable electron transfer properties.

While deciding the type of electrode material is important, selectivity to the analyte is still required. Electrode modification typically involves either chemically attaching, adsorption or the layering of substrate/s onto the surface of the electrode (Snell et al, 1979). By combining modifications, one can obtain a highly selective sensor, however possibly hindering electron transfer as well. It is desirable to obtain both selectivity and electron transfer while minimizing the complexity of the modification. Table 1.2 depicts some of these modifications from related biosensor research in order to detect OP pesticides.
Table 1.2: Results of Related Biosensors

<table>
<thead>
<tr>
<th>Electrode Type</th>
<th>Electrode Modification</th>
<th>Electrochemical Method</th>
<th>Substrate</th>
<th>Analyte</th>
<th>Sensitivity ($\mu$A/$\mu$M)</th>
<th>Linear Range ($\mu$M)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPE*</td>
<td>Carbon Thick Film</td>
<td>Amperometric</td>
<td>OPH</td>
<td>Methyl Parathion</td>
<td>0.00283</td>
<td>5-40</td>
<td>Mulchandani et al, 1999</td>
</tr>
<tr>
<td>Glassy Carbon Disks*</td>
<td>CNT - Enzyme Encapsulation</td>
<td>Amperometric</td>
<td>OPH</td>
<td>Paraoxon</td>
<td>0.025</td>
<td>0.25-4</td>
<td>Deo et al, 2005</td>
</tr>
<tr>
<td>Glassy Carbon*</td>
<td>MC and CB</td>
<td>Amperometric</td>
<td>OPH</td>
<td>Paraoxon</td>
<td>0.198</td>
<td>0.2-8</td>
<td>Lee et al, 2010</td>
</tr>
<tr>
<td>Carbon SPE</td>
<td>Prussian Blue Layer</td>
<td>Amperometric</td>
<td>AChE</td>
<td>Paraoxon - Methyl-Parathion</td>
<td>70° - 135°</td>
<td>14-173° - 7-26°</td>
<td>Suprun et al, 2005</td>
</tr>
<tr>
<td>Packed Tube Electrode*</td>
<td>CNT-Najol Paste</td>
<td>CV - Square Wave</td>
<td>AChE</td>
<td>Chlorphenvinphos</td>
<td>4.91°</td>
<td>0.5-4.5</td>
<td>Oliveira et al, 2011</td>
</tr>
</tbody>
</table>

* Working Electrode of a TES
°Sensitivity is based on % inhibition (%/concentration unit)
"Concentration expressed in ppb

1.6 Purpose

In this research OPH, an enzyme which has high affinity toward OP compounds, was used to detect the OP pesticide Malathion. PNTs were used to encapsulate OPH, to provide structural support for the enzyme as well as to retain enzyme activity. Therefore the objective of this experiment was to test the effectiveness of PNT encapsulation of OPH to detect OPs. Gold Screen-Printed Electrodes (AuSPEs) were used as a three electrode system for a potentiometric test. It is proposed that use of SPEs in conjunction with PNT-encapsulated OPH will promote development of a fast, portable, inexpensive, and precise OP detector that can be used in the field.
Chapter 2

Materials and Methods

2.1 Materials

Organophosphorus Hydrolase (OPH) was obtained from Lybradin. Diphenylalanine, for making peptide-nanotubes, was obtained from Bachem. Mono and Dibasic Potassium Phosphate were obtained from Fisher Scientific. HFP (1,1,1,3,3,3-hexafluoro-2-propanol), Horseradish Peroxidase (HRP), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), and Nafion, 5 wt. % were obtained from Sigma-Aldrich. Bovine Serum and Triton X-100 were both obtained by Atlanta Biological. The potentiostat used, Princeton PARSTAT 2273, was obtained from Advanced Electrical Systems (Figure 2-1). Gold Screen-Printed Electrodes were obtained from Pine Research Instrumentation (Figure 2-2).
2.2 Free Enzyme Electrode Preparation

In the first method, the enzyme was directly applied to the working electrode. To compare a sensor with the encapsulated enzyme, it is imperative to have tests done on a sensor with free enzymes as a reference. This configuration was tested for a few reasons. One was to confirm that OPH successfully detects the presence of malathion. Since only the enzyme is on the electrode, this is the best choice in finding a desired potential window due to the catalyzed electrochemical reaction without any other species added. Along with the potential window, the test provides an idea of what to expect for the subsequent tests that have additional substances on the electrode.

