



**STABILIZING ACETYLCHOLINESTERASE ON CARBON ELECTRODES
USING PEPTIDE NANOTUBES TO PRODUCE EFFECTIVE BIOSENSORS**

THESIS

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Abstract

A biosensor for the detection of organophosphates (OPs) in water was created by encapsulating acetylcholinesterase (AChE) enzyme in peptide nanotubes (PNTs) and attaching the encapsulated enzyme on a carbon screen printed electrode using Nafion®. Sensor operation is based on the fact that acetylthiocholine (ASCh) substrate, in the presence of AChE, will be transformed to thiocholine, which can be oxidized by the electrode, producing a measurable signal. This signal will be inhibited in the presence of OPs, with the extent of inhibition proportional to the OP concentration.

In this study, three versions of the sensor were used to detect the OP malathion. In one version, AChE was placed directly on the electrode. In the second version, AChE was encapsulated in a PNT, and placed on the electrode. And in the third version, PNT encapsulated AChE was attached to the electrode using Nafion. The stability of the sensors was measured over 50 days of storage at 4°C in a phosphate buffer solution. Cyclic voltammograms were taken in an ASCh and phosphate buffer solution, and the peak oxidation was used to measure the performance of the sensor. Amperometric studies were also conducted at 310 mV vs Ag/AgCl to measure the response of the sensors to malathion, when ASCh was present.

This research demonstrates that the use of PNTs and Nafion® allows the sensor to remain stable for a much longer period than similar biosensors which rely on adsorption alone. In fact, the sensors that were constructed without PNTs or Nafion did not remain stable under the storage conditions. The biosensors constructed with PNTs, and with PNTs and Nafion, lost 17.3% and 14.2% of their activity, respectively, after 50 days.

Detection limits as low as 48 nM of malathion were obtained using PNTs, and 102 nM using PNTs and Nafion®.

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To my wife, my companion on this most delightful journey

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Finally, I also thank my fantastic wife. Thank you for your seemingly limitless love, support, and patience.

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STABILIZING ACETYLCHOLINESTERASE ON CARBON ELECTRODES USING PEPTIDE NANOTUBES TO PRODUCE EFFECTIVE BIOSENSORS

I. Introduction

Background

Organophosphates (OPs) are acetylcholinesterase (AChE) inhibitors with a broad range of potency and toxicity. While the term organophosphate strictly applies to esters of phosphoric acid, it is also usually used for similar compounds, such as esters of phosphorous and phosphinic acid, and xanthate esters. OPs have been widely used in agriculture as pesticides and are also used as deadly nerve agents in chemical weapons.

Water may become contaminated with OPs via several routes. Agricultural runoff can easily be contaminated by the overuse of pesticide. Plans for the cleanup of nerve gas attacks involve hosing affected areas down with bleach and water, which may also lead to water contamination. A technology to measure OPs in water is needed in order to prevent contamination from reaching drinking water supplies, as well as to protect the health of decontamination workers and the public.

Chromatography and spectrometry are the classical analytical chemistry methods used to measure OPs. These methods require large, sensitive equipment, which in turn require personnel trained in their use and a laboratory environment, making the process time consuming and expensive. The US Air Force has portable gas chromatograph HAPSITE systems, which can be used to detect OPs in the field, in both air and water. However, these systems are heavy, expensive, require specially trained personnel, and can take up to 30 minutes per measurement.

Such long measurement times can directly impact a unit's ability to maintain mission readiness when responding to a chemical agent attack. The Air Force standard for mission capability restoration is to resume the primary mission within 2 hours after the end of a chemical attack (AFM 10-2503, 2011). This does not allow much time for many measurements to be taken. Additionally, very low concentrations of the most potent nerve agents, such as VX, that are below the limit of detection of the HAPSITE, may still be dangerous. Such undetectable concentrations can cause negative health effects and even death, depending on exposure time.

In the absence of definitive measurements to show that it is safe for personnel to remove protective equipment, unit commanders are forced to be conservative and leave protective measures in place. Such measures can be detrimental to a unit's effectiveness, especially if needed for long periods. Faster, more accurate measurements would allow commanders to remove unnecessary protective equipment sooner.

A new, inexpensive technology is needed that can be used *in situ* to provide data in real time. Biosensors using AChE bound to screen printed electrodes (SPE) to detect OPs may be able to fill this need. However, the process by which the AChE is bound to the electrode, as well as the binding agents used, can affect the sensor's performance. Peptide nanotubes (PNTs) have been used to protect enzymes and bind them to electrodes with minimal enzyme deactivation (Park et al, 2010). Nafion, a stable, biocompatible, Teflon based polymer has been used in the production of glucose biosensors to increase enzyme stability and shelf life (Norouzi et al, 2010; Ren et al, 2012). Figure 1 shows, conceptually, a biosensor with these components.

