CO-ELECTRODEPOSITION OF GOLD NANOPARTICLE AND REDUCED-GRAPHENE OXIDE IN APPLICATION OF ACHETYLCHOLINESTERASE BIOSENSOR

Mr. Chiranthanin Mahayotheecharak

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อิเล็กโตรเดพอซิชันร่วมของอนุภาคนาโนทองและรีดิวซ์กราฟีนออกไซด์สำหรับการประยุกต์ใช้ในไบโอเซนเซอร์ชนิดอะซีติลโคลีนเอสเทอเรส

นายจิรันธนิน มหาโยธีจารักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรมหาบัณฑิต
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Thesis Title	CO-ELECTRODEPOSITION OF GOLD NANOPARTICLE AND REDUCED-GRAPHENE OXIDE IN APPLICATION OF ACHETYLCHOLINESTERASE BIOSENSOR
By	Mr. Chiranthanin Mahayotheecharak
Field of Study	Chemical Engineering
Thesis Advisor	Professor MUENDUEN PHISALAPHONG, Ph.D.
Thesis Co Advisor	Assistant Professor Lerdluck Kaewvimol, Ph.D.

Accepted by the FACULTY OF ENGINEERING, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Engineering

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.....
Thesis Advisor
(Professor MUENDUEN PHISALAPHONG, Ph.D.)

.....
Thesis Co-Advisor
(Assistant Professor Lerdluck Kaewvimol, Ph.D.)

.....
Examiner
(Associate Professor KASIDIT NOOTONG, Ph.D.)

.....
External Examiner
(Chanchana Thanachayanont, Ph.D.)

จิรันธนิน มหาโยธีจารักษ์ : อิเล็กโตรเดพอซิชันร่วมของอนุภาคนาโนทองและรีดิวซ์กราฟีนออกไซด์สำหรับการประยุกต์ใช้ในไบโอเซนเซอร์ชนิดอะซีติลโคลีนเอสเทอเรส. (CO-ELECTRODEPOSITION OF GOLD NANOPARTICLE AND REDUCED-GRAPHENE OXIDE IN APPLICATION OF ACHETYLCHOLINESTERASE BIOSENSOR) อ.ที่ปรึกษาหลัก : ศ. ดร.เหมือนเดือน พิศาลพงศ์, อ.ที่ปรึกษาร่วม : ผศ.เลิศลักษณ์ แก้ววิมล

เทคนิคการตรวจวัดสารปนเปื้อนเช่นยาฆ่าแมลง มีความเป็นที่ต้องการเนื่องจากประเทศไทยเป็นประเทศที่ใช้ยาฆ่าแมลงอย่างแพร่หลาย เทคนิคที่กำลังได้รับความสนใจในปัจจุบันคือเทคนิค ไบโอเซ็ฯเซอร์ชนิดอิเล็กโตรเคม เทคนิคนี้มีความสามารถที่จะตรวจวัดการปนเปื้อนอย่างรวดเร็ว และได้ผลเป็นตัวเลขที่เข้าใจได้ง่าย ไม่เหมือนกับเทคนิคอื่นๆที่พึ่งการเปลี่ยนสี ด้วยความที่กเป็นเทคนิคที่ใช้ง่ายมีราคาไม่แพงทำให้มีความเป็นไปได้ในการนำไปใช้ในพื้นที่จริง ไม่ต้องพึ่งบุคลากรที่ถูกฝึกฝนเป็นพิเศษ ในงานนี้ สกรีนปริ้นท์อิเล็กโทรดจะถูกปรับสภาพพื้นผิวด้วยวัสดุอื่น คือ รีดิวซ์กราฟีนออกไซด์และอนุภาคนาโนทองเพื่อเพิ่มประสิทธิภาพของ อิเล็กโทรดที่ทำจากคาร์บอน และนำไปสร้างเป็นไบโอเซ็นเซอร์ชนิดอะซีติลโคลีนเอสเทอเรส เมื่อน้ำไปวัดยาฆ่าแมลงชนิดคลอไพริฟอส มีความไวต่อการตอบสนองจาก 0.016mg/mL ถึง 1mg/mL และ 1mg/mL ถึง 25 mg/mL และค่าความเข้มข้นต่ำสุดที่วัดได้คือ 0.3503 mg/mL

สาขาวิชา	วิศวกรรมเคมี	ลายมือชื่อนิสิต .....
ปีการศึกษา	2562	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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Chiranthanin Mahayotheecharak : CO-ELECTRODEPOSITION OF GOLD NANOPARTICLE AND REDUCED-GRAPHENE OXIDE IN APPLICATION OF ACHETYLCHOLINESTERASE BIOSENSOR. Advisor: Prof. MUENDUEN PHISALAPHONG, Ph.D. Co-advisor: Asst. Prof. Lerdluck Kaewvimol, Ph.D.

A rapid pesticide detection technique is highly desirable due to the widespread usage of many pesticides in the agricultural industry. An emerging technique that shows promising potential is an electrochemical biosensor. The technique allows sensitive and rapid quantification of an analyte. Due to its simplicity, it could be a potential alternative to traditional method. In this work, we utilised a composite materials consist of partially reduced graphene oxide (rGO) and gold nanoparticle (AuNPs) to fabricate a disposable AChE biosensor on a Screen-Print Carbon electrode. The composite rGO and AuNPs possess high electron conductivity while AuNPs also exhibit desirable biocompatibility. The fabricated biosensor was used to detect an organophosphate pesticide called Chlorpyrifos. The observed linear ranges of the fabricated biosensor was 0.016mg/mL to 1mg/mL and 1mg/mL to 25 mg/mL. The limit of detection was 0.3503 mg/mL. The AChE biosensor was sucessfully fabricated with rGO/AuNPs as support materials.

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Chiranthanin Mahayotheecharak

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Chapter 1: Introduction

1.1 Motivation

In recent decades, a rapid technique has been developed as a preliminary procedure to measure trace amount of pesticide (Ivanov, Evtugyn et al. 2000). In Thailand, as pesticides usage is widely spread in every possible agricultural region, pesticides exposure affected many farmers and farm workers (Tawatsin et al, 2015). The organophosphate and carbamate pesticides could be detected with a tool called biosensor. The biosensor works by utilization of Acetylcholinesterase (AChE) with which pesticides form irreversible complex. This tool could detect pesticide in shorter amount of time while the traditional technique of detection requires lengthy amount of time and much more sophisticated equipment such as Gas Chromatography (Albero et al, 2016) and HPLC (Fu et al, 2009). The usage of biosensor could reduce the amount of labour necessary to screen for pesticide contamination. Although the traditional method is more precise and accurate, a more rapid technique is highly desired for in-field preliminary detection.

The enzyme AChE is traditionally immobilized onto an electrode surface in a biosensor while the surface is often modified with performance enhancing materials to improve sensitivity and biocompatibility. Gold nanostructure has been utilized as an immobilization platform for enzyme due to excellent biocompatibility (Orlando et al, 2016). The usage of Gold nanoparticle has demonstrated improved sensitivity and stability of the recognizing element (Deng et al, 2016). Gold nanoparticles possess physical characteristics that could potentially solve many limitations of the biosensor.

Biochemical Engineering Lab, Chulalongkorn University has been extensively investigating potential raw materials that could pose real benefits to the application of enzymatic biosensor. A member of the group has studied the use of mesocellular foam silica support and AuNPs in Glucose biosensor. The results showed that mesocellular foam silica with AuNPs has a significant increase in enzyme loading in comparison to the mesocellular foam silica without AuNPs coverage. It was also discovered that the presence of AuNPs had positive impact on the oxidation peak shown in cyclic voltammetry. The generated current in chronoamperometry is also affected by the presence of AuNPs due to an increase in enzyme loading. We currently are interested in the influence that AuNPs provides to the biosensor matrix.

Reduced graphene oxide is also an attractive candidate in the field of biosensing (Li et al 2013). It is a cost-effective choice of materials while possessing characteristics similar to that of graphene. Pristine graphene contains no functional groups in their chemical structure however graphene oxide has plenty of functional groups. rGO is considered more suitable due to the nature of the chosen enzyme immobilization technique which utilize glutaraldehyde as a crosslinker.

In this work, the objectives are to reduce the detection time in comparison to previous clay-based iteration of the AChE biosensor and investigate the performance of biosensor in the presence of gold nanoparticle and reduced-graphene oxide. The gold nanoparticles will be electrochemically co-deposited with reduced graphene oxide onto a screen-printed carbon electrode. The enzyme AChE will be immobilized via a covalent interaction with a crosslinker called glutaraldehyde.

1.2 Objectives of this work

To fabricate and evaluate the performance of AChE biosensor based on co-electrodeposition of gold nanoparticle and reduced-graphene oxide for pesticide detection.

1.3 The Scope of Work

Electrode Modification

1.3.1 Investigation of co-electrodeposition process

- Vary [$HAuCl_4$] (0.625 mM to 1 mM)
- Vary Electrodeposition cycles (1 cycles to 5 cycles)

Characterization

- Electrochemical : Cyclic Voltammetry with 0.1 M KCl +5 mM $[Fe(CN)_6]^{3-/4-}$
- Imaging : Scanning Electron Microscopy

1.3.2 Investigation of Enzyme Immobilization

- Vary [AChE] (15U/mL to 90U/mL)

Characterization

- Cyclic Voltammetry in presence of substrate (acetylthiocholine)

1.3.3 Evaluation of Biosensor for Pesticide detection

- LOD, Linear range for Standard, response time, storage stability

Chapter 2 : Theoretical Framework

2.1 Biosensor

Biosensor is a tool used to detect trace amounts of analytes with a receptor as bio-recognizing element. Generally, Biosensors are composed of 3 parts; a receptor element, a transducer interface and signal detector.

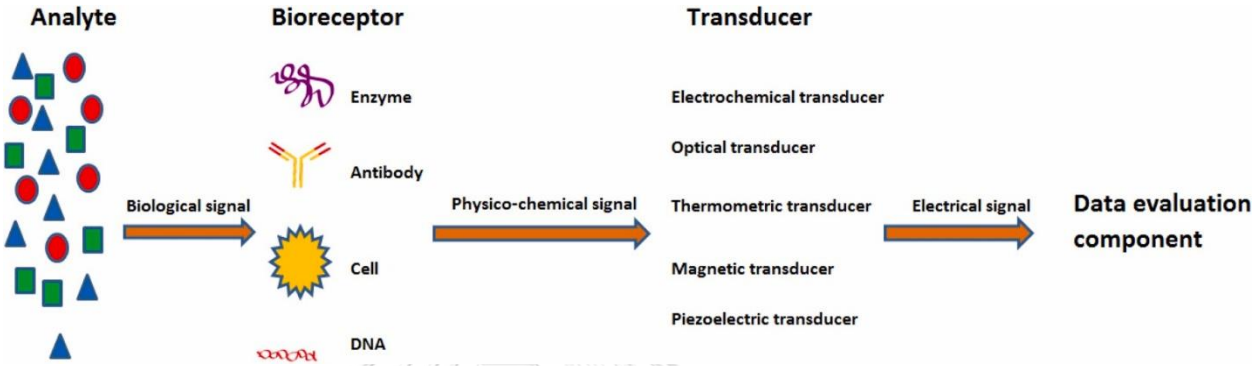


Image 1. Biosensor Schematic (Sabu et al, 2019)

Bioreceptors such as Enzyme, Antibody, Cell and DNA have high selectivity to targets. They are also capable of producing a signal which could be ions, electrons, heat, gases or mass transfer that could be interpreted into electrical signal.

The detector translates the changes that occur when the analyte interact with the receptors on the sensor into an electrical signal. The signal is proportional to the amount of the changes in the system. The signaling is then transcribed into a digital signal which is easy to understand and readily used.

2.1.1 The component of Amperometric Biosensor

There are 3 electrodes in a chemical cell of an amperometric sensor.