A ~1 mg/mL solution of OPH was prepared using DI water. Then 3.5 μL of the well mixed enzyme solution was applied onto the working electrode, and left to dry under a vacuum hood. Once dried, 3 μL of 0.5 % wt Nafion, made by dilution, was applied on top to act as a binding agent.
2.3 OPH Encapsulated PNT Electrode Preparation

For the second biosensor set-up, OPH was encapsulated in PNTs and then applied to the working electrode. This preparation shows the effects of the enzyme encapsulation on the electrochemical detection of malathion. Even though the encapsulation is meant to retain enzyme activity, the CV test is still necessary to see whether there is any change in performance of the electrode.

To start, PNTs were synthesized in two short steps. First step was dissolving 100 mg diphenylalanine into 1mL of HFP. Then 20 µL of the dissolved diphenylalanine was added to 980 µL of DI water (Park et al, 2011A and 2012). The solution was added into a plastic tube, and the tube was shaken by a vortex mixer for 30 seconds to form PNTs.

For enzyme encapsulation, the PNT solution was dried by inert gas flowing over it in a fume hood. The dried PNT was then mixed with a 1 mg/mL solution of OPH by a vortex mixer. The OPH-PNT solution was then placed in a carousel that was in a refrigerated incubator, to have continuous rotation at 5 °C for one day. Then the OPH-PNT solution was applied to the working electrode, followed by 3 µL of 0.5 % wt Nafion for binding.

2.4 HRP Encapsulated PNT Electrode Preparation

For the last biosensor set-up, HRP was first encapsulated in the PNTs followed by the attachment of OPH on the PNT outer walls and Nafion added for binding onto the
working electrode. This method was used to increase the sensitivity of the CV tests. Increased sensitivity will occur only if peroxides are present at any point in the reaction mechanisms. There has been no claim of the presence of peroxides for non-enzymatic hydrolysis of malathion. By performing this test, more insight on the enzymatic hydrolysis of malathion can be obtained.

To prepare a solution of HRP, HRP was dissolved into a 5.8 pH Phosphate Buffer solution (PBS) at 1 mg/mL. HRP was encapsulated following the OPH encapsulation procedure described above. Once encapsulated, the HRP-PNTs were then washed before application. To do this, centrifugation was first carried out at 4000 rpm for 10 minutes to separate the encapsulated HRP-PNT from bulk solution. The HRP-PNTs were then washed three times with 1 mL of the PBS to remove the excess non-encapsulated enzymes (Park et al, 2012). After the final wash, 1 mL of the PBS was added to HRP-PNT which was agitated for uniform mixing. 3.5 µL of this solution was applied onto the working electrode of a bare SPE and left to dry. Subsequently, 3.5 µL of 1 mg/mL OPH solution was added onto the working electrode/HRP-PNT and left to dry. Finally 3 µL of 0.5 % wt Nafion was applied.

2.5 CV Tests

The electrolyte or working solution used in this research was 50 mM and pH 7.4 PBS, prepared by using both mono- and di-basic potassium phosphate and adjusting pH with NaOH. This solution has been known to become contaminated by micro-organisms (Ohio University, 2013), so the solution was prepared fresh every week.
The apparatus was set up so that the potentiostat was linked to the electrode port via the potentiostat cable clamped to Gamry’s cable, which was then placed into a 20 mL vial, as seen from Figures 2-1 and 2-2. The amount of working solution used in each test is 10 mL. Once the electrodes were prepared, they were placed into the port and then carefully dipped into the working solution.

The potentiostat’s parameters were set to run from -0.20 to 1.00 V then in the reverse direction at a scan rate of 50 mV/s. This potential range was chosen by narrowing down from a larger potential window. The peaks seen in the stated range were the only ones affected by the addition of malathion so it was unnecessary to use a larger potential range.

Two scans were performed for each prepared electrode in this analysis. The first scan was when the electrode was tested in only the working solution. The second and last scan was after malathion was mixed into the working solution.