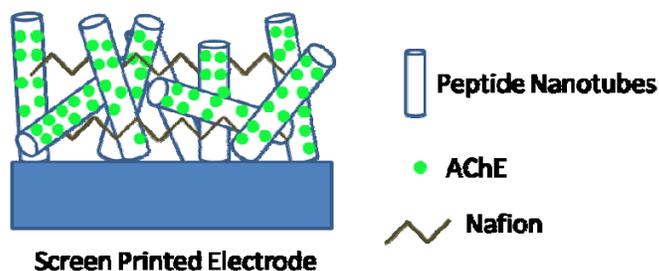


Fig. 1: A schematic diagram of a biosensor using peptide nanotubes to bind AChE to a screen printed electrode with Nafion as a binding agent

Carbon SPEs are a stable, inexpensive, disposable transducer to serve as a platform for developing a biosensor. PNTs provide a biocompatible, electrically conductive surface that serves to protect the AChE. Nafion[®] is cast over the top as a sort of net to bind everything together. Sensor operation is based on the fact that acetylthiocholine (ASCh) substrate, in the presence of AChE, will be transformed to thiocholine, which can be oxidized by the electrode, producing a measurable signal. This signal will be inhibited in the presence of OPs, with the extent of inhibition proportional to the OP concentration.

Research Objectives

The objective of this research is to measure the performance of biosensors which utilize AChE bound to screen printed electrodes. The performance parameters to evaluate such a biosensor will be determined, as well as a means to measure and characterize these parameters. A sub-objective is to determine whether the use of peptide nanotubes and Nafion in the biosensor production increases the stability of the enzyme and the sensor shelf life.

Scope and Approach

A literature review was conducted to find the current practices used to produce biosensors and determine the relative efficacies of using various methods and compounds to bind biosensing enzymes to electrodes. In particular, the review focused on the use of peptide nanotube and Nafion binders to produce biosensors with improved performance characteristics. Additionally, the literature review determined how the biosensor will most likely be applied, and what performance parameters should be optimized for this application. Finally, the review determined what potentiometric and amperometric measurements can be taken to quantify these parameters and compare the results of different production methods.

Biosensors using three different designs were constructed using AChE bound to SPEs to measure concentrations of malathion in water. The first design simply adsorbed AChE to the SPE. The second used PNTs deposited on the SPE to encapsulate AChE. The third used PNT encapsulated AChE deposited on the SPE as well as Nafion to bind the PNTs together.

The performance of the biosensors was tested using potentiometric measurements including cyclic voltammetry before and after a storage period. Potentiometric and amperometric measurements were taken in the presence of various concentrations of the target OPs.

II. Literature Review

Biosensors

Biosensors can potentially be used to detect OPs in water. Biosensors are analytical devices incorporating a biological material attached to a physical transducer to sense a target substrate or substrates.

Biosensors are being increasingly used for detection of specific targets in solutions (Andreescu and Marty, 2006). Biosensors are used to measure glucose levels in blood for diabetes patients (Tudorache, 2007). The food industry uses various types of biosensors to detect pathogens in food during processing (Amine et al, 2006). AChE-based biosensors are emerging as an extremely sensitive means of toxicity monitoring in environmental, food processing, and military applications (Andreescu and Marty, 2006; Tudorache et al, 2007).

Cholinesterase biosensors

Cholinesterases (ChE) are enzymes that hydrolyze the neurotransmitter acetylcholine, which transmits nerve impulses across cholinergic synapses. Both acetylcholinesterase and butyrylcholinesterase (BuChE) have been used in biosensors. While they have similar structures, BuChE preferentially hydrolyzes butyryl choline. AChE preferentially hydrolyzes acetylcholine and esters of thiocholine. Many of these esters, especially acetylthiocholine (ASCh), have been used as substrates for various AChE biosensors. ASCh is particularly useful because, once broken down by the enzyme, thiocholine can be oxidized by an electrode, generating a measurable signal. This oxidation of thiocholine occurs nominally at 410 mV vs. Ag/AgCl (Andreescu,

2001). If a graphite paste is used as the working electrode, TCNQ (7,7,8,8-tetracyanoquinonedimethane) can be used as a mediator. This allows the oxidation of thiocholine to occur at 110mV vs. Ag/AgCl (Bonnet, 2003, p210)

AChE biosensors detect OPs indirectly, by inhibition. An electrochemical measurement is taken with the biosensor in a solution of substrate before it is exposed to the OP. Once an OP is introduced into the system, it will inhibit the AChE. This reduces the rate at which the substrate is broken down, and thus the rate at which the signal is generated by the products of the substrate hydrolysis.

Enzyme Immobilization

The electrode type and material and enzyme immobilization technique used during production of a biosensor are key to how it will function and perform. Many methods have been developed for attaching AChE to electrodes of various types.

Adsorption is the easiest and simplest means of immobilizing an enzyme on an electrode. It is the least immediately denaturing, as it relies on weak bonds such as Van der Waals or electrostatic forces, and does not require functionalization of the enzyme or covalent bonding between the enzyme and electrode. It does not create any diffusion barriers that other entrapment techniques may. However, because there is no covalent bonding, the orientation of the enzyme as it adsorbs to the electrode is not controlled, leaving some of the enzyme oriented in a way that the active site is inaccessible to a substrate in solution. Also, because the adsorptive forces are weak, leaching is generally observed in a stirred environment (Bonnet et al, 2002). Desorption of enzyme can also occur due to changes in temperature, pH, or ionic strength (Sassolas et al, 2012).

