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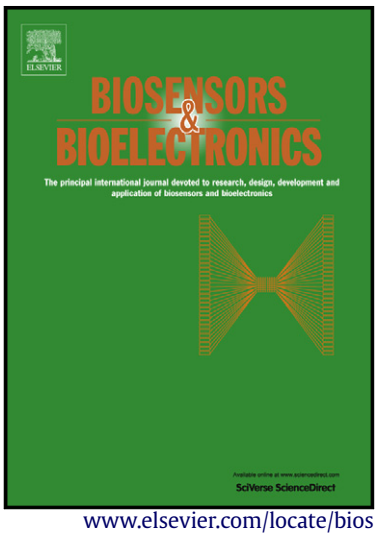

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Organophosphate vapor detection on gold electrodes using peptide nanotubes

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Abstract

Peptide nanotubes (PNTs) encapsulating horseradish peroxidase and surface coated with acetylcholinesterase (AChE) were attached to gold screen printed electrodes to construct a novel gas phase organophosphate (OP) biosensor. When the sensor with the AChE enzyme is put in contact with acetylthiocholine (ATCh), the ATCh is hydrolyzed to produce thiocholine, which is then oxidized by horseradish peroxidase (HRP). Direct electron transfer between HRP and electrode is achieved through PNTs. The signal produced by the electron transfer is measured with cyclic voltammetry (CV). The presence of an OP compound inhibits this signal by binding with the AChE enzyme. In this study, gas phase malathion was used as a model OP due to the fact that it displays the identical binding mechanism with acetylcholinesterase (AChE) as its more potent counterparts like sarin and VX, but has low toxicity, making it more practical and safer to handle. The CV signal was proportionally inhibited by malathion vapor concentrations as low as 12 ppbv. Depending on the method used in their preparation, the electrodes maintained their activity for up to 45 days. This research demonstrates the potential of applying nano-modified biosensors for the detection of low levels of OP vapor, an important development in countering weaponized organophosphate nerve agents and detecting commercially-used OP pesticides.

Keywords: organophosphate vapor, peptide nanotubes, acetylcholinesterase, horseradish peroxidase, malathion, cyclic voltammetry

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1. Introduction

Since their inception in 1937, chemical warfare nerve agents such as sarin and *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (commonly known as VX) have posed some of the deadliest threats in both conventional warfare and terrorist tactics (Upadhyayula, 2011). Current equipment for the field detection of organophosphate nerve agents requires a large portable 30 kg gas chromatograph/mass spectrometer (GC/MS) (Diehl-Faxon et al., 1996; Goltz et al., 2011). This equipment requires trained personnel, costs several hundred-thousand dollars, and requires complicated sample preparation and time-consuming analysis (Goltz et al., 2011). A feasible and far more practical alternative for field detection of organophosphates (OPs) makes use of electrochemical biosensors, which are relatively simple to make and can be easily tailored to suit specific requirements (Arduini et al., 2012; Upadhyayula, 2011).

Indirect detection typically uses acetylcholinesterase (AChE) with an amperometeric transducer to monitor the decrease in concentration of choline, which is a product of acetylcholine (ACh) hydrolysis, due to the presence of an enzyme inhibitor such as an OP (Liu and Lin, 2006).

Previous studies have demonstrated various methods of using an acetylcholinesterase- (AChE-) based biosensor to detect OP compounds in the liquid phase (Crew et al., 2011; Stevens, 2012). While these liquid phase studies have important applications for pesticide detection in agricultural runoff and OP concentrations in water used for chemical agent decontamination, limited research has been performed to investigate the application of AChE-based biosensors to detect OP nerve agents in the atmosphere. Arduini et al. (2007) successfully developed and tested a Prussian blue-

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modified silver screen-printed electrode (SPE) using butyrylthiocholine (BTCh) and butyrylcholinesterase (BChE) in the presence of sarin gas. Using amperometric techniques similar to those used in the liquid-phase studies, they were able to detect sarin in the gas phase at concentrations as low as 12 ppbv in the laboratory, which is lower than the 40 ppbv sarin concentration that represents an immediate danger to life and health. However, a field deployable prototype developed in 2012 was unable to achieve the sensitivity needed for a marketable OP biosensor (Arduini et al., 2012). The ultimate goal of this line of research is to create a small and affordable sensor that can detect in real-time OP compounds in the air below the lethal limit of 30 ppbv for VX (Hulet et al., 2007) or 40 ppbv for sarin (Arduini et al., 2010). Such a device could be carried by a combatant in the field, affixed to points on the perimeter of a base or camp, or attached to a small unmanned aerial vehicle (UAV) for forward reconnaissance.

In this study, a gold screen-printed electrode (SPE) was used because it has been shown to have a higher sensitivity and better reproducibility than carbon SPEs and is cost-effective for mass production unlike glassy carbon electrodes (Xu et al., 2004; Xue et al., 2012). Besides, a gold SPE was preferred due to its strong affinity toward peptide nanotubes (PNTs) that were used to encapsulate horseradish peroxidase (HRP) enzyme in this study.

