# DEVELOPMENT OF ACETYLCHOLINESTERASE (AChE) BIOSENSOR FOR DETERMINATION OF CARBARYL PESTICIDE IN *Brassica oleracea* Var. *capitata* L. (CABBAGE) AND *Brassica oleracea* Var. *italica* L. (BROCCOLI)



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**THESIS SUBMITTED IN FULFILLMENT FOR DEGREE OF MASTER OF SCIENCE**

**BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH**

**2018**

**UNIVERSITI MALAYSIA SABAH**

BORANG PENGESAHAN STATUS TESIS

JUDUL : DEVELOPMENT OF ACETYLCHOLINESTERASE (AChE) BIOSENSOR FOR DETERMINATION OF CARBARYL PESTICIDE IN *Brassica oleracea* Var. *capitata* L. (CABBAGE) AND *Brassica oleracea* Var. *italica* L. (Broccoli)

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# ACKNOWLEDGEMENT

First and foremost, I am thankful to the Almighty God for granted upon me, the patience, strength and good health in order to complete my MSc by research with the title of Development of AChE biosensor for determination of carbaryl pesticide in *Brassica oleracea* var. L. *capitata* (cabbage) and *Brassica oleracea* var. L. *italica* (broccoli). Without His blessings, surely I would not be able to complete this research study.

A huge appreciation goes to both my supervisor and co-supervisor, Assoc. Prof. Dr. Md. Shafiquzzaman Siddiquee and Prof. Dr. Sharifudin Md. Shaarani respectively for their guidance and advises to me throughout this study. I wish to thank you for sharing your skills and expertise in this field of study. I would like to express special thanks to my supportive husband, Baktiar and my lovable childrens, Wafdan and Hannani for always comforting and cheering me up. Not to forget, my sincere thanks to my parents, Hj Anwar and Hjh. Chahaya for their encouragement that aided me in the completion of this study.

I would also like to take this opportunity to record my sense of gratitude to the lab assistants (Mr. Mony and Mrs. Marlenny) for providing guidance and assistances throughout my research. Last but not least, a big thank you to my colleagues, Rovina, Aini, Azriah and Syahadatain for sharing their knowledges and also to all friends who have willingly helped me out with their abilities.

Samsidar Binti Anwar
12 September 2018

# ABSTRACT

Carbaryl pesticide widely applied as insecticide to protect crops against pests in agriculture. Overuses of carbaryl are hazardous as it interrupts human central nervous system by causing signal transmission to be impaired. Because of the hazardous effects resulted by the prolonged exposure to the pesticide, rapid detection of pesticide is highly concerned. Various detection techniques have been developed including chromatographic based method, capillary electrophoresis and spectroscopy. Such techniques are provided excellent sensitivity and accuracy but require expensive instrument, competent handler besides takes longer time of process. In this study, a new electrochemical method was developed based on immobilization of acetylcholinesterase (AChE) enzyme via glutaraldehyde (GA) as a cross linking agent onto glassy carbon electrode (GCE) modified by the homogenously dispersed of multi-walled carbon nanotubes (MWCNTs) and gold nanoparticles (AuNPs) in chitosan (CS) for detection of carbaryl pesticide. The combination of all these nanomaterials are provided great platform for the AChE activity due to their excellent electrochemical properties. The morphological characteristics of hybrid nanomaterials were observed by using SEM, EDX and TEM. The electrochemical behaviours showed the current signals increasing in order: bare GCE < CS/GCE < CS/MWCNTs/GCE < CS/MWCNTs/AuNPs/GCE. The optimal conditions of the developed biosensor were found of pH 7 for Tris-HCl buffer (0.1 M), 20 s for reaction time, 0.1 V/s for scan rate and 6 min for inhibition time. Based on the inhibition of carbaryl on AChE activity, the DPV was detected in the ranges from 0.1 to 8 mg/L. Low detection limit was found of $4.95 \times 10^{-3}$ mg/L with good repeatability, reproducibility and storage stability on the carbaryl detection. The determination of carbaryl in real vegetable samples of *Brassica oleracea var.* L. *capitata* (Cabbage) and *Brassica oleracea var.* L. *italica* (Broccoli) showed good recovery rates between 73.7 to 102.8% with relative standard deviation less than 7%. Thus, the developed AChE biosensor is showed a rapid and high sensitive tool for determination of carbaryl in vegetable samples.