In one study, Bonnet et al. (2003) found that rinsing the electrodes for 30 min in 1M NaCl, then 5 min in a solution of 0.1 M phosphate buffer, 0.2 M NaCl, 2×10^{-3} M MgSO₄, and 0.1% Tween, minimized leaching during measurements, leading to good operational stability. The electrodes also had good storage stability, losing only 1.6% activity over 50 days when stored under vacuum at 4°C. The study found that when used to detect chlorpyrifos ethyl oxon (CP-o), the sensor showed an I₂₀ (the concentration of target inducing 20% inhibition) of 5 ng/l (Bonnet et al., 2003).

Another study found that adsorption and stabilization of AChE to a planar gold electrode was greatly enhanced by coating the surface with gold nanoparticles. The I₁₀ (the concentration of target inducing 10% inhibition) detection limit for carbofuran was estimated to be 33 nM. The sensors were stored at 4°C, but storage stability was only measured over a 1-week period (Shulga, 2007).

Covalent binding is the most often used method to immobilize ChEs to electrodes (Andreescu and Marty, 2006). It involves modifying the electrode with a cross-linker that will bind to the enzyme on one end, and the electrode surface on the other. Binding provides greater operational stability to the electrode, and the enzyme will not leach off the surface during use. However, because this method causes significant enzyme denaturing, a significantly greater amount of enzyme must be used to create a comparable signal to other immobilization methods (Nunes et al, 2004; Adreescu and Marty, 2006). A study by Lin, et al (2003) has also shown that carbon nanotubes (CNTs) may be used as a cross-linker. The study used a carbon electrode coated with CNTs, which were then oxidized to form carboxyl groups. AChE was bound to these groups, and the electrodes were found to generate higher signals with less enzyme than other cross-linking methods.

This is most likely due to the conductive properties of the CNTs. A detection limit of 0.05 μM methyl parathion was achieved using this method (Lin et al., 2003).

A self-assembled monolayer (SAM) may be used to link enzymes to noble metal electrode surfaces (Andreescu and Marty, 2006). This process uses hydrocarbon chains with a hydrophilic function group, usually a carboxyl group, at one end. The molecules are deposited on the electrode surface and form a packed monolayer, held together by Van der Waals forces between the hydrocarbon chains. Enzymes can then be bound to the functional groups. SAMs made from longer chain molecules are generally more stable, but present a barrier to electron transmission to the electrode surface. Shorter chains present less of a barrier, but are generally less ordered and less stable (Sassolas et al, 2012).

Physical entrapment methods have also been used in biosensor construction. Enzymes, along with any mediators and additives, may be entrapped in sol-gel matrices or a photopolymer in a simple, one step fabrication process. Entrapment has been used to immobilize enzymes on screen printed and solid electrodes. While entrapment provides increased storage and operational stability, it also creates a diffusion barrier for substrates to reach the enzyme, which limits the sensor performance (Andreescu, 2006).

Encapsulation is similar to entrapment, but rather than immobilizing the enzyme in a random matrix, it uses ordered structures such as PNTs or liposomes. Encapsulation also has the advantage of protecting the enzyme, leading to increased stability. PNTs have better electric conduction properties than sol-gel or polymer matrices, leading to higher signal generation. However, encapsulation can also create diffusion barriers, slowing the electrode's response time (Park, 2010). These types of electrodes using

PNTs show great potential for stable and biocompatible encapsulation, which may maintain the enzyme activity for a longer time (Park et al, 2010).

Performance

Stability, both storage and operational, is the most desirable characteristic a sensor can have. Storage stability refers to a sensor's ability to retain its activity after a storage period. It is typically measured by determining the activity before and after a 50-day period, and expressed as the percent of the initial activity the sensor retained after the 50 days. Operational stability refers to how stable the sensor is in the environment in which it is intended to operate. Biosensors may only be able to operate stably over a small range of pH values, or in either aqueous or non-aqueous solutions. Operational stability is generally measured simply by taking consecutive measurements and observing whether the signal degrades with each measurement.

The ability to detect the target chemical or chemicals of interest is the primary requirement of any biosensor. For AChE biosensors, which detect indirectly, this ability is measured in inhibition. Cyclic voltammetry measurements are taken on a sensor in identical solutions before and after exposure to the target compound. The peak currents before, i_o , and after, i_i , are used to calculate the inhibition percent using $I\% = (1 - i_i/i_o) \times 100\%$.

In most articles, I_{20} (concentration of target inducing 20% inhibition) is used as a reference value for inhibition tests (Bonnet, 2003; Amine, 2005). The limit of detection for a sensor is sometimes estimated as the I_{10} value (Park, 2010). The inhibition of sensors is also qualitatively shown in many studies by using chronovoltic amperometry.

While this test can show a drop in current when an OP is introduced, quantitative data and models of how the current changes with respect to concentration of OP and time have not been done.

Storage and Operating Conditions

Operating conditions for the sensor will vary as widely as the environments in which military operations are conducted. Water temperature, pH, and salt content cannot be anticipated for every location at which a measurement needs to be taken. An ideal sensor would work in a wide range of these parameters. The general nature of military operations gives preference to an inexpensive, disposable electrode over an expensive, permanent but fragile electrode. Thus, carbon screen printed electrodes were chosen for this research.