Fig. 1 shows the surface modified electrode used in this study. The surface of the working electrode was modified with peptide nanotubes to increase the surface area for enzyme attachment, which has demonstrated an increase of the response in potentiometric detection (Stevens, 2012). Horseradish peroxidase (HRP) was encapsulated within the hollow structure of the peptide nanotubes (PNTs). This has been

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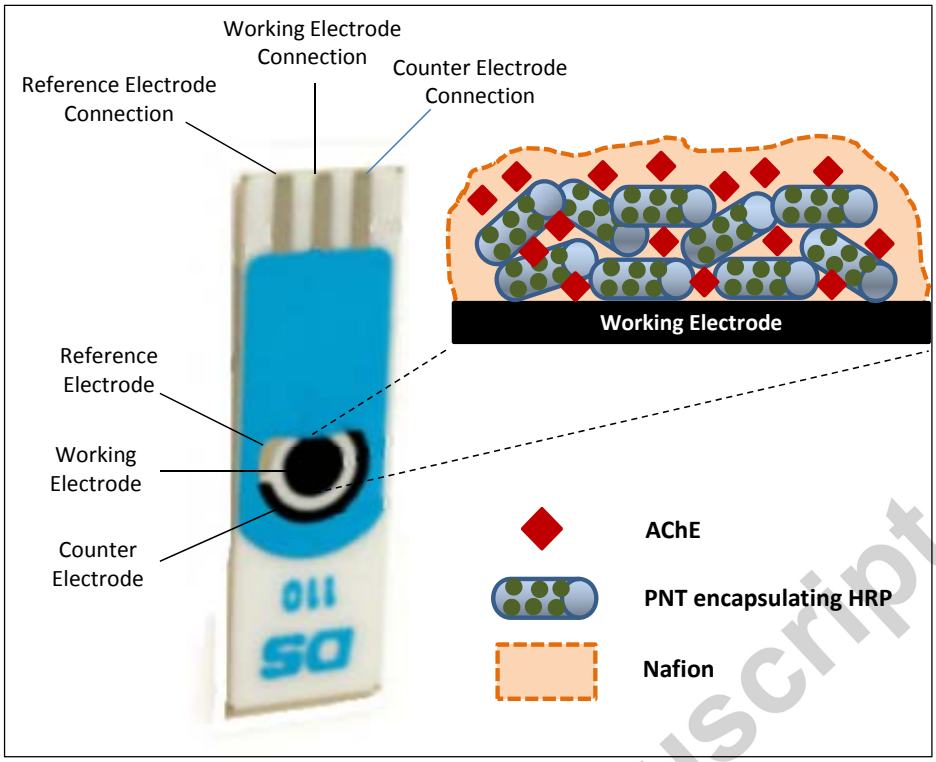

Fig. 1. Schematic depiction of the peptide nanotube (PNT)-modified gold electrode containing encapsulated horseradish peroxidase (HRP) and surface attached AChE enzymes. Nafion serves as a binder to hold the nanotubes to the electrode surface.

shown by Park et al. (2012a) to increase the signal in amperometric detection, thus decreasing the volume of enzyme required and the response time of the redox reaction. Encapsulating the HRP rather than introducing free HRP into solution protects the enzyme, thereby increasing the enzyme activity and longevity (Park et al., 2012b). The use of HRP also decreases the required voltage in the working electrode. PNT is a self-assembly of peptides with a diameter of 1 - 100 nm and micrometer length (Fernandez-Lopez et al., 2001; Scanlon and Aggeli, 2008). Due to their excellent biocompatibility, PNTs have been investigated for potential applications such as drug delivery, tissue-engineering scaffolds, batteries, capacitors and biosensors (Beker et al., 2010; Park et al. 2011). Peptide monomers, for example, diphenylanlanine (Phe-Phe), bolaamphiphile

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glycylglycine dicarboxylate, and amphiphilic alanine- arginine oligopeptide, have been shown to form PNTs of aromatic dipeptides, bolaamphilies, or surfactant-like peptides under specific conditions (Gazit, 2007; Hamley et al., 2013). Nafion, used as a protective layer over the AChE-treated PNT-modified electrode, was shown to assist in immobilizing the enzyme onto the PNTs, especially when in solution (Stevens, 2012).

In this study, acetylthiocholine (ATCh) is used as a substrate. The AChE catalyzes the hydrolysis of ATCh to produce acetic acid and thiocholine (Badea et al., 2006). Thiocholine is then oxidized by HRP to produce dithiocholine (Alfonta et al., 2000; Suprun et al., 2005). The direct electron transfer between HRP and the electrode is facilitated by PNTs. Transfer of electrons in this sequence of reactions is detected and measured by a potentiostat.