2.6 Enzyme Longevity

The activity of the OPH encapsulated inside the PNT was determined using an ABTS assay (Park et al, 2012). In order to determine if encapsulation retains activity, the activity and shelf-life of free OPH was first tested. The encapsulated OPH was then tested for activity and shelf-life.

There are four key solutions used in this method: ABTS solution, enzyme diluent, enzyme solution, and H$_2$O$_2$ solution. To start, a 0.1 M PBS was prepared using KH$_2$PO$_4$, and the pH was adjusted to 5.0 using 1 M NaOH. The ABTS solution is prepared by
adding 0.14g ABTS to 30mL of 0.1 M PBS, and is freshly prepared for each use. To make the enzyme diluent, 0.544g KH$_2$PO$_4$ (to make a 40mM solution), 0.25g Bovine serum, and 0.5 mL of Triton X-100 was added into 100 mL DI water, and pH was adjusted to 6.8 using the 1 M NaOH solution. For the enzyme solution, 10 mg of OPH or OPH encapsulated within PNTs was added into 1 mL of cold diluent solution. The enzyme solution was freshly made for each test. H$_2$O$_2$ solution was prepared by adding 0.5 mL of 30% (w/w) H$_2$O$_2$ into 49.5 mL DI water to make a 0.3% (w/w) solution.

Two stock solutions were formulated, Test and Blank. The Test stock was made from 2.9 mL of ABTS solution and 0.05 mL of enzyme solution. The Blank stock consisted of 2.9 mL of ABTS solution and 0.05 mL of enzyme diluent. Using UV Spectroscopy, each stock solution was tested to measure absorbance (Ab). Once the Ab equilibrated, each sample was returned to their respective stock solution. In both stock solutions, 0.1 mL of H$_2$O$_2$ solution was added and immediately mixed by inversion. Again, 1 mL of each stock was placed in to the UV Spectrometer. Initial Ab and time was recorded, and then measured again after 25 minutes. Activity of the enzyme is calculated from Equation 1, where ε is the millimolar extinction coefficient of oxidized ABTS at 405nm (Park et al, 2012).

\[
\text{Activity} = \frac{(\text{Abat 25min} - \text{Abat thebeginning})}{(\text{Finalmixturevolume})(\text{dilutionfactor})} \times \frac{25}{\left(\varepsilon \right)(\text{Volume of enzyme solution added})}
\]
Chapter 3

Results and Discussion

3.1 Free Enzyme Analysis

For the first electrode modification, only OPH was attached to the electrode with Nafion. From this test, an electrochemical reaction is expected to occur from the addition of malathion to the electrochemical cell. The reaction will result in a change in current peak height and can be seen by comparing the CV scans from a blank electrolyte solution and the solution with malathion added.
In Figure 3-1, the CV comparison shows that with the addition of malathion, to make a 100 µM malathion working solution, there was an increase in current response from the blank from -45 to -95 µA around 0.6 V, indicating an electrochemical reaction had taken place. This current peak increase occurs on the reverse scan, from 1.0 to -0.2 V, an oxidative scan. Since the reaction occurred on the oxidative scan, it is an oxidation reaction, likely resulting from the oxidation of the characteristic leaving group.
Figure 3-2: Calibration curve for OPH only applied onto electrode.

The calibration curve, Figure 3-2, relates the concentration of malathion to the average percentage increase of the CV peak current at 0.6 V for the malathion curve compared with the blank solution curve. Average peak increase was calculated by the following equation:

\[
\text{Percent Increase} = \left( \frac{\text{Malathion Peak Current}}{\text{Blank Peak Current}} - 1 \right) \times 100\% \tag{2}
\]

The trend line represents the relationship of the average percent increase of current from each concentration tested. This relationship is linear, i.e. higher concentrations of malathion result in higher percent increases for the range of concentrations from 10 to 100 µM. The slope of the trend line is associated with the
sensor's sensitivity, and is 0.8288 (%/ µM). The $R^2$ value is 0.844 and the error bars represent 95% confidence of percent increase for each concentration. The $R^2$ and error bars are not quite to be desired and further improvements on techniques can diminish some of the human and systematic errors.