Storage conditions will also vary depending on the environment. While installations in the U.S. may have vacuum storage capability, it is doubtful that forward bases would. Therefore, a sensor with unprotected enzymes that must be stored at 4°C under vacuum is not a practical choice. Most bases are likely to have electrical and refrigeration capabilities. If sensors using encapsulation can be kept in solution at 4°C they would be preferred, and sensors that could be kept in solution at ambient temperature would be ideal. For this reason, encapsulation in PNTs was the immobilization method chosen to be studied for stability in this research.

III. Scholarly Article

Stabilizing acetylcholinesterase on carbon electrodes using peptide nanotubes

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Abstract

A biosensor for the detection of organophosphates (OPs) in water was created by encapsulating acetylcholinesterase (AChE) enzyme in peptide nanotubes (PNTs) and attaching the encapsulated enzyme on a carbon screen printed electrode using Nafion®. Sensor operation is based on the fact that acetylthiocholine (ASCh) substrate, in the presence of AChE, will be transformed to thiocholine, which can be oxidized by the electrode, producing a measurable signal. This signal will be inhibited in the presence of OPs, with the extent of inhibition proportional to the OP concentration.

In this study, three versions of the sensor were used to detect the OP malathion. In one version, AChE was placed directly on the electrode. In the second version, AChE was encapsulated in a PNT, and placed on the electrode. And in the third version, PNT encapsulated AChE was attached to the electrode using Nafion. The stability of the sensors was measured over 50 days of storage at 4°C in a phosphate buffer solution. Cyclic voltammograms were taken in an ASCh and phosphate buffer solution, and the peak oxidation was used to measure the performance of the sensor. Amperometric studies were also conducted at 310 mV vs Ag/AgCl to measure the response of the sensors to malathion, when ASCh was present.

This research demonstrates that the use of PNTs and Nafion® allows the sensor to remain stable for a much longer period than similar biosensors which rely on adsorption alone. In fact, the sensors that were constructed without PNTs or Nafion did not remain stable under the storage conditions. The biosensors constructed with PNTs, and with PNTs and Nafion, lost 17.3% and 14.2% of their activity, respectively, after 50 days. Detection limits as low as 48 nM of malathion were obtained using PNTs, and 102 nM using PNTs and Nafion®.

Key words: Acetylcholinesterase, biosensor, peptide nanotubes, disposable, inhibition

1. Introduction

Organophosphates (OPs) are acetyl cholinesterase (AChE) inhibitors with a broad range of potency and toxicity. They have been widely used in agriculture as pesticides and are also used as deadly nerve agents in chemical weapons.

Chromatography and spectrometry are the classical analytical chemistry methods used to measure OPs. Unfortunately, these methods require large, sensitive equipment, which in turn require personnel trained in their use and a laboratory environment, making the processing time consuming and expensive.

Biosensors offer new opportunities to develop a system that may be used to detect OPs in water. They are becoming increasingly popular for detection of specific target compounds in solutions [1]. Biosensors are used to measure glucose levels in blood for diabetes patients[2]. The food industry uses various types of biosensors to detect pathogens in food during processing [3]. AChE-based biosensors are emerging as an

extremely sensitive means of toxicity monitoring in environmental, food processing, and military applications [1,2].

Cholinesterases (ChE) are enzymes that hydrolyze the neurotransmitter acetylcholine (ACh), which transmits nerve impulses across cholinergic synapses. Both acetyl cholinesterase and butyryl cholinesterase (BuChE) have been used in biosensors. While they have similar structures, BuChE preferentially hydrolyzes butyryl choline. AchE preferentially hydrolyzes acetyl choline and esters of thiocholine. Many of these esters, especially acetyl thiocholine (ASCh), have been used as substrates for various AchE biosensors. ASCh is particularly useful because, once broken down by the enzyme, thiocholine can be oxidized by an electrode, generating a measurable signal. This oxidation of thiocholine normally occurs at 410 mV vs Ag/AgCl [1].

The electrode type and material and enzyme immobilization technique used during production of a biosensor are key to how it will function and perform [1,4]. Peptide nanotubes (PNTs) have recently become a useful tool in constructing nanoscale devices [4,5]. PNTs are biocompatible, very stable, and electrically conductive. The goal of this work was to demonstrate the use of PNTs and Nafion® to immobilize AchE on carbon screen printed electrodes for use as OP sensors, and determine how these materials affect the stability of the sensors.

2. Materials and Methods

2.1. Materials

AChE (Type V-S, from electric eel, 500 U/mg) and acetylthiocholine chloride were obtained from Sigma-Aldrich (St. Louis, MO) and stored at -10°C. The dipeptide

H-Phe-Phe-OH and Albumin from bovine serum (BSA) were also obtained from Sigma-Aldrich (St. Louis, MO) and stored at 4°C. 1,1,1,3,3,3-hexafluoro-2-propanol (HFP), (99.8% purity) was obtained from Sigma-Aldrich (Milwaukee, WI). Nafion® 117 solution (~5% in a mixture of lower aliphatic alcohols and water) was obtained from Sigma-Aldrich (Allentown, PA) and used as received. All other chemicals were of analytical grade and used as received. Deionized water was prepared through reverse osmosis.