1.) Working Electrode

The working electrode is part of the transducer layer where electrical signal is detected. It is sensitive to changes due to the reactions in the chemical cells. Working

electrode should have high capability to conduct electrons. It should possess certain resilient to chemical degradation to retain the best performance of the biosensor. The electrode is often made out of noble materials such as platinum, gold or silver.

2.) Reference Electrode

The reference electrode is an electrode with well characterized electrical potential where the potential stays constant throughout the operation. The method to keep the potential fixed is by employing a system of redox reactions. The reference electrode is used in a half cell (reference cell) to determine the potential of the other half cell (working cell). The electrode is often made with silver.

3.) Counter Electrode

The counter electrode functions as an anode or a cathode opposite that of the reaction in the working half cell. Traditionally, it is made with a conductive material and has high surface ratio to make sure that the reaction speed is affecting the reaction in the working half cell.

2.1.2 Mechanism of the Electrochemical Biosensor

The biosensor relies on 2 components to measure the trace amounts of target analytes; they are bioreceptors and transducer. The analytes are detected by a highly selective bioreceptors; an enzyme is designed to detect a very specific analytes and often bind to one domain of targets only. Once the target is bound, the biosensor relies of a series of chemical reactions to detect the target. The reactions either release or consume electrons which is monitored by the transducer layer. The electron change is proportional to the concentration of the analyte present in the biosensor.

A generalized model could be found on Figure 2. The first step is often the binding of the target molecule to the bioreceptor. The target is then converted into a product by the bioreceptor, an enzyme. This reaction is traditionally a reduction-oxidation reaction where the target is oxidized by the enzyme. The enzyme becomes reduced and ready to transfer electrons to the electron mediator. The electron passes through the mediator to the electrode surface causes electrical response in the detectors. The electrical responses are often continuous because the electron transfer is proportional to the concentration of the target analytes in the chemical cell.

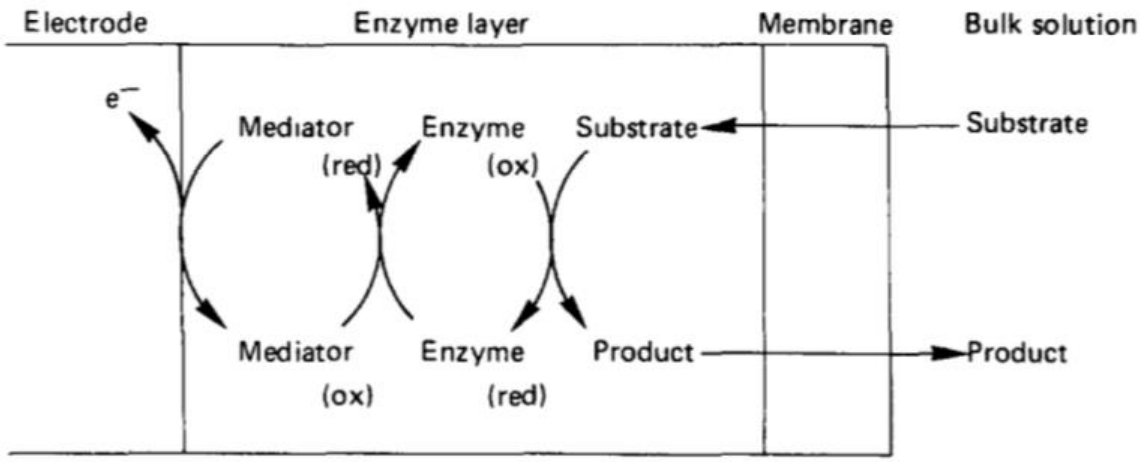


Fig 1. Schematic representation of electrode surface reaction (Davis, 1984)

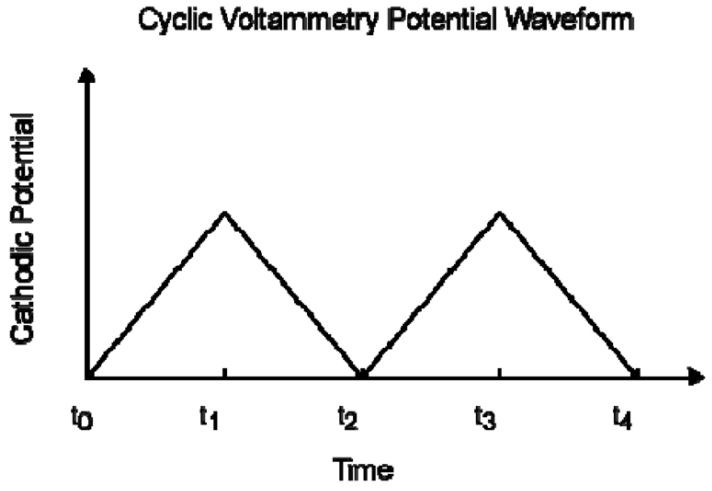
2.1.3 Electrochemical Measurements

Voltammetry is a technique that relies on potential differences in the system. A potential is applied to the cell, this changes the potential of the electrode, perturbing the systems on the surface of the electrode. The molecules present on the surface of the electrode respond to this potential difference and reactions occur. The reactions often designed to generate electrons which is measured by the detector.

2.1.3.1 Cyclic Voltammetry (CV)

Cyclic Voltammetry is a technique that is very important in the field of Electrochemistry. The technique gives insights into the behaviors of the reactions where the observed peaks correspond to a specific step in the reactions.

CV applies a certain voltage to the chemical cell in a repeating cycle. Then the measurement is taken to record the generated current from the reactions. The potential of the working electrode will be compared to the reference electrode. When a voltage is applied in a constant scan rate, it is considered forward scan while vice versa is considered backward scan. The forward and backward scan have identical scan rate but the voltage is the opposite pole. Both scans together are measured in a closed



cycle, hence the name.

Figure 2. Voltammetry Potential Waveform(Yang, 2009)

2.1.3.2 Cyclic Voltammogram

When the generated current is plotted against applied potential, the graph is called Cyclic Voltammogram. The anodic peak is traditionally used to call the highest applied voltage point (E_{pa}). The cathodic peak is called the lowest applied voltage point (E_{pc}). The height of the peak however corresponds to the current generated by the system. The anodic current (i_{pa}) is the current generated when anodic voltage is

applied and vice versa is the cathodic current (i_{pc}). The generated current is dependent on the concentration of the target analytes introduced to the system.

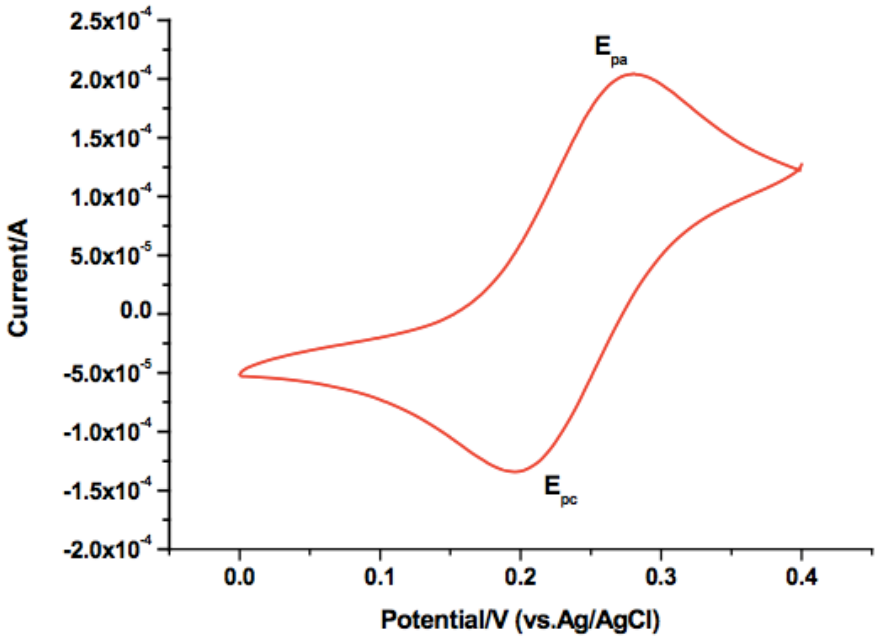


Figure 3. An Example of Cyclic Voltammogram(Yang, 2009)

The most significant features of this particular figure are the peak potential (E_{pa} and E_{pc}) and the peak currents (I_{pa} and I_{pc}), they are used for characterization of the modified electrode surface.

2.1.4 Parameters that affect the performance of the electrochemical technique

There are a few parameters that are used to describe electrochemical systems.

2.1.4.1 The linearity

When a relationship between the electrical signal and the concentration of analyte is established, the linearity of the calibration curve describes the reliability of the measurement. An accurate detection is only made when the calibration curve is well established.

2.1.4.2 Limit of Detection (LOD)

The limit of detection represents the lowest concentration that could be detected by the biosensor.

2.1.4.3 Sensitivity

The sensitivity of a sensor refers to how accurate it is when the target analyte is introduced to the sensor. The sensitivity shows the changes in the target analyte and how the electrical signal is detected as a result of said change. The slope of an amperometric graph tells how much of the analyte is being detected. The detection is described as sensitive when the slope of the amperometric graph is large.

2.1.4.4 Selectivity

The selectivity of a biosensor systems refers to the capability to distinguish target molecules from interferences in the bulk solution. Often time when a measurement is made, the sample includes many undesirable interferences. The biosensor traditionally has a specific range of detection where it is very good at detection and out of this range, the electrical signal could not be trusted because the signal could be generated because of interferons not the target molecules.

2.1.4.5 Reproducibility

Reproducibility refers to how close are the results from different batch of measurements from the instrument. In the same experimental setting, a measurement made one after another could potentially be different. The conditions of experiments are often designed to minimize these differences.

2.1.4.6 Response time

The response time to describe how long it takes for a perturbed system to return to equilibrium or steady state. Different biosensors often have different response time.

2.2 Acetylcholinesterase Biosensor

The Acetylcholinesterase biosensor was developed to detect organophosphate and carbamate pesticides. The biosensor relies on an indirect method to detect the pesticides. In general, direct detection describes a process where the binding of analyte to receptor produces electrical signaling.

2.2.1 Reactions that govern the detection process

AChE Biosensors work on the premises that pesticides (target) selectively bind to the enzyme AChE and deactivate it. At first, the biosensor generates an electrical signal with a substrate called acetylthiocholine relying on a series of reactions.

Reaction 1:

$$\textbf{Acetylthiocholine chloride} + H_2O \xrightarrow{AChE} \textbf{Thiocholine} + \textbf{Acetic acid}$$

Reaction 2:

$$\textbf{2 Thiocholine}_{(red)} \xrightarrow{Voltage} \textbf{Thiocholine}_{(ox)} + 2H^+ + 2e^-$$

The enzymes first cleave acetylthiocholine into choline and acetate. The produced thiocholine is then oxidized electrochemically via the electrode potential difference. When the target molecule is introduced to the sensor, it interrupts the first reaction. Instead of binding to Acetylthiocholine, AChE binds irreversibly to the pesticides and less current is generated.

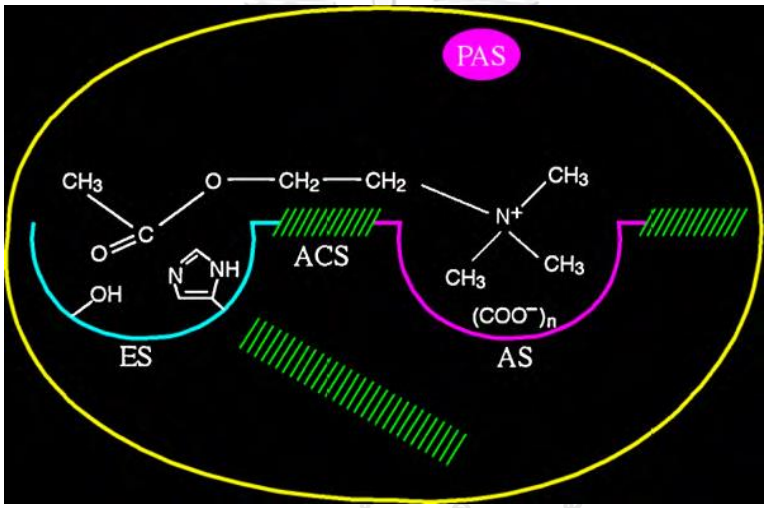
The inhibition procedure is traditionally calculated as explained below:

$$\text{Inhibition}(\%) = 100\% \times \frac{\text{ip,control} - \text{ip,experimental}}{\text{ip,control}}$$

Where ip, control stands for peak currents obtained without pesticide inhibition and ip, experimental is for currents with pesticide inhibition.