# ***ABSTRAK***

## ***PEMBANGUNAN AChE BIOSENSOR UNTUK PENGESANAN RACUN PEROSAK KARBARIL PADA Brassica oleracea* var. L. *capitata (KUBIS) DAN Brassica oleracea* var. L. *italica (BROKOLI)***

*Racun perosak karbaril digunakan secara meluas sebagai insektisida untuk melindungi tanaman daripada perosak dalam pertanian. Penggunaan berlebihan karbaril adalah berbahaya kerana ia mengganggu sistem saraf pusat manusia dengan menyebabkan penghantaran isyarat menjadi terjejas. Disebabkan kesan berbahaya yang disebabkan oleh pendedahan yang berpanjangan kepada racun perosak, pengesanan pantas racun perosak sangat diambil berat. Pelbagai teknik pengesanan telah dibangunkan termasuk kaedah berasaskan kromatografi, elektroforesis kapilari dan spektroskopi. Teknik sedemikian memberikan sensitiviti dan ketepatan yang sangat baik tetapi memerlukan instrumen yang mahal, pengendali yang kompeten selain proses mengambil masa lebih lama. Dalam kajian ini, kaedah elektrokimia baru dibangunkan berdasarkan enzim asetonilkolinesterase (ACHE) melalui glutaraldehid (GA) sebagai agen penyambung silang ke elektrod karbon berkaca gelas (GCE) yang diubahsuai oleh nanotiub karbon multiwall (MWCNTs) dan nanopartikel emas (AuNPs) dalam kitosan (CS) untuk pengesanan racun perosak karbaril. Kombinasi kesemua nanomaterials ini menyediakan platform yang hebat untuk aktiviti AChE kerana sifat elektrokimia yang sangat baik. Ciri-ciri morfologi nanomaterial hibrid telah diperhatikan dengan menggunakan SEM, EDX dan TEM. Tingkah laku elektrokimia menunjukkan arus elektrik bertambah menurut turutan: GCE <CS / GCE <CS / MWCNTs / GCE <CS / MWCNTs / AuNPs / GCE. Kondisi optimum biosensor yang telah dibangunkan mendapati pH 7 untuk buffer Tris-HCl (0.1 M), 20 s untuk masa tindak balas, 0.1 V / s untuk kadar imbasan dan 6 min untuk masa inhibisi. Berdasarkan perencatan karbaril pada aktiviti AChE, DPV bagi karbaril pada kepekatan berbeza dikesan di antara 0.1 hingga 8 mg / L. Had pengesanan didapati sebanyak 4.95 x* $10^{-3}$ *mg / L dengan pengulangan yang baik, kebolehulangan dan kestabilan penyimpanan pada pengesanan karbohidrat. Penentuan carbaryl dalam sampel sayuran sebenar Brassica oleracea var. L. capitata (Kubis) dan Brassica oleracea var. L. Italica (Broccoli) menunjukkan kadar pemulihan yang baik antara 73.7 hingga 102.8% dengan sisihan piawai relatif kurang daripada 7%. Oleh itu, biosensor AChE yang dibangunkan menunjukkan alat sensitif yang cepat dan tinggi untuk menentukan karbamat dalam sampel sayur-sayuran.*

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# LIST OF SYMBOLS

| | |
|---|---|
| **%** | Percentage |
| **°C** | Degree celcius |
| **cm** | Centimetre |
| **g** | gram |
| **h** | hour |
| **M** | molar |
| **mg** | milligram |
| **mg/mL** | Milligram per milliliter |
| **min** | minute |
| **mL** | milliliter |
| **mM** | millimolar |
| **mm** | millimeter |
| **nm** | nanometer |
| **U/mL** | Units per milliliter |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **ACh** | Acetylcholine |
| **AChE** | Acetylcholinesterase |
| **ATCl** | Acetylthiocholine chloride |
| **AuNPs** | Gold nanoparticles |
| **CS** | Chitosan |
| **CV** | Cyclic voltammetry |
| **DPV** | Differential Pulse Voltammetry |
| **GA** | Glutaraldehyde |
| **GCE** | Glassy carbon electrode |
| **f-MWCNTs** | functionalized-Multiwalled carbon nanotubes |
| **LOD** | Limit of detection |
| **MRLs** | Maximum residue limits |
| **MWCNTs** | Multiwalled carbon nanotubes |
| **NMs** | Nanaomaterials |
| **RSD** | Relative standard deviation |

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# CHAPTER 1

# INTRODUCTION

This chapter provides important overview for the whole current research. Application of pesticides, development of enzyme based biosensor and related problems are briefly explained. The significance of conducting this study is described and all objectives of the research are stated in this chapter.

## 1.1 Background of Research

The utilization of pesticides has tremendously increased since past few decades. Pesticides are depicted as substances or chemical designated for destroying, combating, controlling or mitigating any pest including insect, rodent, fungus, weeds and nematodes, aquatic plant or animal life bacteria or other microorganisms (FIFRA, 2012). Due to the increasing of the human population worldwide, there is a growing interest in agricultural fields, mainly vegetable production for fresh market consumption (Jallow *et al.*, 2017). However, the presences of insect and weed pests have adversely affected the crops yields. As a consequence, farmers have mislaid a lot of income and struggling to fulfill the demands. Desperately, in order to surpass the problem, pesticides have been applied extensively to address threats of these destructive pests, saving the crops and also improving the quality of the agriculture products. In fact, the applications of pesticides are indeed highly required for crop protection and accomplish the demands. Insecticides, herbicides and fungicides are the main forms of pesticides that are mostly used in agriculture to mitigate various agricultural pests (Songa and Okonkwo, 2016). Although these pesticides are not completely protecting the crops, however it could help in reducing losses due to the damage caused by pests.