Carbon screen printed electrodes with a 2 mm diameter carbon working electrode, a carbon counter electrode, and a Ag/AgCl reference electrode were purchased from Pine Research Instrumentation (Durham, NC).

2.2. Apparatus

All electrochemical experiments were conducted using a PARSTAT 2273 potentiostat connected to a notebook computer. Experiments were conducted in a Pine Research Instrumentation compact voltammetry cell.

2.3. Methods

PNTs were created by dissolving the dipeptide in the HFP at a concentration of 100mg/mL, then diluting 20µL of this solution in 1 mL of water. PNTs self-assembled under these conditions.

Three electrode types were tested. For the simplest, adsorbed type, 2 µL of 1000U/mL AChE solution were deposited on the working electrode surface and allowed to dry for 1 hour before use. For the second type, PNT modified electrodes, 2 µL of the prepared PNT solution were first deposited on the working electrode and allowed to dry for 2 hours, followed by 2 µL of 1000U/mL AChE solution. For the final type, Nafion®

modified electrodes, 2 μ L of the PNT solution, the AchE solution, 1% w/v BSA, and Nafion solution, were deposited and allowed to dry for 2 hours each.

To measure stability, cyclic voltammetry measurements were taken in a testing solution of 0.1M KCl, 0.1M phosphate buffer solution at pH 7 containing 1mmol ASCh. This solution was purged with N₂ for 20 minutes before use. Peak currents corresponding to the oxidation of thiocholine were measured for each sensor. The sensors were then stored in a pH 7, 0.1M phosphate buffer solution at 4°C for 50 days, and cyclic voltammetry measurements were taken every 10 days. The peak currents measured initially, i_o , and after storage, i_i , were used to calculate the enzyme activity degradation.

The detection ability of the sensor was measured using cyclic voltammetry and amperometry. These studies were conducted immediately after creation of the adsorption based sensors, and after 12 hours of storage for encapsulated sensors. Cyclic voltammetry measurements were taken in a 0.1M KCl, 0.1M phosphate buffer solution at pH 7 with 1mmol ASCh and the peak oxidation currents of thiocholine were measured for each sensor. The sensors were rinsed for 1 minute with a 0.1M phosphate buffer solution. The sensors were then exposed to various concentrations of malathion for 10 minutes, and rinsed with phosphate buffer again. Cyclic voltammetry measurements were taken again in the same manner as before exposure. The peak currents before, i_o , and after, i_i , are used to calculate the inhibition percent using $I\% = (1 - i_i/i_o) \times 100\%$. The limit of detection is estimated as the concentration which causes 20% inhibition.

3. Results and Discussion

Figure 1 shows cyclic voltammograms (CVs) performed with bare electrodes and PNT encapsulated AChE electrodes in 0.1M KCl, 0.1M phosphate buffer solution in the presence of 1 mmol ASCh. A clear oxidation peak can be seen at ~ -0.3 V with the AChE electrode while the response of the bare electrode is minimal. The CV is measuring the oxidation of thiocholine, which is a hydrolysis product of the enzymatic reaction of ASCh with AChE. Thus, since the bare electrode does not have AChE, there is no oxidation peak.