2. Materials and Methods

2.1 Materials

AChE-type V-S from electric eel, 500 U/mg; HRP, and malathion (> 95%) were purchased from Sigma Aldrich (Milwaukee, WI) and stored at -10 °C. ATCh and H-Phe-Phe-OH were purchased from Sigma Aldrich (St. Louis, MO), and stored at 4 °C. 1,1,1,3,3,3-hexafluoro-2-propanol (99.8% purity) was purchased from Sigma Aldrich (Milwaukee, WI) and stored at 4 °C. Nafion© 117 solution (approx. 5%) was purchased from Sigma Aldrich (Allentown, PA). Deionized water was generated in the lab via reverse osmosis.

Gold SPEs (model DRP-250) with a 4-mm-diameter gold working electrode and the electrode-potentiostat interface cable were purchased from Metrohm USA (Riverview,

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FL). The jacketed compact voltammetry cell was purchased from Pine Research Instrumentation (Durham, NC).

2.2 Apparatus

All electrochemical measurements were conducted using a Parstat 2273 Advanced Electrochemical System and PowerSuite© Software from Princeton Applied Research. The liquid media were dried using a Fisher Scientific Isotemp Vacuum Oven, model 280A. PNT solution was agitated using a Cole-Parmer 8890 Sonicator. Eppendorf Centrifuge 5810R was used for centrifuging the PNT solution.

Experiments were conducted in a Pine Research Instrumentation jacketed compact voltammetery cell. The temperature within the cell was adjusted and maintained using a Cole-Parmer Polystat Temperature Controller. Malathion concentration was determined using an Agilent Technologies 6890N Network GC Systems model gas chromatograph mass spectrometer (GC-MS).

2.3 Methods

PNTs were synthesized by dissolving 100 mg of H-Phe-Phe-OH in 1 ml of 1,1,1,3,3,3-hexafluoro-2-propanol (Reches and Gazit, 2003). This mixture was swirled gently by hand for a few seconds and then placed in a sonicator for five minutes to ensure complete dissolution.

To encapsulate HRP inside the PNTs, 1 ml of the PNT solution was dried overnight in a vacuum oven. Added to this was 1 ml of 50 mM phosphate buffer solution

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containing 1 mg HRP. This mixture was vortexed briefly and then incubated in a shaker at 5 $^{\circ}$C, 120 rpm for one week. This allowed the HRP to lodge inside the hollow structure of the PNT via capillary action (Park et al., 2010). The mixture was then centrifuged at 4,000 rpm for 1 hour. The liquid was withdrawn with a micropipette, and the PNT mixture was washed with 4 ml of 5.8 M phosphate buffer solution and then centrifuged again for 1 hour. This process was repeated twice more. After the liquid was withdrawn the third time, 2 ml of 5.8 M phosphate buffer solution was added, and the PNTs containing the encapsulated HRP were refrigerated. This process was confirmed to have encapsulated the HRP via scanning transmitting electron microscopy (STEM) analysis (Park et al., 2010).

Malathion vapor concentration was determined by comparing peak areas on a GC-MS of known liquid concentrations of malathion to peak areas of unknown vapor concentrations. A calibration curve was established using 10 points of known-concentration malathion standards from 0.1 ppm to 100 ppm. Malathion vapor was obtained by pipetting 2 mL of 95% pure malathion into a 40-ml amber vial with a gas-tight septum cap. Malathion (vapor pressure 4×10^{-5} mm Hg at 30% at room temperature) was allowed to equilibrate with the atmosphere inside the vial until saturation. A locking gas-tight syringe was then used to transfer 2 mL of the gas inside the vial into the GC-MS via a direct injection port.

Vapor concentration of a sample was adjusted by injecting a known volume of gas saturated with malathion at its vapor pressure (known concentration) into a vial of known volume purged with nitrogen at a constant temperature. The resulting concentration was then calculated using an ideal gas law after equilibration was achieved.

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The sensors were prepared by first depositing 5 µL of PNTs containing the encapsulated HRP on the working electrode, which was then allowed to dry in a hood at room temperature and pressure (average of 65 °C and 745 mmHg). Then, 5 µL of 1000 U/mL AChE was deposited on top of the PNTs and allowed to dry. Over that, 5 µL of Nafion® was deposited and allowed to dry. The electrode was then placed into a voltammetry flask filled with phosphate buffer solution, and cyclic voltammetry measurements were taken. The electrodes were then removed from the phosphate buffer solution and inserted into an identical vial with phosphate buffer solution containing 1 mmol ATCh, and cyclic voltammetry measurements were taken again. The electrodes were then immediately transferred into a vial purged with nitrogen or into a vial containing 25 ppbv malathion vapor, and cyclic voltammetry measurements were taken again.