2.2.2 Acetylcholinesterase

Acetylcholinesterase is present in the neuromuscular junctions. Its primary function is hydrolysis of choline esters first described in 1914



(Rosenberry, 1975).

Figure 4. Active site where acetylthiocholine binds to AChE showing anionic site and esteratic site(Dvir et al, 2010).

The enzyme possesses very high specific activity which is remarkably close to a diffusion controlled reaction (Sussman et al, 1987). This means that the reaction rate is so fast that the limiting step in the reaction is almost entirely the diffusion rate of the substrate. The termination of nervous transmission relies on the hydrolysis of acetylcholine. When exposed to pesticides, they bind irreversibly to the active sites

causing extreme toxicity. The inability to efficiently break down crucial substrate such as acetylcholine can lead to dramatic effects.

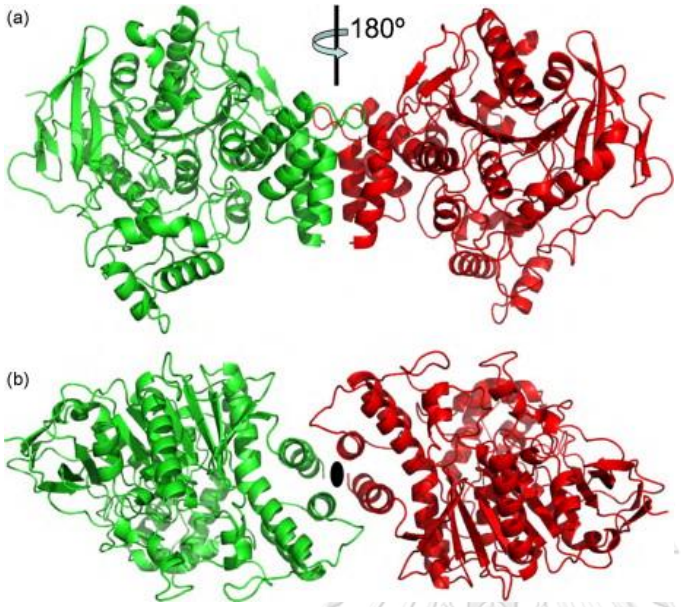


Figure 5. Cartoon representation of AChE dimer connected via 4 alpha-helices(Dvir et al, 2010).

2.3 Gold nanoparticle

Gold Nanoparticle has been the center of attention for the past decade, they possess special characteristics that bulk materials do not. These characteristics depend heavily on the synthesis and shapes of the AuNPs. Spherical AuNPs possess specific colour at specific core size in the range of 100 nm to 2 nm and show absorption peak at around 500 nm (Jain et al, 2006). AuNPs is found to be highly biocompatible, has high surface-volume ratio and is not toxic (Murphy et al, 2008).

Gold has an intriguing appeal to many in the biosensing fields possessing many characteristics that have potential in the biosensor application. As metal, its presence can improve electrical conductivity compared to its carbon-based counterparts. It has high specific surface area and also exhibit biocompatibility which both are desirable in the construction of a robust Acetylcholinesterase biosensor

(Orlando et al, 2016). Gold-base electrode has a weakness which is limited enzyme immobilization method. Gold nanoparticle could potentially reduce the cost of operation from utilization of gold and still provide the biosensor system with the desired characteristics. When used in conjunction with other polymers where enzymes could be more readily immobilized could present great advantage to the fabrication of AChE biosensor. These are current trends that researchers have tried to utilise gold and other materials to create a biosensor.

2.3.1 Surface Chemistry of AuNPs

The surface chemistry of AuNPs is widely studied and better understood than other nanoparticles. Negatively charged proteins and DNA could be attracted to the surface of a citrate-capped AuNPs because of the existing positively charged Au^{+} ions (Delong et al, 2012). Small organic ligands could be easily linked to AuNPs through Thiol or N-containing linker reagent (Pensa et al, 2012). There are reports of successful manipulations of chemical functional groups on AuNPs (Delong et al, 2012). The charges on the surface could be made positive, negative, mixed, or neutral. Thiolates describe the linkage of Thiol group to a metal surface through Au-S bonding. This bond has been utilized as one of the approaches of immobilizing enzyme molecules to the surface of AuNPs.

2.4 Reduced Graphene Oxide

Graphene oxide is not a naturally occurring compound. It was first discovered 150 years ago. The synthesis of GO happened when an attempt to treat graphite with a mixture of potassium chlorate and nitric acid by an oxford chemist Benjamin C. Brodie in 1859. In 1957, Hummers and Offeman developed a more efficient process while also being safer and faster. The investigation into GO spreads wide to study its

structure which elucidate its functionalized carbon structures including hydroxyl, epoxy and partially hydroxyl, carboxyl, ketone, and ester at the edge of the sheet.

2.4.1 Hummer's Method to Synthesize Graphene Oxide (Alam et al, 2017)

Hummers and Offeman utilized concentrated sulfuric acid, sodium nitrate and potassium permanganate while keeping the temperature lower than 40 degrees Celsius. The process takes around 2 hours and the resulting product is highly oxidized. Also It was discovered that Hummer's product has the oxidation only on the exterior while the interior of the bulk graphite is not oxidized into graphene oxide. There have been many modifications made to the original Hummers method and it is still currently the most common way to synthesize GO. This method produces GO with the thickness of 1 nm and the width of 1 micron. The reported chemical structure is C:O:H = 4:2.95:2.5 however the separation process and the purification are still time-consuming.

2.5 Electrodeposition

Electrodeposition or electroplating is a process where metal particle is decorated onto an electrode surface via application of reducing voltage in a galvanic cell. In general, an electrode surface is inserted into a solution which has the desired metal species in the form of an electrolyte. The metal species is generally positively charged and attracted to the cathode which is negatively charged. The cathode is the designated electrode where the metal species is deposited while the anode is generally inert. Gold electrodeposition relies on Au precursor solution which contains ionic form of Gold. The potentiostat applies reducing voltage to drive the reaction :

Au^{3+}(aqueous) + $3e^{3-}$ reducing potential> Au(solid). The purpose of electrodeposition is to coat a surface with metal species to improve the performance of the electrode surface or to prevent surface corrosion.

Chapter 3 : Literature Review

3.1 Materials for application in biosensor

Nanomaterial has been a topic of attention for the past decade owing to the fact that they possess special characteristics that bulk materials do not have. Both organic and inorganic materials have been utilised to modify electrode in attempts to improve the performance of a biosensor. There are advantages to both sources as inorganic materials could be carefully selected to improve electron conductivity and organic materials could be selected to improve stability of the bio-recognizing element such as enzymes. Different materials are often chosen to eliminate limitations that current biosensors possess.

3.1.1 Carbon nanotube-Based Electrochemical Enzyme Biosensor

A study was conducted to investigate the performance of an AChE biosensor fabricated from Multiwalled Carbon Nanotubes and AChE liposome bioreactor (ALB) (Yan et al, 2013). GCE was coated with layers of materials alternating between MWCNTs, Chitosan, and final layer being ALB. It was found that at 6 layers, the performance of the biosensor was optimized. With dichlorvos as a model pesticide, the detection limit was found to be 0.68 ± 0.076lg/L with 2 linear ranges of 0.25 to 1.75 μM and 2.00 to 10.00 μM. After 30 days, 100% of initial activity was retained. Carbon nanotubes are small and robust. They possess good mechanical strength, great electrical conductivity, and chemical stability. Also, there is space cavity is available for enzyme entrapment. These features render CNTs an attractive supporting candidate.

3.1.2 Polymer-based Electrochemical Enzyme Biosensor

Silk fibroin (SF) was also used to make an AChE biosensor for detecting pesticide (Xue et al 2012). A AChE-SF/MWNTs/GCE was used to detect methyl parathion and has linear range of 3.5×10^{-6} to 2.0×10^{-3} M with a detection limit of $5.0 \times 10{-}7$ M. Also, the linear range of carbaryl was between 1.0×10^{-7} to 3.0×10^{-5} M with a detection limit of $6.0 \times 10{-}8$ M. The electrode retained 80% of activity after 30 days. Silk fibroin is biopolymer with high biocompatibility while also possessing great mechanical property and it is a non-toxic material which is desirable for enzyme immobilization support.

3.1.3 Graphene-based Electrochemical Enzyme Biosensor

CdS-graphene composite was used to construct an enzymatic biosensor with acetylcholinesterase (Wang et al 2010). The group found that the composite outperform electrode with solely CdS nanocrystal. The electrode has good stability, retaining 8% activity after 20 days. The detection limit for carbaryl was 0.7 ng/mL with a linear range of 2 ng/mL to 2 μg/mL.

Porous Reduced Graphene Oxide was used with Chitosan as an immobilization matrix for AChE biosensor (Li et al 2013). Porous rGO was used due to high specific surface and low mass transfer resistance and Chitosan for biocompatibility for biomolecules. The presence of pRGO in the electrode reduce electron transfer resistance in the fabricated electrode compared to bare GCE and CS/GCE. The biosensor has a detection limit of 0.5 ng/mL for carbaryl with a linear response range of 0.001 to 0.05 μg/mL. It also retained 83% of enzyme activity after 30 days.

Graphene and Graphene oxide possess appealing features such as high conductivity and high surface area. They are very cost-effective in production process. Although chemical properties of graphene oxide (GO) are inferior to pristine graphene, the defects in GO are actually beneficial due to the presence of functional groups. These defects increase the hydrophilicity of GO. This means that GO could be dispersed in water with sonification which render its manipulation easier.

There are obvious advantages of certain categories of materials over other kinds in various aspects. Biopolymers are used due to compatibility with bio-recognizing element when longevity is desired. A metal composite would be a candidate to improve sensitivity or conductivity over a less electroactive materials. Also certain materials with special properties in a nanostructure are also employed for that specific characteristics. For example, materials with high surface-to-volume ratio such as graphene oxide and gold nanoparticle.

3.2 The influence of Electrode surface modification

3.2.1 Electrode Surface modification with Drop-Cast method

There are a few common strategies that researchers employ to modify the electrode surface. One is to prepare materials separately and drop them onto the electrode layer by layer. Thereby constructing a multilayer transducer interface. This is a very flexible approach and has high applicability to different kinds of materials.

There has been growing attention to materials in the form of nanoparticles in the recent decade. The main character has been gold nanoparticles due to their inert nature and high biocompatibility suitable for housing biomolecules such as enzymes. Gold nanoparticles in application of Biosensors has been very promising. Many

research groups have demonstrated the potential application of Gold nanoparticle in many different sensor fields such as optical and electrochemical. The promises of GNPs are such as to improve electron conductivity and retention of enzymatic activity.