### 3.2 OPH Encapsulation Analysis

For this electrode modification, OPH was encapsulated into the PNTs and then attached to the electrode by Nafion. From this analysis, the change of sensor performance due to the encapsulation of OPH can be observed.

![Figure 3-3: CV of OPH-PNT applied onto the electrode.](image)
Similar to Figure 3-1; Figure 3-3 shows that the addition of malathion results in an increase of the current peak from -45 to -109 µA, again indicating an electrochemical reaction was taking place. This shows that the use of PNTs to encapsulate OPH does not provide overall hindrance to the electrochemical reaction.

![Figure 3-4: Calibration curve for OPH-PNT applied onto electrode.](image)

The calibration curve, Figure 3-4, relates the malathion concentration to the average percentage increase of the CV peak current at about 0.6 V for the malathion solution compared with the blank solution. Just as Figure 3-2 illustrates: higher concentrations of malathion result in higher percent increases for the range of concentrations investigated.
The sensitivity of this calibration is 0.994 (%/ µM), which is higher than that of the free enzyme sensor, which was 0.8288 (%/ µM), showing that there is an increase of sensitivity by encapsulating OPH. This trend line shows an $R^2$ value of 0.936, an improvement as compared to the free enzyme calibration, which was 0.844. By running Excel's T-Test function to compare data from Figures 3.2 and 3.4, the test indicated that there was no significant statistical difference. Again, upon improvement of techniques, the error bars will become narrower and the difference between graphs grows more significant.

### 3.3 HRP Encapsulation Analysis

For this method, HRP was first encapsulated into the PNTs. After encapsulation, HRP-PNT was then applied onto the working electrode, OPH added to the outside of the HRP-PNTs, and then Nafion was added as a binder. HRP was used because it was expected to facilitate the redox reaction between the OPH and malathion or a reaction in the presence of any peroxide that might be produced in the electrochemical reaction. It is expected that the CV peak position and heights would then be shifted from those in Figures 3-1 and 3-3, which were for OPH enzyme only.
Figure 3-5: CV of OPH/HRP-PNT applied onto the electrode.

Figure 3-6: CV of the comparison of OPH-HRP-PNT and OPH-PNT blank curves.
As expected, Figure 3-5 shows an increase in the peaks from -39 to -85 μA due to the addition of malathion. From this, the malathion's electrochemical reaction still occurs. However, by comparing CV curves of OPH-PNT and OPH-HRP-PNT as seen in Figures 3-7 and 3-8, it can be observed that the position of the peaks along the potential axis did not change upon the use of HRP, thus no change in working potential. There is a reduction of peak current of about 10 μA between the blank curves and about 25 μA between malathion curves due to the use of HRP. This can either mean that there is small facilitation of the redox reaction by HRP consuming electrons from reaction or that HRP is just a layer of resisting electron flow to the electrode. If the first case is true, further tests can be run using HRP to further the sensor enhancement. If the latter is true, then there are other, less expensive, methods to simply hinder electron flow. From the
employed test, there was no significant change from the use of HRP, but insight was left for further study and possible sensor enhancement.

### 3.4 Enzyme Longevity

![Figure 3-8: Longevity comparison of encapsulated and unencapsulated OPH.](image)

The result of the longevity tests, as seen in Figure 3-8, indicates that the encapsulated enzyme maintains activity for around 8 days then slowly loses activity to 80% at day 20. The free enzyme roughly retains activity for 4 days then had completely lost all activity by day 12. The data shows that, by encapsulating the enzyme, storage time after preparation on the electrode can be extended, as compared to free enzyme preparation.
Chapter 4

Conclusion

The use of Organo-Phosphorus Hydrolase (OPH) enzyme on a Screen-Printed Electrodes (SPEs) shows promising results in effecting fast, portable, inexpensive, and precise detection of Organo-Phosphate Pesticides and Nerve agents. From Figures 3-1 and 3-3, it is clear that there is an electrochemical reaction due to the addition of malathion, as demonstrated by the increase of the CV response curve peak.