A longevity experiment for AChE was conducted at fifteen, thirty, forty-five, and sixty days after electrode construction. The electrodes were tested with two different preparation methods and for each preparation method, two different tests were applied for a total of four different tests (Table 1). These different methods were investigated to help determine what options might be available for preparation and use of a viable field-deployable sensor. For the longevity experiments, two sets of electrodes were initially prepared. One set was prepared with just PNTs and another set had PNTs covered with AChE (Table 1). Considering the ultimate goal of developing a field-deployable biosensor, it would be beneficial if the electrode could be prepared with AChE, so in the field it would only be necessary to add ATCh to activate the biosensor. The electrodes were stored dry at 4 °C. At the aforementioned time intervals, the electrodes were tested

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using cyclic voltammetry measurements.

Table 1. Setup conditions for enzyme longevity experiment.

	Preparation 1: Electrodes prepared with PNTs and AChE	**Preparation 2:** Electrodes prepared with just PNTs
Treatment 1 (Wet)	Treated with Nafion and immersed in phosphate buffer solution containing ATCh	Treated with AChE and Nafion and immersed in phosphate buffer solution containing ATCh
Treatment 2 (Dry)	Treated with 5 µL ATCh	Treated with 5 µL AChE and 5 µL ATCh
Residual activity test	Inserted into 25 ppbv malathion gas	Inserted into 25 ppbv malathion gas

Preparation 1: PNTs and AChE/Tested Wet (Treatment 1)

One set of electrodes was prepared with 5 µL of PNTs encapsulating HRP. This was allowed to dry and then 5 µL of AChE was deposited on the PNTs and allowed to dry. The electrodes were then stored for the longevity tests. Just prior to testing, 5 µL of Nafion was added on top of the AChE and allowed to dry. Once dried, the electrode was immersed in a phosphate buffer solution and a cyclic voltammogram was taken. Then the electrode was removed from the phosphate buffer solution and immersed in a phosphate buffer solution with 1 mmol ATCh and then a cyclic voltammogram was again taken.

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Finally, the electrode was inserted into a gaseous environment containing 25 ppbv malathion gas and another cyclic voltammogram was taken.

Preparation 1: *PNTs and AChE/Tested Dry (Treatment 2)*

One set of electrodes was prepared with 5 µL of PNTs encapsulating HRP. This was allowed to dry and then 5 µL of AChE was deposited on the PNTs and allowed to dry. The electrodes were then stored for the longevity tests. Just prior to testing, 5 µL of ATCh was added on top of the AChE and the electrode was immediately inserted into a nitrogen gas environment and a cyclic voltammogram was taken. Then the electrode was removed from the nitrogen gas and placed in a gaseous environment containing 25 ppbv malathion gas and another cyclic voltammogram was taken.

The difference between Treatments 1 and 2 is the method of sample application. In Treatment 1, the electrode was immersed in the buffer solution containing 1 mmol ATCh (inhibitor), whereas in Treatment 2, 5 µL of ATCh was added onto the electrode right before the gas phase testing.

Preparation 2: PNTs Only/Tested Wet (Treatment 1)

One set of electrodes was prepared with 5 µL of PNTs encapsulating HRP. The electrodes were then stored for the longevity tests. Just prior to testing, 5 µL of AChE and 5 µL of Nafion were added on top of the PNTs and after each addition, allowed to dry. Once dried, the electrode was immersed in phosphate buffer solution and a cyclic voltammogram was taken. Then the electrode was removed from the phosphate buffer solution and immersed in a phosphate buffer solution with 1 mmol ATCh and a cyclic

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voltammogram was again taken. Finally, the electrode was inserted into a gaseous environment containing 25 ppbv malathion gas and another cyclic voltammogram was taken.

Preparation 2: PNTs Only/Tested Dry (Treatment 2)

One set of electrodes was prepared with 5 µL of PNTs encapsulating HRP. The electrodes were then stored for the longevity tests. Just prior to testing, 5 µL of AChE and 5 µL of ATCh were added on top of the PNTs and the electrode was immediately inserted into a nitrogen gas environment and a cyclic voltammogram was taken. Then the electrode was removed from the nitrogen gas and placed in a gaseous environment containing 25 ppbv malathion gas and another cyclic voltammogram was taken.

A difference between Prep 1/Treatment 1 and Prep 2/Treatment 1 is the addition timing of AChE and Nafion. In Prep 1, the electrodes were prepared with the HRP-encapsulating PNTs and AChE deposited on top of them, then stored until testing, and Nafion was added right before testing, whereas in Prep 2, AChE and Nafion were added right before testing.

A response vs. concentration test was conducted by injecting a known volume of malathion-saturated gas into a known volume of nitrogen. For this test, a 50-mL gas-tight syringe was used. Varying mixtures of the 25 ppbv malathion gas and nitrogen gas were drawn into the syringe for a total of 50 mL, which was then allowed to sit at 20 $^{\circ}$C for an hour to allow the gases to mix evenly. The entire volume was then injected through a septum into the 25-mL voltammetry flask containing the electrode. The mixture was tested at 0, 10, 25, 50, 70, 75, 80, 90, and 100% of the initial 25 ppbv malathion vapor. At

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each concentration, each electrode was tested first in phosphate buffer, then phosphate buffer with ATCh, and finally in the malathion-nitrogen vapor mix to establish inhibition of the reaction in terms of percent change. At each phase, a cyclic voltammogram was generated. The duplicate experimental results for each concentration were averaged.