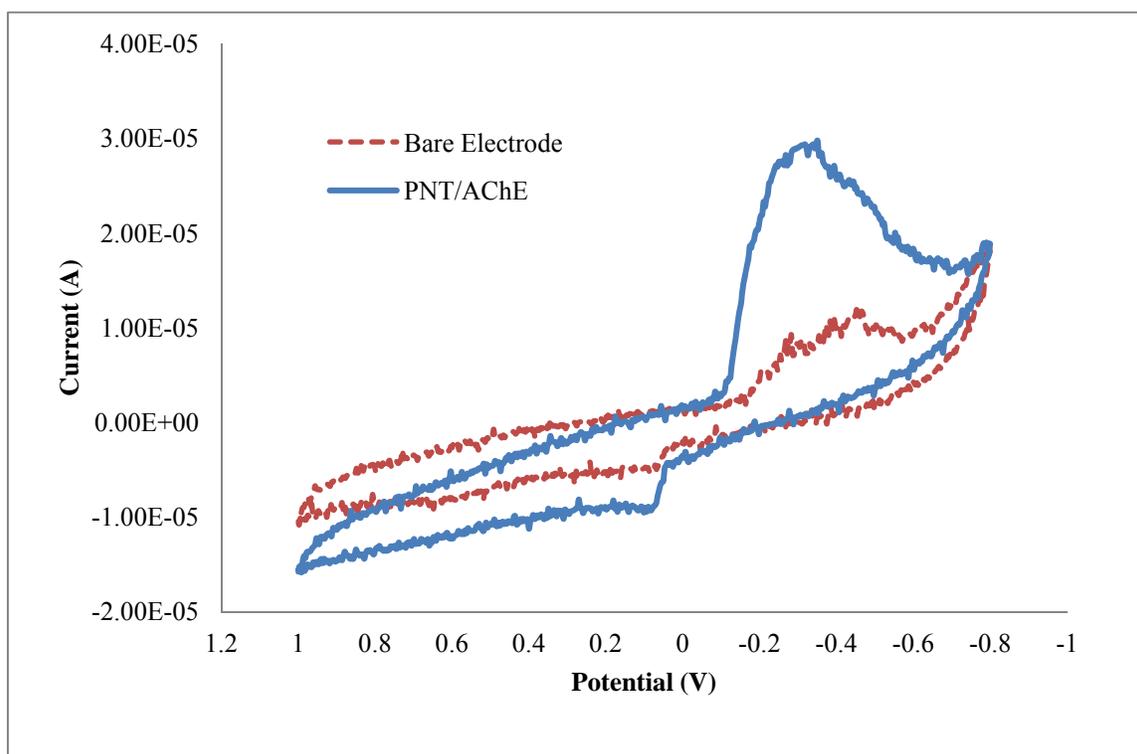


Fig. 1. Cyclic voltammogram of 1mM ASCh in 0.1M KCl, 0.1M phosphate buffer, pH 7 of bare carbon SPE and SPE modified with PNT and AChE. Scan rate: 50 mV/s. Initial scan direction: positive

Consecutive cycles of the adsorbed type electrode in the testing solution show that current peaks degrade for each cycle. This indicates that AChE leaches from the surface,

and the sensors are not operationally stable. After 10 days in storage at 4°C in a 0.1M phosphate buffer solution, pH 7, the adsorbed type electrode gives no response, showing that electrodes with unprotected AChE cannot be stored in this manner.

Figure 2 shows the degradation over 50 days of PNT-modified electrodes and PNT plus Nafion® modified electrodes. 10 electrodes of each type were used in this study. The PNT modified electrodes lost a mean of 17.3% of enzyme activity, with a standard deviation of 5.3% after 50 days. The PNT plus Nafion® modified electrodes lost a mean of 14.2%, with a standard deviation of 5.5%. While the PNT plus Nafion electrodes appear to degrade less, the results are not statistically significant.

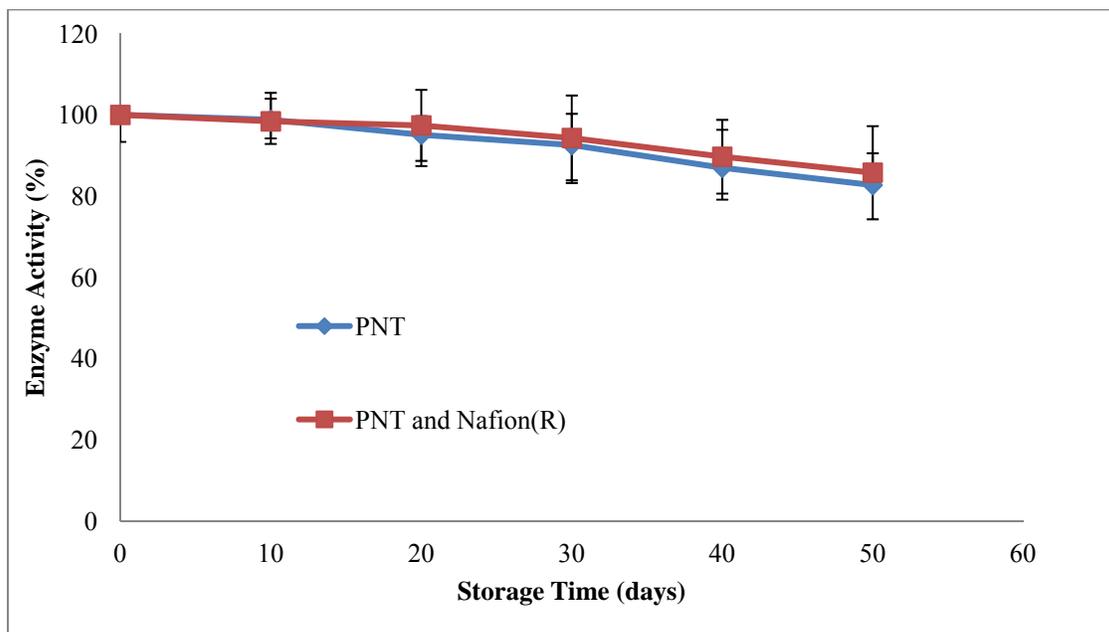


Fig. 2. Enzyme activity loss of PNT and PNT plus Nafion® sensors over time in storage. Experiments conducted in 1mM ASCh in 0.1M KCl, 0.1M phosphate buffer, pH 7.

Inhibition curves for AChE immobilized by PNT and PNT plus Nafion® by malathion are shown in Figure 3. Each of the reported values is the mean of responses of

3 electrodes. The detection limit at 20% inhibition is estimated to be 48 nM and 102 nM for PNT and PNT plus Nafion® electrodes, respectively.