There are reports of GNPs usage in Electrochemical biosensors such as citrate-capped GNPs were electrostatically adsorbed to porous polysaccharide layer. The electrode was used to detect parathion with limit of detection of $5x10^{-15}$ g/mL and the linear response range at $1x10^{-8}$ to $1x10^{-14}$ g/mL. There was significant reduction in the electron transfer resistance compared to those without GNPs. The fabricated electrode has high storage capability having retained 95% of initial activity after 30 days (Deng et al, 2016). The decent retention in enzymatic activity could be synergistic effects of combination between biocompatibility of GNPs and Chitosan with the protective effects of a nafion film. GNPs in a form of a hollow spheres were also used to entrap AChE. The electrode was used to measure trace amounts of methyl parathion. The LOD was 0.13 nM with linear response ranges of 0.5 to 25 nm and 25 to 300 nM. There was obvious improvement to electron transfer capability in the electrode with hollow gold spheres compared to bare electrode (Jiang et al, 2016). A nanocomposite of GNPs and porous carbon was utilized as a support layer for enzyme immobilization. The presence of GNPs was crucial in improving the peak oxidation current up to 2.2 times that of bare BDD electrode. The electrode detects dichlorvos with LOD of $2.99x10^{-13}$ M and linear range of $4.5x10^{-13}$ to $4.5x10^{-9}$ M. The electrode has exhibited stellar storage capability retaining 95.42% of initial activity after 30 days (Wei et al, 2015). A gold nanorod was grown out of GNPs in an attempt to improve enzyme loading capability. The author argued that a Rod having larger

surface-volume ratio could improve enzyme loading but the shape of the gold nanoparticle did not have any effect on the performance of the electrode. The electrode was used to detect paraoxon and dimethoate. The LOD are 0.7 nM and 3.9 nM respectively. The linear response ranges are 1 nM to 5 μM and 5 nM to 1 μM respectively. The electrode itself retained 93% of initial activity after 30 days (Lang et al, 2016). Another interesting approach to Drop-cast method was an encapsulation of Enzymes in a biomimetic polymer layer. GNPs and AChE were prepared as conjugates and grown around it was a silica-based encapsulation. The fabricated electrode was not used to detect any pesticides, but the storage capability is phenomenal. The electrode retained 155% of initial activity after more than 100 days (Buiculescu et al, 2012).

The approach to the usage of GNPs such as Deng's has been very logical, the group was aware of the weakness in their porous polysaccharide support in electron conductivity and employed GNPs to resolve that limitations. In Jiang's case, the use of GNPs was to directly improve the glassy carbon electrode, their aim was to demonstrate that different forms of GNPs also exhibit possible application in Biosensing. Wei's group however were looking for a possibility in synergistic phenomenon from using GNPs and other materials such as ionic liquids. The results were impressive reduction in electron transfer resistance of the fabricated electrode. And with Lang's work, the potential of GNPs expanded to other parameters than just electron conductivity, various morphology of Gold nanomaterials could be tailored to specific usage. Buiculescu's group had shown a more sophisticated approach to retain the enzyme activity within a protective encapsulation structure.

We are interested in using gold nanoparticles in this work because its ease of usage. The GNPs are easy to prepare and the chemical properties are desirable in the application in Biosensing field.

3.2.2 Electrode Surface modification with Electrodeposition

There has been extensive electrode surface modification accomplished and the goals are to improve the performance of the electrode and remove limitations such as enzyme instability and reduce working potential. Another approach to utilize gold nanoparticles is through electrochemical deposition. A voltage cycles are applied to the electrode in the presence of precursor form of gold. The precursor in response to the change in voltage precipitate into nanoparticles directly on the surface of the electrode. In this approach, the formation of GNPs was controlled through concentration of the precursor, the scanning potential, the scanning rate, the deposition time, and the presence of other materials in the solution.

There are reports such as electrodeposition of Gold nanoparticle on gold electrode to increase surface roughness for enzyme adsorption and electrocatalytic ability, the biosensor was used to detect carbofuran with LOD of 33 nM and linear range of 1 to 6 μM (Shulga et al, 2006). There was evidence suggesting the ability to tune the size of the deposited GNPs. A Screen-printed electrode has also been modified via electrodeposition. The SPCE was pretreated with Na_2CO_3 to increase roughness before electrodeposition. The result electrode exhibited 21% larger surface area compared to bare SPCE. The electrode measured trace amounts of methyl parathion with LOD of 0.6 ppb and linear range of 0.2 to 0.1 ppb (Grace et al, 2016). A similar approach was also conducted to investigate the time-dependent electrodeposition of GNPs. The group found that after the deposition time of 10

seconds, the nanostructure continued to increase but the electrochemically active area did not increase. The electrode was used to detect monocrotophos with LOD of 1000 μM and linear range of 50 to 400 μM (Dimcheva et al, 2013). Interestingly, a few groups of researchers had utilised an almost identical method to co-deposit gold nanoparticle with carbon sheet onto an electrode in detection of arsenic(III)(Liu et al, 2013 and Zhao et al 2018) and methylmercury(Yiwei et al, 2017). A GO/$HAuCl_4$ solution was prepared in a Carbonate Buffer Solution. The electrode was then immersed and CV cycles were applied to induce formation of composite GNPs/reduced GO on the electrode surface(Liu et al, 2011, Liu et al, 2013 and Yiwei et al, 2017). In the case of Zhao's work, Phosphate buffer solution was used instead of Carbonate buffer solution with a little modification to the concentration of $HAuCl_4$ and fewer CV cycles.

Electrodeposition is a very facile approach to construct a robust gold nanostructure on the surface of an electrode. The early works were to investigate the influence of various parameters on GNPs formations and the later works present real application in the detection of trace chemicals. The potential of GNPs/rGO are very appealing to the application of pesticide detection.

3.3 Influence of Acetylcholinesterase immobilization process

3.3.1 Immobilization via Self-assembly monolayer

The SAM of enzyme layer relies on the Au-S bond because the -SH group has high affinity for metallic gold surface. The presence of thiol group can help enzyme attach itself to the gold nanoparticle surface. This process was utilized by a few groups to immobilize acetylcholinesterase on their gold-modified electrode.

Shulga et al 2006 used this technique and provide a Km that demonstrated gentle immobilization process. Grace et al 2016 also used this approach and the result biosensor had shown low detection limit. Dimcheva et al 2013 had modified the process by adding a cleaning step before the adsorption and the biosensor had retained 50% activity after a storage period of 30 days. Deng et al 2016 relied partially on this method as they did not use a crosslinker. The retained activity is very high at 95% after 30 days but this is more attributed to the fact that they have protective layer. Wei et al 2015 had fabricated a biosensor that retained 95% of its initial activity after 30 days. The good stability came from a number of factors such as the porous nature of carbon support and ionic liquid matrix.

The SAM technique was shown to work, and AChE could be immobilized onto the gold surface via this facile process.

3.3.2 Immobilization via Crosslinker

Lang et al 2016 utilized glutaraldehyde as immobilization technique and the biosensor retained 93% of the initial activity after a storage period of 30 days. The biosensor did not have any protective layer which suggest that the presence of BSA and Glutaraldehyde helped stabilize the AChE.

3.3.3 Encapsulation

Buiculescu et al 2012 encapsulate AChE and gold nanoparticle inside a silica layer. The enzymatic activity of AChE was higher than its initial value after 4 months. The author attributed this result from the reactivation of encapsulated AChE from dry state to rehydrated state.

There is no one correct way to attach enzyme to a support, but the more suitable approach is certainly debatable. Crosslinkers are very appealing as more of

the presented literature articles which had included any kind of crosslinkers have exhibited excellence storage ability. The usage of crosslinker in the immobilization process could ensure longevity of the fabricated electrode. The possibility of using both electrodeposition and Drop-cast approach together could yield a facile step-by-step fabrication process of a more than adequate electrode in application of pesticide detection.

Table 1. Trend in Biosensor for pesticide detection

	Modification	**LOD**	**Pesticide Target**	**Retained activity**	**Crosslinker**	**References**
Graphene /Graphene oxide	AChE/CdS-Graphene/Chitosan/GCE	0.7 ng/mL	carbaryl	81% after 20 days	None	Wang et al 2010
	AChE-BSA/prGO-Chitosan/GCE	0.5 ng/mL	carbaryl	83% after 20 days	None	Li et al 2013
	AChE/AuDMBG/rGO/GCE	0.35 ppb	triazophos	90.2% after 21 days	Cysteamine	Ju et al 2015
Gold nanoparticles	AChE/AuNPs/Au Electrode	33 nM	carbofuran	N/A	None	Shulga et al 2006
	AChE/AuNPs/SPCE	0.6 ppb	methyl parathion	N/A	None	Grace et al 2016
	AChE/AuNPs/Graphite	1000 µM	monocrotophos	50% after 30 days	None	Dimcheva et al 2013
	AChE/AuNRs/GCE	0.7 nM	paraoxon	93% after 30 days	Glutaraldehyde	Lang et al 2016
	AChE/AuNRs/GCE	3.9 nM	dimethoate	93% after 30 days	Glutaraldehyde	Lang et al 2016
	AChE/AuNPs/Nano-PPCPE	5 x 10-15 g/ml	parathion	95% after 30 days	None	Deng et al 2016

Chapter 4: Materials and Methods

4.1 Reagents

Graphene oxide, $HAuCl_4.3H_2O$, 25% Glutaraldehyde solution, Acetylcholinesterase from Electrophorus electricus, 5,5-dithio-bis-[2-nitrobenzoic acid] (DTNB) and Acetylthiocholine chloride were purchased from Sigma Aldrich, Singapore. Screen-Printed Carbon Electrode(SPCE) was obtained from Quasense, Thailand. Sodium hydrogen phosphate (Na_2HPO_4), and Sodium dihydrogen phosphate (NaH_2PO_4) were obtained from Ajax Finechem. All reagents were of analytical grade and used as-obtained. All aqueous solutions were prepared with deionized water.

4.2 Methods

4.2.1 Co-deposition of reduced-graphene oxide and $HAuCl_4$ onto a screen-printed carbon electrode

A mixture solution of Graphene oxide(GO) and $HAuCl_4$ was first prepared by dissolving $HAuCl_4$ into 0.1M PBS pH9 for final concentration of 0.5 mM. The GO powder was then added to the solution at 1g/L concentration. The solution was finally sonicated with a probe-type sonication for 5 minutes. A 3-pole screen-printed carbon electrode was submerged in the prepared solution and cyclic voltammetry was performed with applied voltage of -1.4V to 0.6V at 50 mV/s scan rate. The electrode SPCE/rGO/AuNPs was washed with DI water and kept in storage for further usage.

4.2.2 Electrochemical characterization of electrode modified with rGO/AuNPs

The investigations of electrochemical properties of the modified electrodes were performed with cyclic voltammetry. The parameters were set at a fixed scan rate of 50 mV/s and voltage from -0.3 V to 0.6 V in an electrolyte solution containing 0.1 M PBS solution pH 7.0 and 10mM $[Fe(CN)_6]^{3-/4-}$. The electrochemical properties of electrodes modified with only rGO or only $HAuCl_4$ were also investigated in similar manners.

4.2.3 Physical characterization of electrode surface

FESEM were performed on bare electrode and electrodes with modifications including electrodeposited rGO, electrodeposited AuNPs, electrodeposited rGO + AuNPs at various concentrations of $HAuCl_4$ (gold precursor). EDS were performed on electrode modified with the selected condition of electrodeposited rGO and 0.5 mM $HAuCl_4$.

4.2.5 Biosensor Fabrication

The modified electrode surface was treated with 3uL of Glutaraldehyde solution at concentration of 1% v/v for 90 minutes. The unbound crosslinkers were washed away with DI water. A stock AChE enzyme solution was prepared at 30unit/mL in 0.1M PBS pH 7. 3uL of AChE solution was dropped onto the working electrode surface and left overnight. Before the usage of electrode now denoted

SPCE/rGO/AuNPs/AChE, the surface was also washed again to remove any unbound enzyme molecules.

4.2.6 Determination of optimum parameters for chronoamperometry

4.2.6.1 Working potential

Chronoamperometry was performed on the prepared electrode in the range of 0 V to 1 V with 50mV/s scan rate to determined optimum oxidation potential.

4.2.6.2 Glutaraldehyde concentration

Chronoamperometry was also performed on electrode prepared with a range of GA concentration to determine the optimum conditions. The prepared concentrations of GA were 0.25%v/v, 1%v/v, 2.5%v/v and 5%v/v.

4.2.6.3 AChE Concentration

Chronoamperometry was also performed on electrode prepared with a range of AChE concentration to determine the optimum conditions. The prepared concentrations of AChE were 10u/mL, 30u/mL, 50u/mL and 70u/mL.