3. Results and Discussion

The cyclic voltammogram in Fig. 2 establishes a baseline oxidation peak for the reaction between AChE and ATCh. The potentiostat measures the oxidation of thiocholine, which is a product of the hydrolysis of the enzymatic reaction with AChE and ATCh. The bottom (thin) line is the response when there is no ATCh present, and therefore there is no reaction (1.6×10^{-6} A). Note that this line is not precisely zero on the y-axis of the grid. This small non-zero response is presumed to be noise from the potentiostat. This measured value for the buffer solution without any ATCh (and therefore presumably no reaction), is used as baseline "zero" in all subsequent calculations. The small peak on the left side of the reduction curve (negative current) is thought to be the result of reductive desorption of thiol group in acetylthiocholine. This result verifies that the interaction between acetylthiocholine and gold surface was negligible. The top (thick dotted) lines of oxidation peaks on the right represent the response in the presence of the reaction (4.8×10^{-4} A). The other dotted lines in between are the progression of CV response in terms of time until the equilibrium (the top dotted line). The equilibrium CV response was usually obtained after 3 – 5 minutes.

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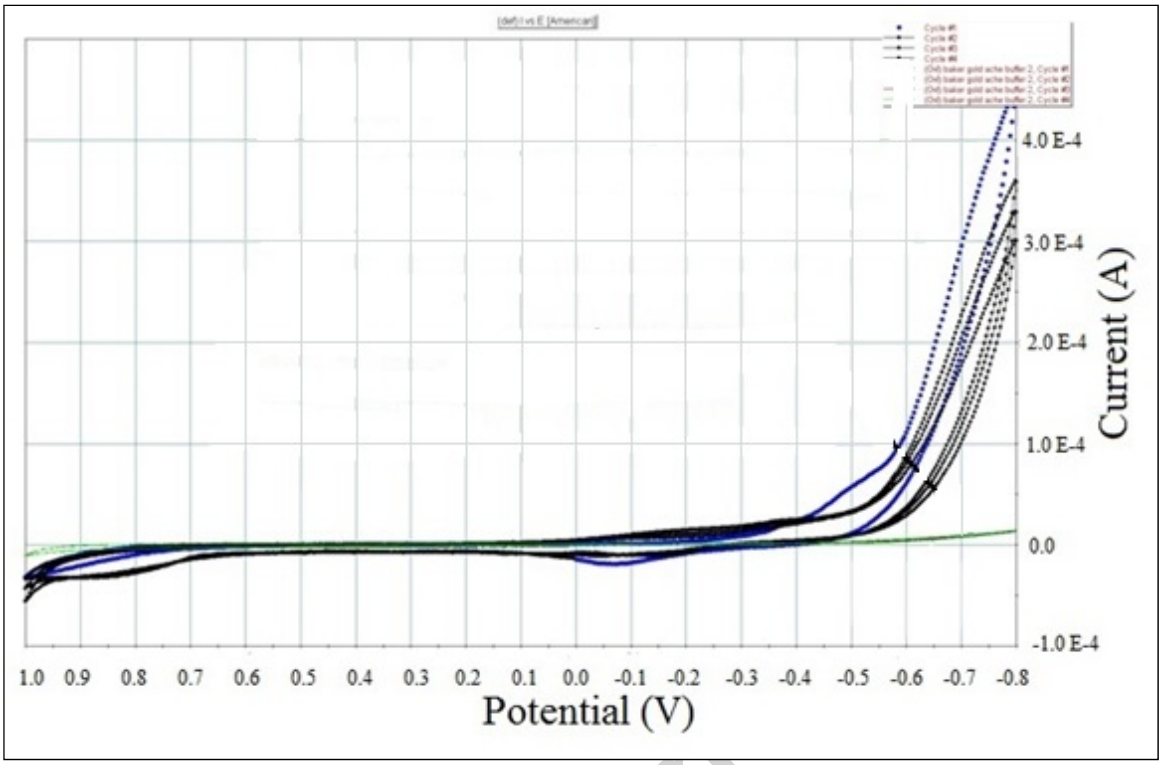

Fig. 2. Cyclic voltammograms for reaction between ATCh and AChE in the liquid phase. The dotted lines terminating between 0.3 and 0.4 mA represent the CV scans of the ATCh and AChE reaction, while the thin solid line terminating near 0.0 mA is the control (no ATCh present).