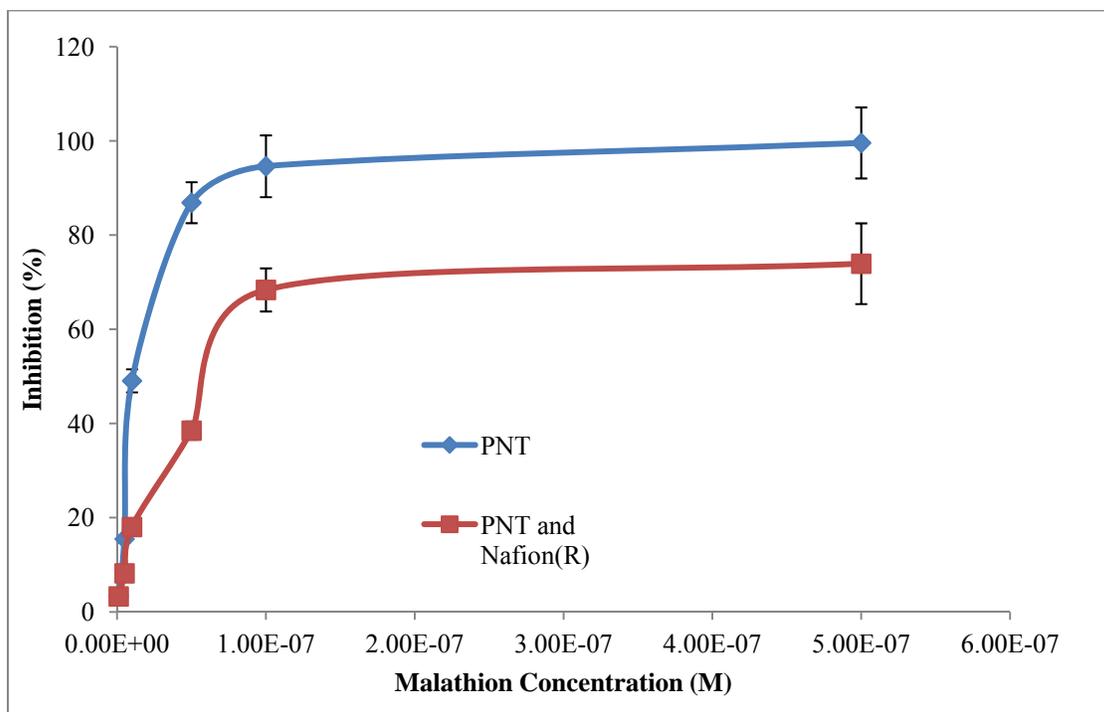


Fig 3. Inhibition curves for AChE immobilized by PNTs and PNTs plus Nafion® by malathion. Experiments conducted in 1mM ASCh in 0.1M KCl, 0.1M phosphate buffer, pH 7.

4. Conclusions

The use of peptide nanotubes to encapsulate AChE deposited on carbon screen printed electrodes greatly enhanced the stability of the enzyme both operationally and over a storage period. After immobilization with this technique, the enzyme maintained its function and showed a rapid response to ASCh. This response allowed for real-time monitoring for enzyme inhibitors at low concentrations. This work shows that the use of PNTs to encapsulate AChE creates a practically usable sensor that can be stored at 4°C without being under vacuum.

References:

- [1] S. Andreescu, J.Marty, *Biomolecular Engineering* 23 (2006) 1–15
- [2] M. Tudorache, C. Bala, *Anal Bioanal Chem* 388 (2007) 565–578
- [3] A. Amine, H. Mohammadi, I. Bourais, G. Palleschi, *Biosensors and Bioelectronics* 21 (2006) 1405–1423
- [4] B.Park, D. Yoon, D. Kim, *Biosensors and Bioelectronics* 26 (2010) 1–10
- [5] S. Scanlon, *Nanotoday* 3 (2008) 22-30

IV. Conclusion

Additional Results and Discussion

Cyclic voltammograms were also performed with electrodes which were then stored at ambient temperature in a 0.1M phosphate buffer solution, pH 7, to study the stability of sensors stored in this way. However, no sensors produced a signal after 10 days of storage.

Amperometric detection of malathion was conducted by first injecting 100 μL of 121 mM ASCh into the electrochemical cell, which contained 12 mL of 0.1 M phosphate buffer, pH 7. After the current from thiocholine oxidation stabilized, malathion was injected into the cell, resulting in a decrease in the oxidation current. Inhibition percentages were calculated using the currents before, i_o , and after i_i , malathion injection using $I\% = (1 - i_i/i_o) \times 100\%$, but no clear correlation between concentration and inhibition calculated in this manner could be found.

Significance of Research

As the US military finds itself in more asymmetric warfare situations, the need for the ability to detect nerve agents becomes more pressing. While several types of biosensors have shown the ability to do so accurately and quickly in a lab setting, they can be very fragile and unstable. These qualities limit their utility and practicality in military field operations.

This research has shown a technique to make AChE biosensors more robust and stable. It has demonstrated a sensor that can be stored in a simple refrigerator and retains adequate enzyme activity to perform detection functions. While it is not a fully functional detector that can be used in the field, it is a step toward that goal.

Future Research

While this research studied the stability of biosensors over time in storage, there are several other factors that could influence stability that were not studied. The pH, salt content, and temperature of the sample could all potentially affect the sensors' performance, and offer opportunities for future research

Amperometric studies were also conducted which showed, qualitatively, the response of the biosensors to substrate and inhibitor injections. Further research into quantifying and modeling these responses would offer insight into the potential for these types of biosensors to distinguish between types of inhibitors and concentrations.