4.2.6.4 pH range

Chronoamperometry was also performed on electrode in various pH value of 0.1M PBS to determine the optimum conditions. The electrolyte solutions were prepared in different values ranging from pH 4.5 to pH 8.

4.2.6.5 ATCL Concentration

Chronoamperometry was also performed on electrode in various concentration of ATCL substrate to determine the optimum conditions. The substrate concentration was 5mM, 7.5mM, 10mM, and 12.5mM.

4.2.7 Sensitivity and Limit of detection of the fabricated biosensor

Sensitivity and limit of detection of the fabricated biosensor were determined with Chlorpyrifos as an inhibitor molecule. The initially chronoamperometry were performed on the fabricated biosensor to establish a baseline current (ΔI_{base}), the electrode was then submerged in a solution of Chlorpyrifos for 5 minutes to inhibit the enzyme. Finally, the inhibited current(ΔI_{in}) was also measured with chronoamperometry.

4.2.8 Stability test

Chronoamperometry was performed on a batch of prepared electrode in a period of 30 days to determine their baseline current (ΔI_{in}).

Chapter 5: Results and Discussions

The objectives of this work is the investigation of the reduced-graphene oxide composite with Gold nanoparticles matrix as a surface modification candidate for AChE biosensor fabrication process. The investigations were split into two different parts which are the modification and detection studies. The modification begun with electrodeposition of rGO and AuNPs and then electrochemical characterization of the modified electrode surface via cyclic voltammetry utilizing redox couple $[Fe(CN)_6]^{3-/4-}$ and physical characterization with FESEM-EDS and HRTEM. The detection part included optimization of parameters for AChE biosensor, determination of the sensitivity and LOD of the fabricated biosensor and its storage stability study.

5.1 Electrochemical characterization of the composite matrix rGO/AuNPs

The focus materials for this process were graphene oxide and gold nanoparticles. The electrode surface modifications relied on electrochemical technique namely cyclic voltammetry. The technique applied a dynamic range of voltage potential to electrode surface while it was submerged in solution containing dispersed GO and $HAuCl_4$. The study started by varying the concentration of stock $HAuCl_4$ to investigate the influences on the electrocatalytic behaviors.

5.1.1 Effects of varying concentration of HAuCl4

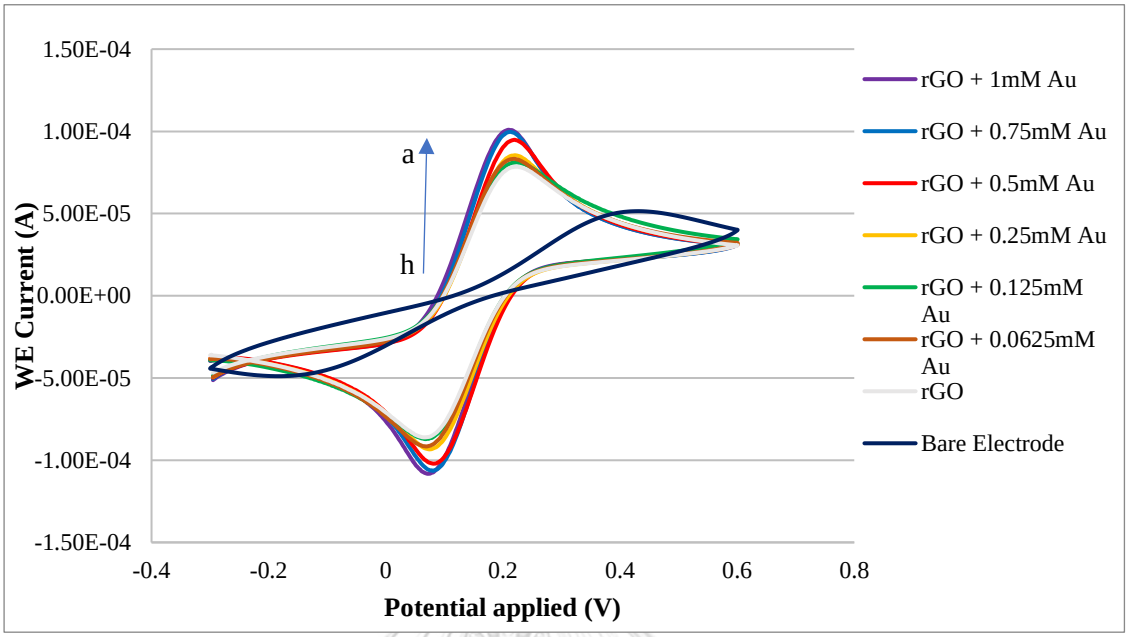


Figure 5.1. CV of electrode with 10mM $[Fe(CN)_6]^{3-/4-}$ prepared in 1g/L GO and various concentrations of $HAuCl_4$, 1 mM (a), 0.75 mM (b), 0.5 mM (c), 0.25 mM (d), 0.125 mM (e), 0.625 mM (f), 0 mM (g), Bare (h).

Electrochemical measurements have provided some insights into the electrocatalytic behaviors of the various modified electrode as shown in figure 5.1. Electrodeposition via cyclic voltammetry of rGO alone has shown observable improvements in electrocatalytic activity, this was in line with other works that suggested that rGO had better electroconductivity to that of GO (Radon et al, 2018). The carbon paste electrode in general was made from graphite powder with pasting liquid, the deposition of rGO provided the electrode surface with better electron conductivity. The electrodeposition of composite materials made from rGO and AuNPs exhibited even superior improvements. The oxidation peak current had evidently increased to 100 µA in the rGO and 1mM $HAuCl_4$ composite compared to

the bare electrode with the oxidation peak current of 51 µA. The peak reduction current was also reduced to – 106 µA in the rGO and 1mM $HAuCl_4$ composite compared to the bare electrode with the peak reduction of – 48 µA. The increases in concentration of precursor gold $HAuCl_4$ with the fixed concentration of colloidal GO sheets had shown the increases in the electrocatalytic activity of the modified electrodes. The two-fold increase in concentration of $HAuCl_4$ did not lead to two-fold increase in peak oxidation current. The 0.5 mM condition was selected as suitable due to sustainability reasons. Further increase in $HAuCl_4$ led to size increases of the deposited AuNPs which lead to reduced specific surface area.

5.1.1 Effects of varying CV cycles

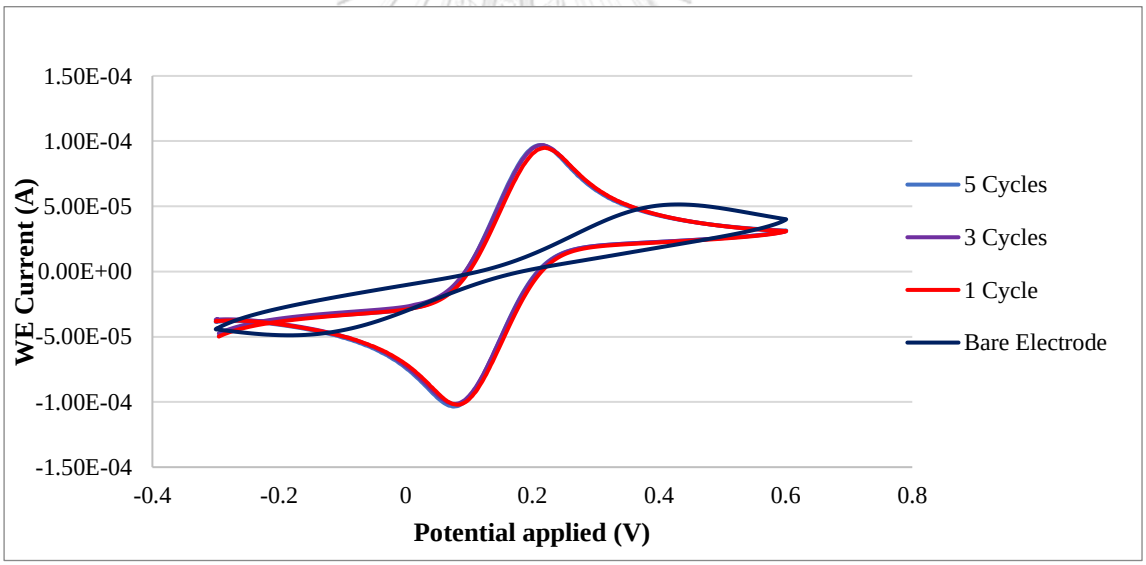


Figure 5.2. Effects of CV cycle numbers on Electrode electrocatalytic activity.

An investigation on CV sweep cycles was also performed on the electrode with 0.5 mM $HAuCl_4$ condition, figure 5.2. There were no observable benefits to the electrocatalytic behaviors with the increasing cycle numbers. Others had shown that

increasing sweeping cycles lead to improvements in peak currents but there was an observable plateau point where the increased sweeping cycles no longer lead improvements in electrocatalytic behaviors of the GCE (Yan et al, 2013 and Zhao et al 2018). In previous literature, a group had speculated a process where rGO film and AuNPs formed on a GCE surface where dispersed GO sheet were electrochemically reduced and lost their hydrophilicity leading to deposition on the electrode surface (Yan et al, 2013). With similar electrodeposition process but different surface morphology, in this work SPCE were utilized which had rough surface compared to glassy carbon electrode, the surface morphology was likely the key factor that affected the sweep cycle counts. In this work, the electrodeposition sweep was fixed at 1 cv cycle.

5.1.3 EIS of modified electrode

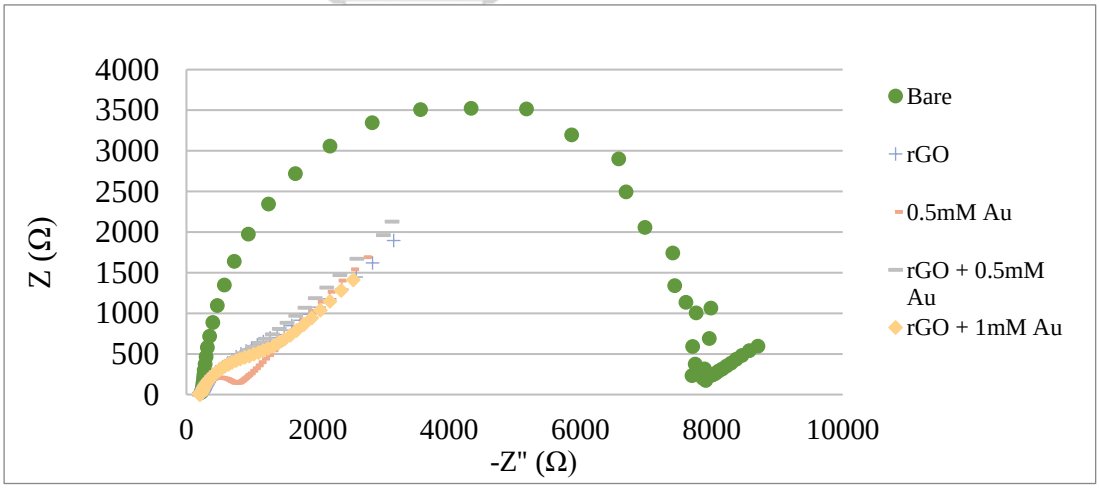


Figure 5.3. EIS of electrode modified with rGO, 0.5mM Au, rGO+0.5mM Au and rGO+1mM Au in 10mM $[Fe(CN)_6]^{3-/4-}$.

Furthermore, an electrical impedance spectroscopy was performed to determine the electrodes electron transfer resistance values. In figure 5.3,

electrochemical impedance spectroscopy also showed results that support the cyclic voltammetry results. The conditions which were investigated in this order; bare electrode, rGO, 0.5mM $HAuCl_4$, composite rGO/0.5mM $HAuCl_4$ and lastly composite rGO/1mM $HAuCl_4$. The electron transfer resistances of the prepared electrodes were determined to be 7.64 kΩ, 133 Ω, 363 Ω, 44 Ω, and 35 Ω, respectively. The modified electrode showed order of magnitudes improvements compared to that of bare electrode. The EIS results were consistent with the cyclic voltammetry results where the composite rGO/AuNPs modified SPCE were superior to bare electrode. The electrode with higher concentration of $HAuCl_4$ showed slightly better electron transfer resistance but not in order of magnitude differences.