The next step of the experiment was to determine if malathion vapor could be observed to inhibit the enzymatic reaction between AChE and ATCh. The thin film of phosphate buffer containing ATCh that remained on the electrode after it was removed from the solution continued the reaction. Ideally, there would be no decrease in reaction when the sensor is placed in a nitrogen atmosphere. The 44% decrease in the peak heights from 4.8×10^{-4} A to 2.7×10^{-4} A shown in Fig. 3a is assumed to be due to the baseline current change after the electrode was placed in the gas phase from the liquid solution. Then, when the electrode was placed in the 25 ppbv malathion vapor, the response of the electrode dropped over 99% from the initial current in the liquid phase 4.8×10^{-4} to

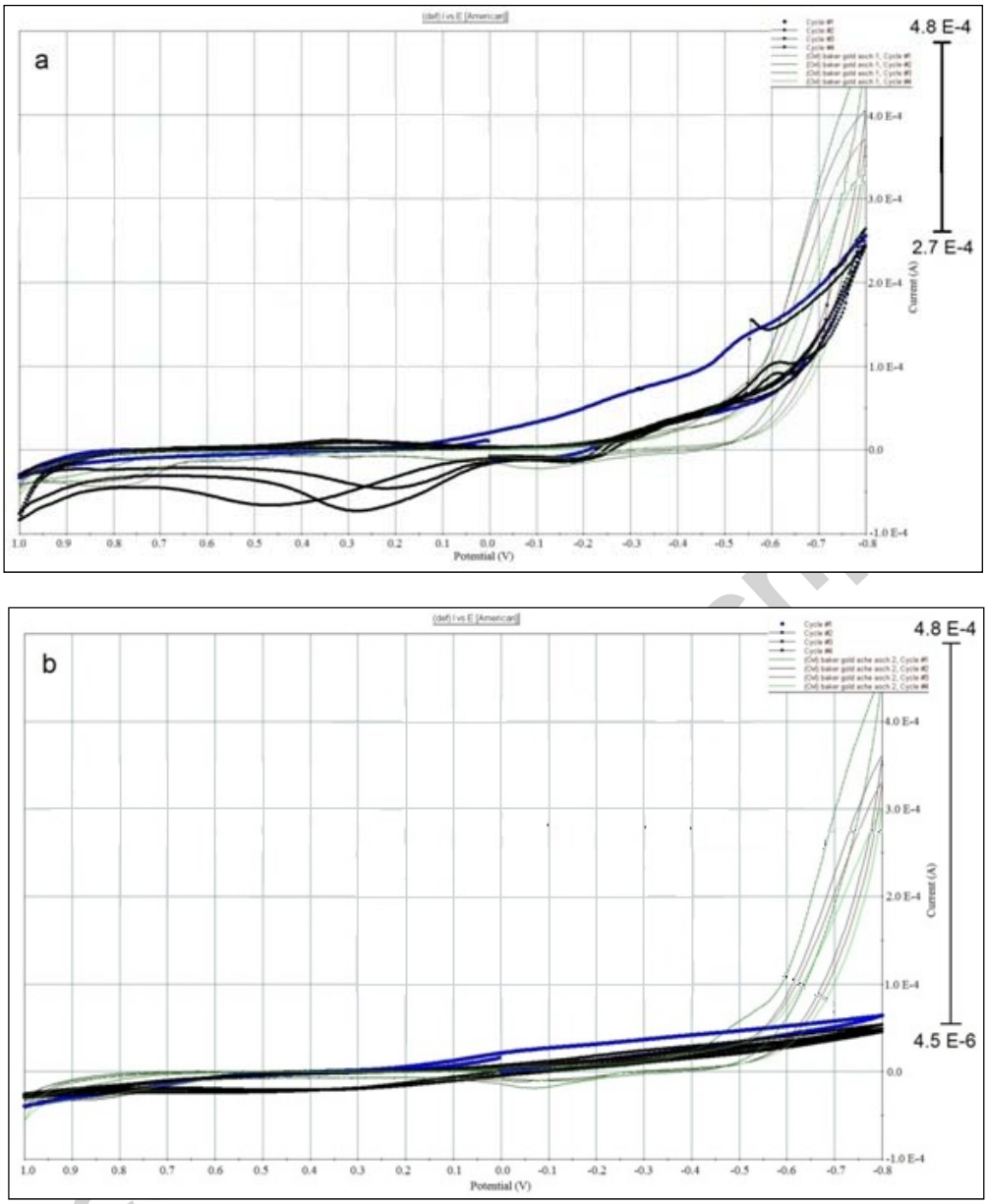

Fig. 3. CV scans of the ATCh and AChE reaction in either nitrogen gas or 25 ppbv malathion vapor. The highest lines in both CV scans represent the current response of the AChE-deposited electrode in phosphate buffer containing 1 mmol ATCh. (a) The thick solid lines represent the response in nitrogen gas. (b) The thick solid linesrepresent the response in nitrogen gas saturated with malathion vapor.

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4.5×10^{-6} A, and decreased 98.3% from the gas-phase baseline of 2.7×10^{-4} (Fig. 3b). This 98.3% decrease in the current was due to the inhibition of AChE enzyme by malathion. We concluded from this result that it was, in fact, the malathion vapor that inhibited the reaction between the enzyme and the substrate.