Applying this technology to produce a stable biosensor to detect OPs in the gas phase is another area for future research. Such a sensor would provide a significant benefit to the military.

Bibliography

- Amine, Aziz, Hasna Mohammadi, Ilhame Bourais, and Giuseppe Palleschi. "Enzyme inhibition-based biosensors for food safety and environmental monitoring," *Biosensors and Bioelectronics*, 21: 1405–1423 (2006).
- Andreescu, Silvana, Thierry Noguer, Vasile Magearu, and Jean-Louis Marty. "Screen-printed electrode based on AChE for the detection of pesticides in presence of organic solvents," *Talanta*, 57: 169–176 (2002) .
- Andreescu, Silvana, and Jean-Louis Marty. "Twenty years research in cholinesterase biosensors: From basic research to practical applications," *Biomolecular Engineering*, 23: 1–15(2006).
- Bonnet, Christine, Silvana Andreescu, and Jean-Louis Marty. "Adsorption: an easy and efficient immobilisation of acetylcholinesterase on screen-printed electrodes," *Analytica Chimica Acta*, 481: 209–211 (2003).
- Department of the Air Force. *Operations in a Chemical, Biological, Radiological, Nuclear, and High-Yield Explosive (CBRNE) Environment*. AFM 10-2503, p52. Washington: HQ USAF, 7 July 2011.
- Lin, Yuehe, Fang Lu, and Joseph Wang. "Disposable Carbon Nanotube Modified Screen-Printed Biosensor for Amperometric Detection of Organophosphorus Pesticides and Nerve Agents," *Electroanalysis* 16, Nos. 1-2: 145-149 (2004).
- Norouzi, Parviz, Farnoush Faridbod, Bagher Larijani, and Mohammad R. Ganjali. "Glucose Biosensor Based on MWCNTs-Gold Nanoparticles in a Nafion Film on the Glassy Carbon Electrode Using Flow Injection FFT Continuous Cyclic Voltammetry," *International Journal of Electrochemical Science*, 5: 1213-1224 (2010).
- Nunes, Gilvanda S., Gérard Jeanty, and Jean-Louis Marty. "Enzyme immobilization procedures on screen-printed electrodes used for the detection of anticholinesterase pesticides Comparative study," *Analytica Chimica Acta*, 523: 107–115 (2004).
- Park, Byung-Wook, Do-Young Yoon, and Dong-Shik Kim. "Recent progress in biosensing techniques with encapsulated enzymes," *Biosensors and Bioelectronics*, 26: 1–10 (2010).
- Pohanka, Miroslav, Petr Dobes, Lucie Drtinova, and Kamil Kuc̆a. "Nerve Agents Assay Using Cholinesterase Based Biosensor," *Electroanalysis*, 21, No. 10: 1177 – 1182 (2009).

- Ren, Juan, Wentao Shi, Kai Li, and Zhanfang Ma. "Ultrasensitive platinum nanocubes enhanced amperometric glucose biosensor based on chitosan and nafion film," *Sensors and Actuators B: Chemical*, 163: 115–120 (2012).
- Sassolas, Audrey, Loïc J. Blum, and Béatrice D. Leca-Bouvier, "Immobilization strategies to develop enzymatic biosensors," *Biotechnology Advances*, 30: 489-511 (2012).
- Scanlon, Shane, and Amalia Aggeli. "Self-assembling peptide nanotubes," *Nanotoday*, 3, No.3-4: 22-30 (June-August 2008).
- Shulga, Olga, and Jon R. Kirchhoff. "An acetylcholinesterase enzyme electrode stabilized by an electrodeposited gold nanoparticle layer," *Electrochemistry Communications*, 9: 935–940 (2007).
- Tudorache, Madalina , and Camelia Bala. "Biosensors based on screen-printing technology, and their applications in environmental and food analysis," *Analytical and Bioanalytical Chemistry*, 388:565–578 (2007).

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14. ABSTRACT A biosensor for the detection of organophosphates in water was created by immobilizing acetylcholinesterase (AChE) on a carbon screen printed electrode using peptide nanotubes (PNT) and Nafion®. This sensor was used with acetylthiocholine (ASCh) substrate to detect the inhibitory effects of malathion. The stability of the encapsulated enzyme was measured over 50 days of storage at 4°C in a phosphate buffer solution. The oxidation of thiocholine, a product of the enzymatic reaction of ASCh and AChE, was used to measure the activity of the encapsulated enzyme. Cyclic voltammograms were taken in an ASCh and phosphate buffer solution, and the peak oxidation was used to measure the performance of the sensor. Amperometric studies were also conducted at 310 mV vs Ag/AgCl to measure the response of the sensors to ASCh and malation. This research demonstrates that the use of PNTs and Nafion® allows the sensor to remain stable under these conditions for a much longer period than similar biosensors relying on adsorption alone, losing 17.3% and 14.2%, activity, respectively, after 50 days. Detection limits of as low as 48 nM of malation were obtained using PNT, and 102 nM using PNT and Nafion®.					
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