The relation between percent increase of peak current and malathion concentration is linear for both calibration curves, Figures 3-2 and 3-4. Table 4.2 shows the summary of the calibration in comparison to the data of related biosensors. There was no statistical significant difference in data between using free enzyme and encapsulated enzyme according to Excel's T-test function. However, there was still a slight increase in sensor sensitivity. With the further improvements on technique, statistical difference in data as well as sensitivity can be more pronounced.
Table 4.1: Comparison to the Results of Related Biosensors

<table>
<thead>
<tr>
<th>Electrode Type</th>
<th>Electrode Modification</th>
<th>Electrochemical Method</th>
<th>Substrate</th>
<th>Analyte</th>
<th>Sensitivity (nA/µM)</th>
<th>Linear Range (µM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPE*</td>
<td>Carbon Thick Film</td>
<td>Amperometric</td>
<td>OPH</td>
<td>Methyl Parathion</td>
<td>2.83</td>
<td>5-40</td>
<td>Mulchandani et al, 1999</td>
</tr>
<tr>
<td>Glassy Carbon Disks*</td>
<td>CNT Encapsulation</td>
<td>Amperometric Flow-Injection</td>
<td>OPH</td>
<td>Paraoxon</td>
<td>25</td>
<td>0.25-4</td>
<td>Deo et al, 2005</td>
</tr>
<tr>
<td>Glassy Carbon*</td>
<td>MC and CB</td>
<td>Amperometric</td>
<td>OPH</td>
<td>Paraoxon</td>
<td>198</td>
<td>0.2-8</td>
<td>Lee et al, 2010</td>
</tr>
<tr>
<td>Carbon SPE</td>
<td>Prussian Blue Layer</td>
<td>Amperometric</td>
<td>AChE</td>
<td>Methyl Parathion</td>
<td>70°-135°</td>
<td>14-173', 7-26°</td>
<td>Suprun et al, 2005</td>
</tr>
<tr>
<td>Packed Tube Electrode*</td>
<td>CNT-Najol Paste</td>
<td>CV - Square Wave</td>
<td>AChE</td>
<td>Chlorphenvinphos</td>
<td>4.91°</td>
<td>0.5-4.5</td>
<td>Oliveira et al, 2011</td>
</tr>
<tr>
<td>Gold SPE</td>
<td>Free Enzyme PNT Encapsulation</td>
<td>CV</td>
<td>OPH</td>
<td>Malathion</td>
<td>0.8288°, 0.9356°</td>
<td>10-100</td>
<td>This work</td>
</tr>
</tbody>
</table>

* Working Electrode of a TES
Sensitivity is based on % inhibition (%/concentration unit)
Sensitivity is based on (% increase of peak current/concentration)
° Concentration expressed in ppb

It was also shown from this test of encapsulated HRP resulted in little change in detector response. Small reduction of current from the electrochemical reaction was observed. Further tests can still be employed to possibly further the sensor enhancement, making the use of HRP more practical for field application.

The enzyme longevity assay, as seen in Figure 3-7, clearly demonstrates the advantage in terms of enzyme activity retention from encapsulating the enzymes in PNTs. This was expected from what has been seen in previous research (Park et al, 2010, 2011A and 2012).
Chapter 5

Future Work

While this research confirms the ability to detect OP compounds using these biosensors, there is still a plethora of research to complete this study. The concentrations used for calibration is above that which is regulated by EPA, 0.6 µM, and thus an extension of tests can be ran to cover the regulated concentration. Further improvement of the techniques and experience in electrochemical testing can reduce human and systematic errors thus providing more pronounced calibrations. Further experiments on the use of HRP are possible. One being the addition of a small amount of hydrogen peroxide to the working solution can forcefully provide sensor enhancement (Park et al 2011B). Another minor experiment is to have the HRP on the outside of PNT wall and OPH encapsulated.

Other electrochemical techniques can be explored that may give more accurate and sensitive results. Amperometry is commonly used for detection, as seen from Tables 1.2 & 4.1. Amperometry is performed by monitoring current through time at a working potential obtained from a CV test. Once initial current is stable, the analyte is injected into working solution and then the response current is monitored.
Both cyclic voltammetry and amperometry can be used to calculated concentration from current. In order to calculate concentration, reaction kinetics such as diffusion and reaction rates are required. Unfortunately there is limited data on the reaction between enzymes and the OP compounds, thus simple calibrations due to signal response are often employed.
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