The longevity test indicated that the time elapsed was much less significant than the method used. Those electrodes that were prepared using the wet method (Treatment 1) showed an *increase* in sensitivity (response) over time (Fig. 4). Those treated with the dry method (Treatment 2) showed no response after 15 days (Fig. 4). Notably, when comparing the responses of the sensors prepared using the wet method, we see that even when the AChE enzyme was attached to the electrode weeks earlier (Preparation 1; PNT+AChE), the response was the same as when the electrode was treated with AChE just before testing (Preparation 2; PNTs only).

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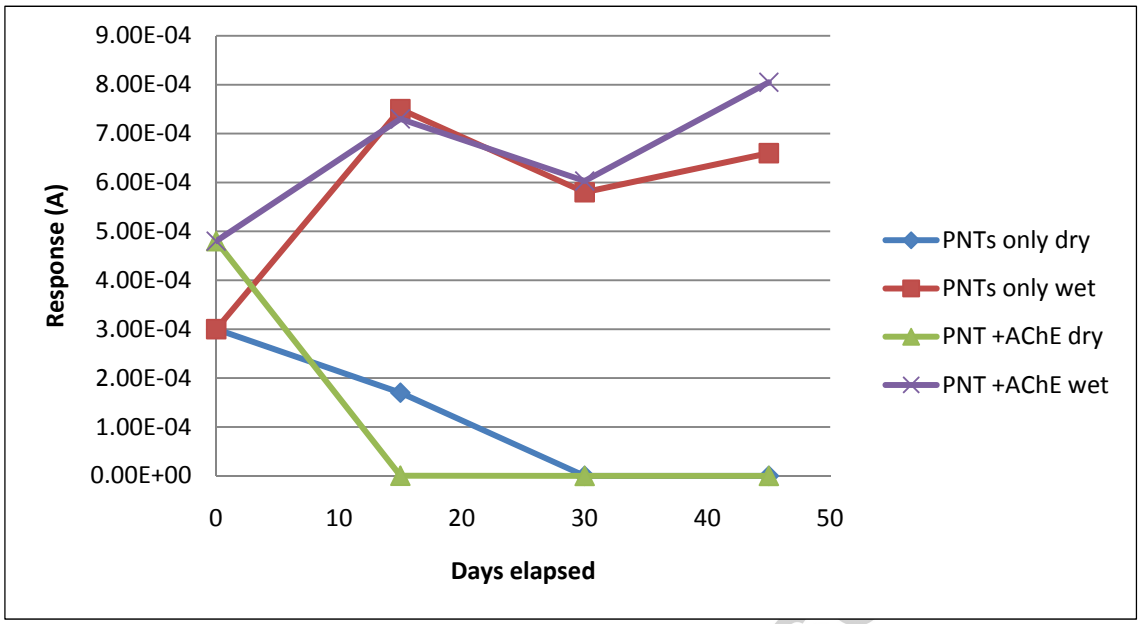

Fig. 4. Results of the longevity test indicate that the preparation method has the more significant effect on sustained viability (preparation methods shown in Table 1). Y-axis shows drop in current due to inhibition by 25 ppbv malathion.

During this longevity experiment, the electrodes were tested in 25 ppbv malathion gas. Those electrodes that were tested by the wet method (Treatment 1) maintained their initial reactivity over 45 days. Those electrodes that were tested by the dry method showed no response after 15 and 30 days for the electrodes prepared by Prep 1 and Prep 2, respectively. Therefore, regardless of the preparation method, either Prep 1 or Prep 2, the sensor maintained the enhanced sensitivity longer when it was dipped in the buffer solution containing ATCh before being tested for gas phase detection than when ATCh was directly applied to the electrode. The sensor prepared wet by Prep 1 (PNT+AChE) showed slightly higher sensitivity than the sensor prepared wet by Prep 2 (PNT only). When the ATCh was directly applied to the electrode (i.e., dry method or Treatment 2), the sensor prepared using Prep 2 (PNT only) initially showed a lower response, but it

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maintained its performance about 15 days longer than the one by Prep 1 (PNT+AChE).

Our results were comparable to those reported by Arduini et al. (2012), although their test was over a much longer duration. At times greater than 60 days, Arduini et al. (2012) did show some decrease in enzyme activity.

The final aspect of this study was to establish a response versus concentration curve for the biosensor. Results are shown in Fig. 5. Based on the data shown in Fig. 5, it was determined that the inhibition response at concentrations below 12.5 ppbv was not related to the concentration, so a detection limit of 12.5 ppbv was established and a linear correlation was demonstrated for malathion concentrations between 12.5 and 25 ppbv ($R^2 = 0.86$), which are a range of concentrations below the lethal concentration for all known OPs (Arduini et al., 2012).