5.2 Physical Characterization via FESEM

The surface morphology of various modified electrode was investigated with FESEM at 35000x magnification and EDS. The electrodes were modified with rGO and $HAuCl_4$ at different concentrations.

5.2.1 FESEM of various modified electrodes

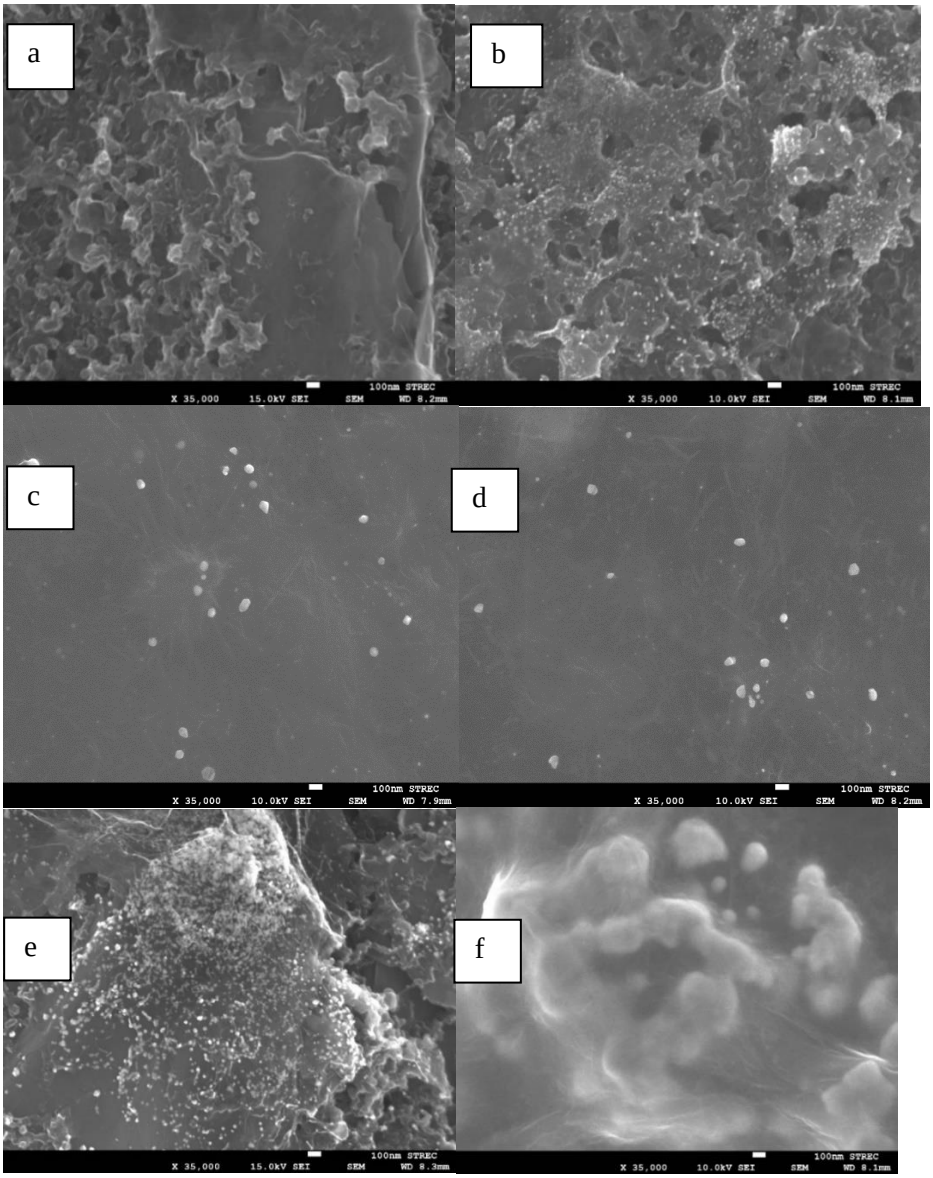


Figure 5.4. FESEM of Modified electrode surface at magnification of x35000; a) rGO, b) rGO/0.0625mM $HAuCl_4$, c) rGO/0.125mM $HAuCl_4$, d) rGO/0.25mM $HAuCl_4$, e) rGO/0.5mM $HAuCl_4$, f) rGO/1mM $HAuCl_4$, g) Bare electrode.

The FESEM images of modified electrode surface had shown that there were AuNPs spread across the surface of the matrix and if carefully observed (figure 5.4 b,c,d and e). Some of the AuNPs appeared to be under a transparent structure (figure 5.4 c,d,e and f). The condition with lowest concentration of $HAuCl_4$ had shown smallest AuNPs particle diameter (figure 5.4 b). As the concentration of $HAuCl_4$ increased the surface AuNPs exhibited larger diameter (figure 5.4 c and d) but also not very good dispersion. The condition that showed most populated and dispersed AuNPs were condition with 0.5mM $HAuCl_4$ (figure 5.4 e). At 1mM $HAuCl_4$ the particles that formed were no longer nanoparticle (figure 5.4 f). The images corresponded to the previously shown cyclic voltammogram results that as more AuNPs were deposited, the oxidation peak current also increased. The results also were in accordant with another work, as they increased the concentration of $HAuCl_4$, the oxidation peak current increased until a certain point where the AuNPs size became too large that the specific surface area reduced and the oxidation peak current was lower (Yi et al, 2019).

The transparent structure was most likely to be sheets of reduced-graphene oxide. The surface modified with only rGO showed such transparent structure (figure 5.4 a). It was speculated that $HAuCl_4$ were first deposited as AuNPs then GO sheet got reduced and deposited to the surface covering the previously deposited AuNPs then more AuNPs were formed on top of the rGO sheets during the CV deposition (Yan et al, 2013). The process had created an intercalated structure of AuNPs and rGO.

The AuNPs size in rGO/0.5mM $HAuCl_4$ could be used to explain the slight shift to the right in CV results from Figure 5.1 where increased specific surface area might have influenced the oxidation current and reduction current of $[Fe(CN)_6]^{3-/4-}$ compared to the CV peaks of the other $HAuCl_4$ concentrations.

5.2.2 EDS of electrode modified with rGO/0.5mM $HAuCl_4$

Element	Weight%	Atomic%
C K	97.97	99.87
Au M	2.03	0.13
Totals	100.00	

Table 5.1. The percent weight of the elements presented on electrode modified with rGO and 0.5mM $HAuCl_4$

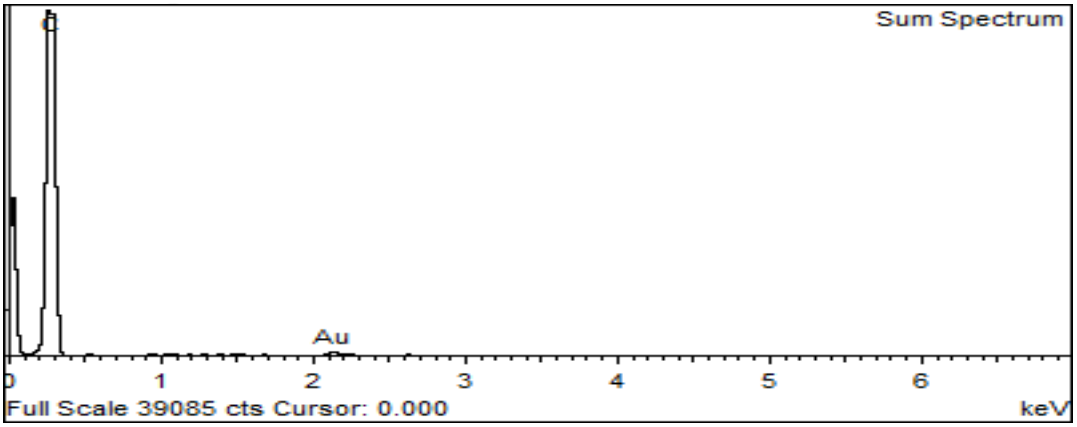


Figure 5.5. The EDS of the electrode modified with rGO and 0.5mM $HAuCl_4$

The EDS results (figure 5.5) showed presence of gold and carbon elements on the modified electrode surface and table 5.1 showed that around 2% of the surface structure was confirmed to be gold which meant that the observable AuNPs on (figure 5.4 e) were covered with rGO sheets composed of mostly carbon atoms.

5.2.3 Color mapping of the electrode modified with rGO/0.5mM HAuCl

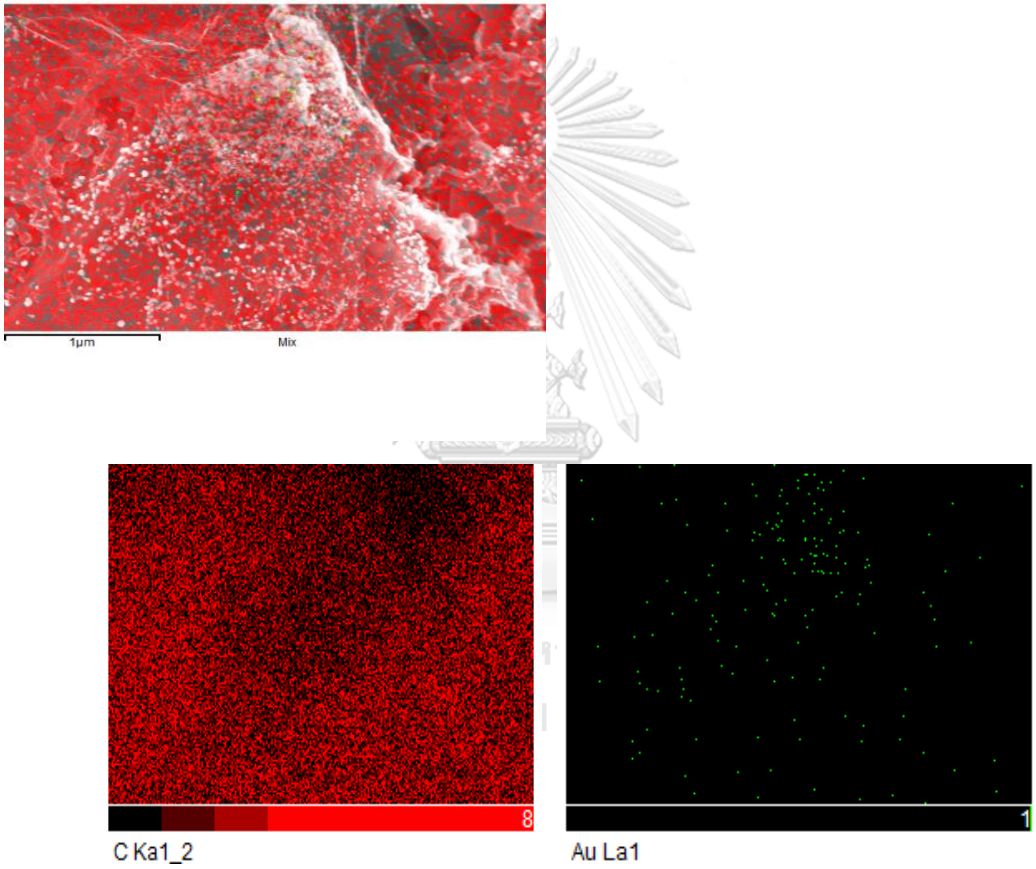


Figure 5.6. The color mapping of the electrode surface showing the positions of carbon and gold.

In the Mapping (figure 5.6), the red dots represented carbon in rGO sheets which dominated the surface of the electrode and the green dots dispersed sparingly on the surface representing the gold particles. These results had shown the

successful co-electrodeposition of rGO and AuNPs from a single step cyclic voltammetry sweep.

5.3 Fabrication of AChE Biosensor

Several parameters were investigated during the fabrication of AChE biosensor. The working potential of the electrode was identified. The concentrations of GA (crosslinker), the concentrations of AChE, working pH, and concentrations of ATCL were also optimized. Finally, the sensitivity and limit of detection were measured with a standard pesticide model, Chlorpyrifos.

5.3.1 Working potential

A series of CV were performed on modified electrodes with and without presence of AChE and its substrate ATCL. In the applied voltage range of 0V to 1V, a peak oxidation was observed in the electrode with AChE adsorbed on the surface and with presence of ATCL. The peak was absent in the electrode without the substrate. In figure 5.7, the oxidation peak was found to be 0.8V and selected as working potential for further chronoamperometry studies.

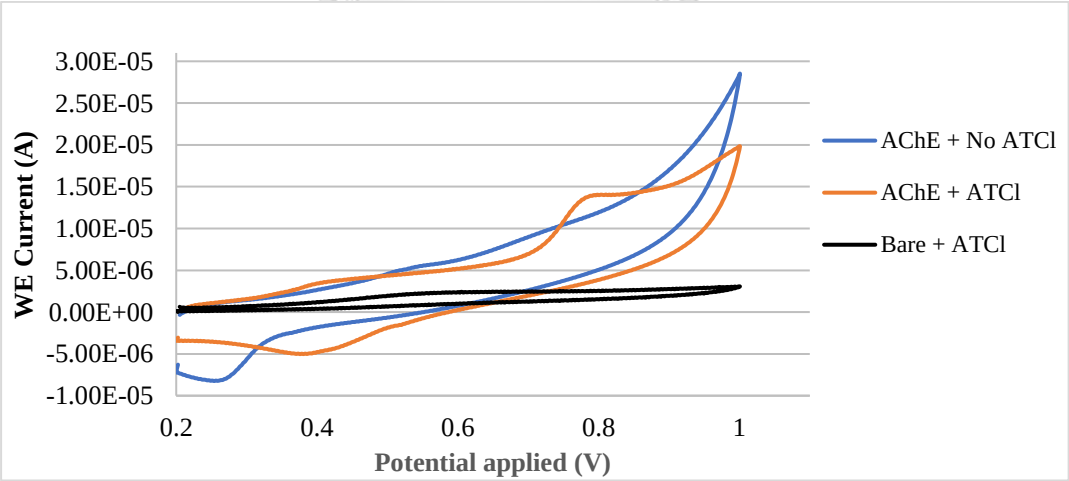


Figure 5.7. CV of bare electrode and modified electrode with AChE and ATCL, with AChE and without ATCl.

5.3.2 Crosslinker

Chronoamperometry was performed on electrodes with different amount of Glutaraldehyde concentration during the process of crosslinking AChE to the matrix. The optimum concentration of GA was found to be 1%, the increase in concentration results in the reduction of oxidation current ΔI. GA is a reactive species which could form permanent bonds that could also permanently inhibit enzyme activities. When concentration of GA was lower than1%, it was likely that the enzyme film was not resilient to the washing process which led to lower peak oxidation current.

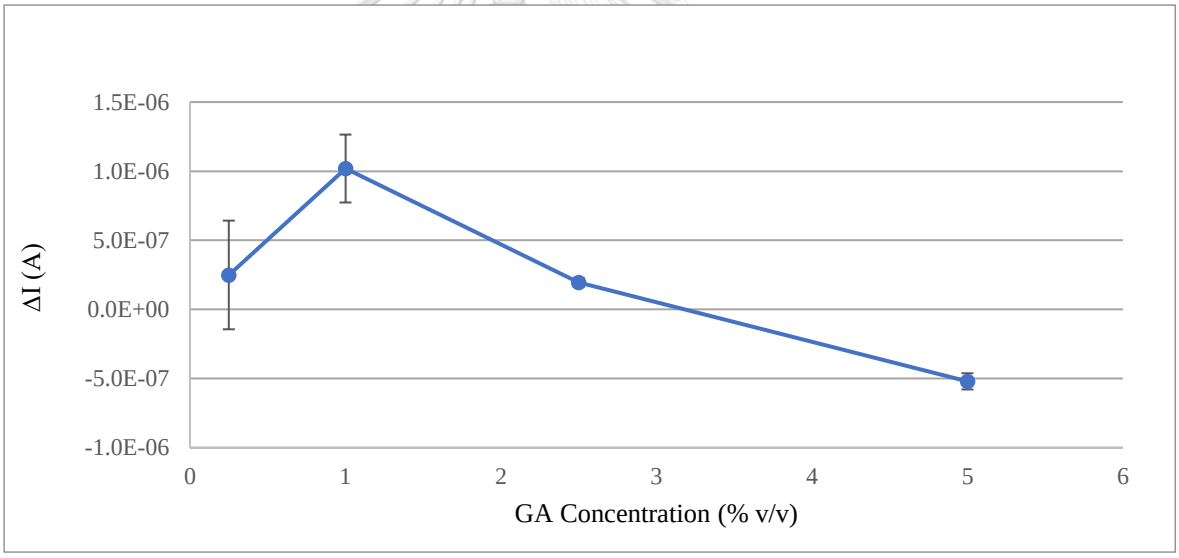


Figure 5.8. The oxidation current ΔI of electrode with varying amount of GA as AChE crosslinker.

5.3.3 The effect of AChE concentrations on oxidation current ΔI of the electrode

The fabricating process required the immobilization of AChE onto the composite matrix of rGO/AuNPs. The enzyme was drop-casted onto the composite

surface that had been functionalized with glutaraldehyde. The unbound enzyme would be later washed off with a buffer solution. The optimum concentration of AChE to be drop-casted was investigated for sustainable purposes. The concentration of AChE was varied from 15u/mL to 90u/mL during the fabrication process. The optimum amount was identified to be 30u/mL as any increased in AChE concentration no longer provide higher oxidation current ΔI. This concentration was selected for further fabrication as it provided the best peak oxidation current with the least amount of enzyme usage.

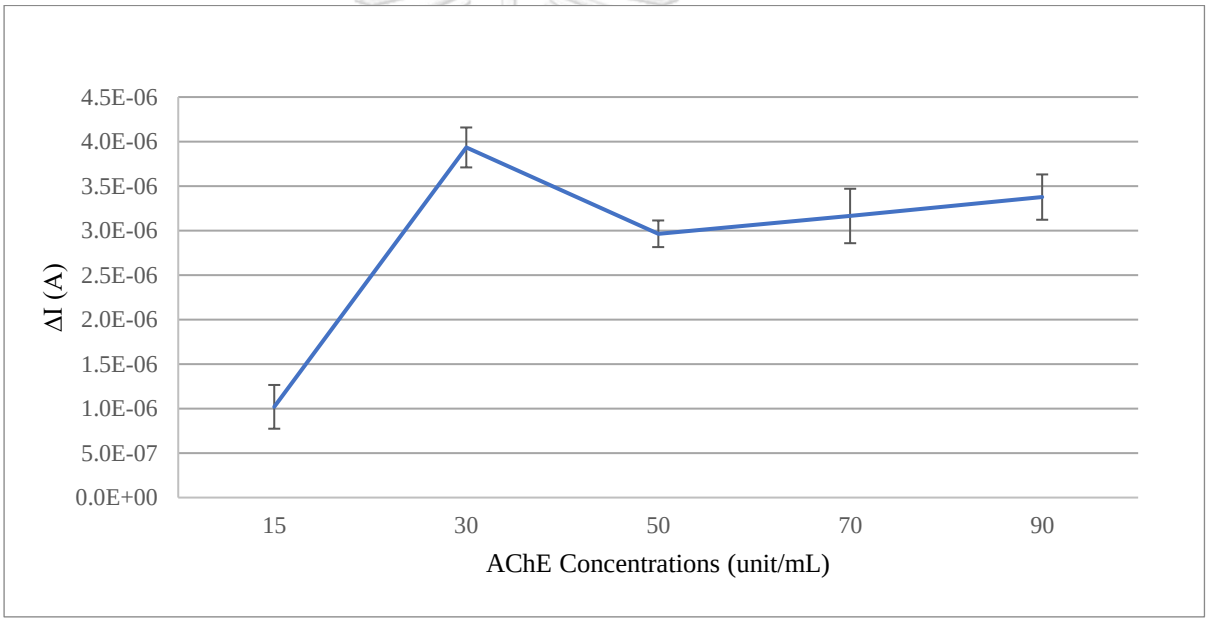


Figure 5.9. The oxidation current ΔI of electrode with varying amount AChE immobilized.

5.3.4 The effect of pH on oxidation current ΔI of the electrode

The fabricated biosensor's operating pH range were investigated by varying the pH value of 0.1 M PBS from pH 4.5 to pH 8 during the chronoamperometry. The optimum amount was identified to be pH 7 as any increase or decrease in pH value

would provide reduced oxidation current ΔI. This was observed also in other works that employed AChE as recognizing elements (Caetano et al, 2008, Jha et al, 2010, Yanping Li et al, 2017 and Yi et al 2019).

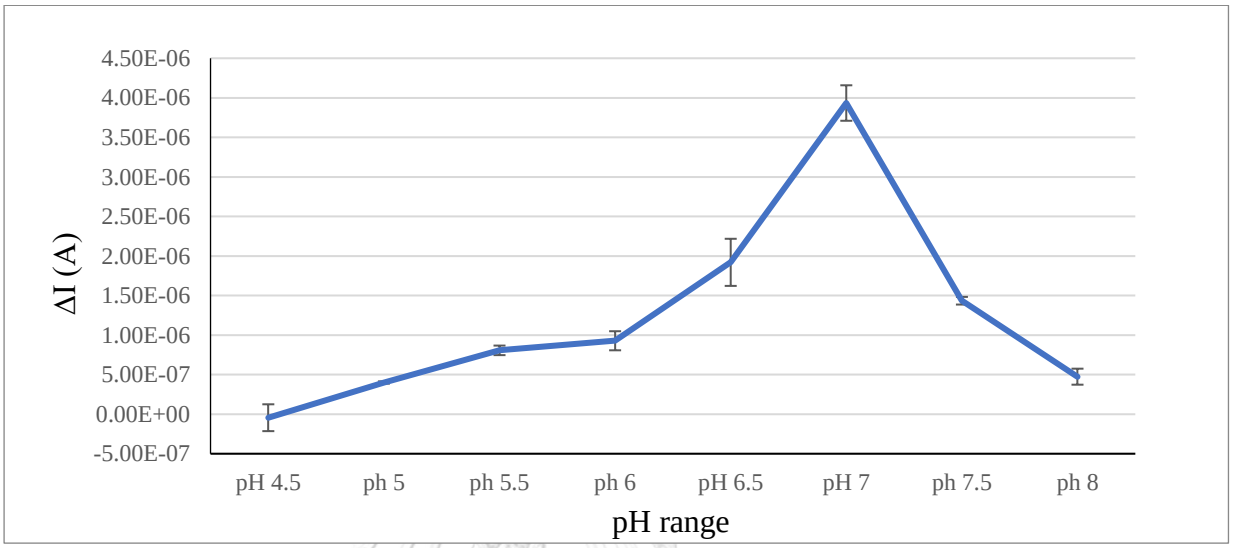


Figure 5.10. The oxidation current ΔI of electrode with varying pH value during chronoamperometry.

5.3.5 The effect of ATCL concentration on oxidation current ΔI of the electrode

The substrate concentration was a parameter that influenced the peak oxidation current of the fabrication biosensor. The concentration of ATCL was investigated to determine the optimal point for the operating condition of the biosensor. The ATCL concentrations were varied from 5mM to 12.5mM during chronoamperometry. The optimum amount was identified to be 7.5mM as any increase or decrease in ATCL concentration would provide slight reduction in oxidation current ΔI.