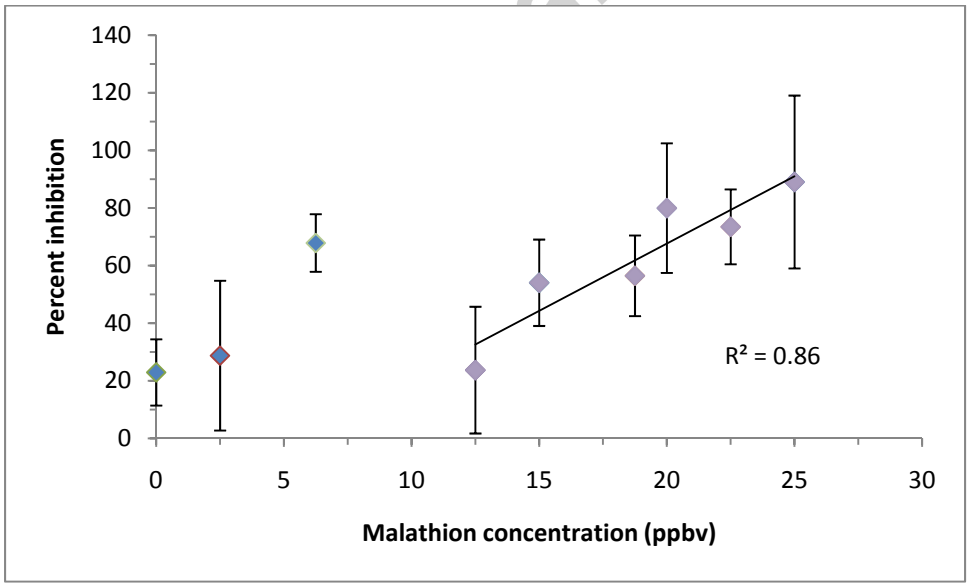

Fig. 5. Biosensor response vs. malathion vapor concentration. Linear fit shown for concentrations greater than 12.5 ppbv (error bars indicate standard deviation of four replicates for each concentration).

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4. Conclusions

Under laboratory conditions, peptide-nanotube-modified biosensors appeared to be a viable method for detecting OP vapors at low concentrations below 25 ppbv. The detection limit for malathion found in this study (12.5 ppbv) was well below the lethal limit for all known OPs and the response time was nearly instantaneous. The longevity test demonstrated that the electrodes prepared by the wet method (Treatment 1) maintained activity for 45 days. The ease and low cost of their construction, their sensitivity, and their rapid response give peptide-nanotube-modified biosensors significant advantages over current detection methods. Based on these tests, we find that field application of the biosensor has the following constraints: (1) the reagents must be refrigerated, (2) the sensor needs to be fully immersed in the buffer solution containing ATCh just prior to use. The results of this study are promising and further research and development of this field-deployable OP biosensor appears worthwhile.

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List of Figures

Fig. 1. Schematic depiction of the peptide nanotube (PNT)-modified gold electrode containing encapsulated horseradish peroxidase (HRP) and surface attached AChE enzymes. Nafion serves as a binder to hold the nanotubes to the electrode surface.

Fig. 2. Cyclic voltammograms for reaction between ATCh and AChE in the liquid phase. The dotted lines terminating between 0.3 and 0.4 mA represent the CV scans of the ATCh and AChE reaction, while the thin solid line terminating near 0.0 mA is the control (no ATCh present).

Fig. 3. CV scans of the ATCh and AChE reaction in either nitrogen gas or 25 ppbv malathion vapor. The highest lines in both CV scans represent the current response of the AChE-deposited electrode in phosphate buffer containing 1 mmol ATCh. (a) The thick solid lines represent the response in nitrogen gas. (b) The thick solid lines represent the response in nitrogen gas saturated with malathion vapor.

Fig. 4. Results of the longevity test indicate that the preparation method has the more significant effect on sustained viability (preparation methods shown in Table 1). Y-axis shows drop in current due to inhibition by 25 ppbv malathion.

Fig. 5. Biosensor response vs. malathion vapor concentration. Linear fit shown for concentrations greater than 12.5 ppbv (error bars indicate standard deviation of four replicates for each concentration).

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Table 1. Setup conditions for enzyme longevity experiment.

	Preparation 1: Electrodes prepared with PNTs and AChE	**Preparation 2:** Electrodes prepared with just PNTs
Treatment 1 (Wet)	Treated with Nafion and immersed in phosphate buffer solution containing ATCh	Treated with AChE and Nafion and immersed in phosphate buffer solution containing ATCh
Treatment 2 (Dry)	Treated with 5 µL ATCh	Treated with 5 µL AChE and 5 µL ATCh
Residual activity test	Inserted into 25 ppbv malathion gas	Inserted into 25 ppbv malathion gas

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- We construct a gas phase organophosphate (OP) sensor using nanotubes and enzymes
- Cyclic voltammetry measures thiocholine oxidation in the presence of the enzymes
- Presence of malathion (our model OP) inhibits the oxidation reaction
- Inhibition proportional to gas phase malathion concentrations as low as 12 ppbv
- Demonstrates PNT-modified biosensors can detect low levels of OP vapor

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