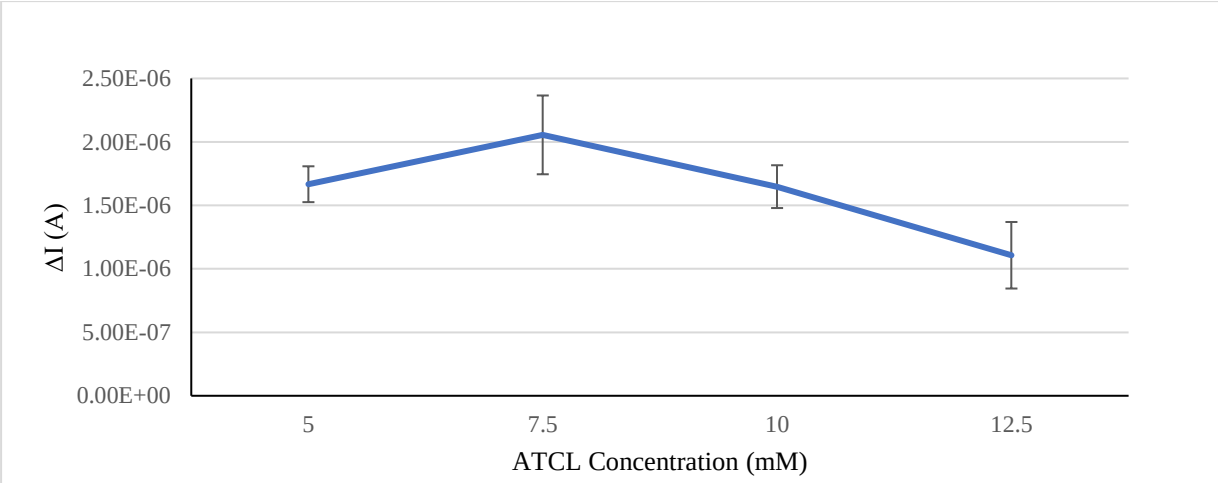


Figure 5.11. The oxidation current ΔI of electrode with varying ATCL concentration during chronoamperometry.

5.3.6 Pesticide Measurement time

The chronoamperometry time of the fabricated biosensor was selected at 300 seconds where decently stable oxidation curve was observed. The decayed electrical signal was due to degradation of the substrate (ATCL).

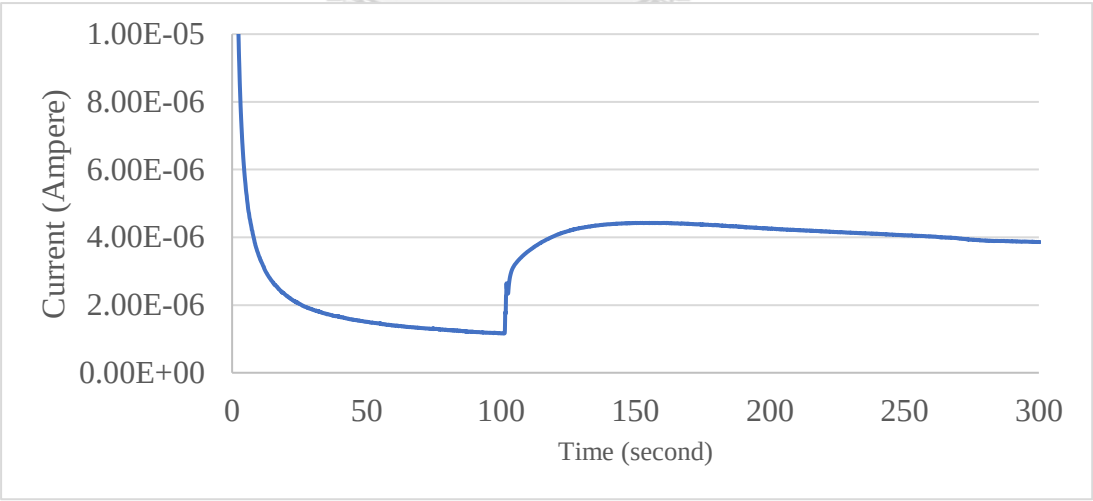


Figure 5.13 Chronoamperometry curve of AChE/GA/AuNPs/rGO/SPCE in 7.5mM ATCL.

The measurement time per sample came from 2 chronoamperometry runs (10 minutes) plus 5 minutes incubation time which resulted in 15 minutes per sample.

5.3.7 Acetylcholinesterase Biosensor; Sensitivity and Limit of Detection

Finally, with all optimized parameters, AChE biosensor sensitivity and LOD were investigated with Chlorpyrifos inhibition method. Organophosphate and carbamate species can target AChE active site and form an unbreakable bond to inhibit its activity. The baseline current (ΔI_{base}) was first measured after that the electrode was submerged in a pesticide standard for 5 minutes. The last step was to measure again, the inhibited current (ΔI_{in}) from the same electrode to construct a calibration curve. With the calibration curve, a sensitivity was calculated and LOD was determined.

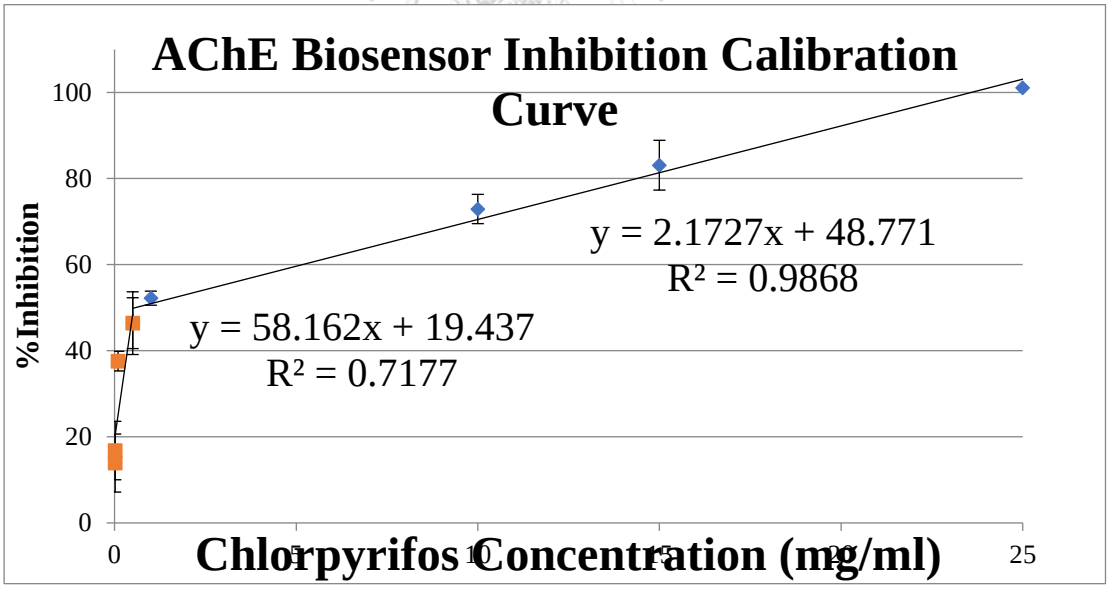


Figure 5.12. The calibration curve for Chlorpyrifos with concentration ranging from 0.02 mg/mL to 25mg/mL and inhibition time of 300 seconds.

The measured LOD was calculated with the 3Sigma method; LOD= (3* sb)/m where sb represent standard deviation of blank and m represent the slope of linear sensitivity from the calibration curve. There were 2 observed sensitivities from two linear ranges; the ranges are 0.016mg/mL to 1mg/mL and 1mg/mL to 25 mg/mL. The limit of detection was calculated from the lower sensitivity which was m=58.162. The calculated LOD was 0.3503 mg/mL.

The fabricated AChE biosensor was stored in a fridge at 4°C for a period of 30 days. The sensor lost around 20% of its initial oxidation current after 14 days and 45% after 30 days.

Materials	**Inhibitor**	**linear range**	**LOD**	**Reference**
ACHE/ERGO/NF/GCE	Methyl parathion	$7.59x10^{-12}$ to $2.65x10^{-9}$ mg/mL	$3.799x10^{-12}$ mg/mL	Jeyapragasam, 2017
AChE/AuNCs/GO–CS/SPCE	Chlorpyrifos	1 x 10^{-2} to 5 x 10^{2} ug/L	3 x 10^{-3} ug/L	Yao, 2019
AChE/e-GON–MWCNTs/GCE	Carbofuran	0.03 to 0.81 ng/mL	0.015 ng/mL	Yanping li, 2017
AChE/e-GON–MWCNTs/GCE	Paraoxon	0.05 to 1 and 1 to 104 ng/mL	0.025 ng/mL	Yanping li, 2017
AChE/Au/MWCNTs/GCE	Paraoxon	0.028 to 1.927 ng/mL	0.028 ng/mL	Jha, 2010
AChE-e-pGON/GCE	Paraoxon	0.3–6.1 ng/mL	0.15 ng/mL	Yanping li, 2016
ACHE/AuNPs/NVRGO/GCE	Malathion	$9.07x10^{-18}$ mg/mL	$9.07x10^{-19}$ mg/mL	Man, 2019
ACHE/AuNPs/NVRGO/GCE	Methyl parathion	$1.44x10^{-11}$ to $1.44x10^{-17}$ mg/mL	$8.73x10^{-17}$ mg/mL	Man, 2019
AChE/Carbon paste electrode	Carbaryl	$2.48x10^{-7}$ to $3.73x10^{-6}$ mg/mL	0.4×10^{-3} mg/mL	Caetano, 2008
AChE/GA/AuNPS-rGO/SPCE	Chlorpyrifos	0.016 to 1 and 1 to 25 mg/mL	0.3503 mg/mL	This work

Table 1. Comparison to other works.

Chapter 6: Conclusions and recommendation

6.1 Co-electrodeposition of rGO and AuNPs

The co-electrodeposition was employed to modify SPCE surface with varying amount of AuNPs ranging from 0.625mM to 1mM. It was found that as the concentration of $HAuCl_4$ increased, the electrocatalytic behaviors also increased until 0.75mM where further increases no longer improved the performance of the electrode. The concentration that was selected as suitable was 0.5mM $HAuCl_4$ where the electrocatalytic activity is only slightly worse that 0.75mM to promote sustainability practice. Upon observation of FESEM images, at lower $HAuCl_4$ concentration, the gold particles were small and sparse. At higher concentration, 0.5mM $HAuCl_4$, the surface was populated with well dispersed AuNPs while at 1mM $HAuCl_4$, the gold formed very large clumps and not well dispersed

6.2 Biosensor Fabrication

The modified SPCE was successfully fabricated into an AChE biosensor. The working potential was 0.8V, optimum crosslinker concentration was 1%, enzyme loading was 30u/mL, working pH was found to be pH 7 and the ATCL substrate concentration was 7.5mM. The biosensor had 2 linear ranges of 0.016mg/mL to 1mg/mL and 1mg/mL to 25 mg/mL. The limit of detection was calculated from the lower sensitivity which was m=58.162. The calculated LOD was 0.3503 mg/mL.

6.3 Recommedation

The limitation of this iteration of AChE was its vulnerability to external influences due to absence of protective layers. The enzyme activity was reduced to

50% after 30 days. I would try to improve this activity by utilizing a different technique to immobilize the enzyme, maybe adding a biopolymer layer as final coating instead of using a crosslinker to bind enzyme to the surface of the electrode. I would recommend investigations into different metal elements for formation of nanocomposite materials such as copper or silver due to the expensive nature of gold.

In this work, a disposable AChE biosensor was successfully fabricated and its sensitivity and LOD were determined. The fabricated biosensor showed fast response and low detection time at only 15 minutes per sample. In comparison to other works, the prospect of disposable AChE biosensor could mean that an on-site detection of organophosphate and carbamate pesticides could readily be conducted. Although, the LOD of this AChE biosensor was not very low but it was lower than pesticide maximum residue limits established by Thai agricultural standard. This disposable AChE biosensor showed great promises in real world application.

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VITA

NAME	Chiranthanin mahayotheecharak
DATE OF BIRTH	27 November 1994
PLACE OF BIRTH	Thailand
INSTITUTIONS ATTENDED	Earlham College
HOME ADDRESS	245 Moo 4 Soi Suksamran Tambol Phochai Nongkhai Thailand 43000