Despite its tough role to prevent attacks from pests, the overuse of pesticide has triggered serious problems. Pesticide may cause toxic to non-target organisms because of their long half-lives that lead to its long persistence in the environment (Smith *et al.*, 2002). Epidemiologists recommended that currently available pesticides in the market may trigger cancer in non-target organism, including human beings. Obviously, farmers and agricultural workers are the most potential group that experience in suffers due to unintentional pesticide poisoning per year (IARC, 2003; Alavanja *et al.*, 2005). The Codex Alimentarius Commission of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have established the maximal residue limits (MRLs) of pesticides in a variety of foods.

Several types of pesticides are commercially available such as carbamates, organophosphorus (OP), pyrethroids and organochlorines (OCs). However, OCs are currently no longer used and also prohibited. In fact, most of countries have banned the usages of OCs pesticides due to its extremely high toxicity and very persistence in the environment (Van Dyk and Pletschke, 2011). For instance, dichlorodiphenyltrichloroethane (DDT) has a half-life span for 3-20 years (Lintellman *et al.*, 2003). Among all classes of pesticides, carbamates are the compound that frequently used due to powerful insectidal action and low persistence in environment (Nikolelis *et al.*, 2008). While, for OP and OC, they are excluded because of their extremely toxic and related with neurological diseases (Hour *et al.*, 1998).

Development in biosensor area has gained much interest from other researchers as to diversify the existing methods for pesticides detection. Biosensor is an analytical device that biological elements tightly integrated onto the physical transducers for the detection of target compounds or analytes (Guilbault *et al.*, 2004). This device converts the biological recognition event to a measurable electrical signal. These biosensor techniques represent as an advanced method for detecting pesticide, complementing the existing methods. Numerous advantages of biosensors are outlined such as rapid, simple, real-time, and sensitive. Moreover, biosensors are more suitable for on-site detection and for field analysis. Various types of biological materials are used and merged with the transducer including enzyme, antibody, microorganism, whole cell or deoxyribonucleic acid (DNA).

Enzymatic biosensors are based on enzymes in intimate contact with the physical transducers. The enzyme is immobilized onto the surface of transducers via cross-linking, physical adsorption, covalent binding, entrapment or other immobilization methods.

Recently, detection of pesticide compounds with enzymatic biosensors particularly based on enzyme inhibition is in growing progress since its first development was reported in 1962 (Guilbault *et al.*, 1962). The principle idea of enzyme inhibition based biosensor is the quantification of the inhibitor (in this case the pesticide) by measuring the activity of enzyme in the absence and presence of the pesticide. Acetylcholinesterase (AChE) enzyme is the main enzyme applied in the construction of enzyme biosensor for detection of carbamate pesticide. Carbaryl, a member of carbamate is capable of inhibiting the AChE enzyme activity resulted by binding between carbonyl group of carbaryl with serine hydroxyl group of AChE. An illustration for schematic description of AChE inhibition by carbaryl is represented in Figure 1.1.



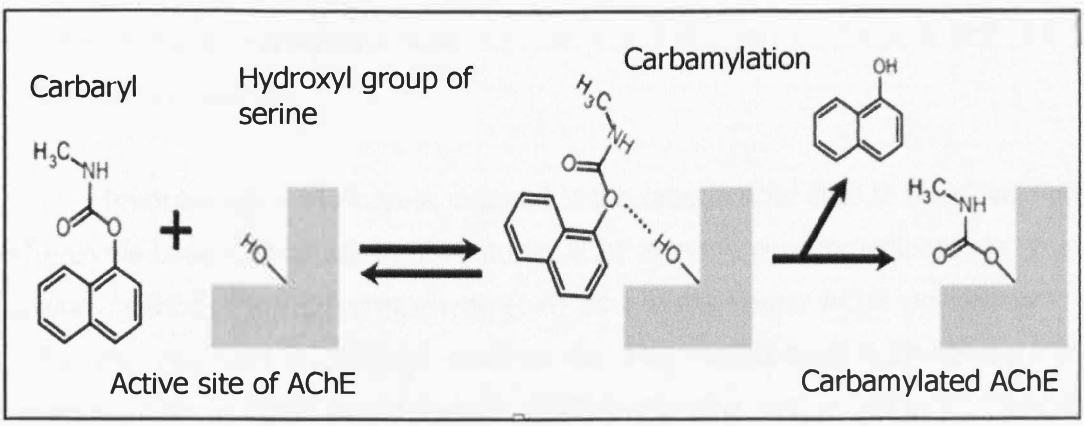


**Figure 1.1 : The schematic description of AChE inhibition by carbaryl.**
Source : Jeon *et al.*, 2013

The utilizations of nanomaterials for developing novel biosensors have been shown great promising tool for the pesticides detection. Chitosan, carbon nanotubes, and metal nanoparticles are some examples of various types of nanomaterials sparked much research interests in the past few years owing to their excellent properties. Chitosan (CS) is used as carrier support for the enzymes and it has some beneficial properties known for its non-toxicity, biocompatibility, biofilm formation capability and solubility in mild acidic solution (Salam *et al.*, 2011). Besides, the presence of many functional groups structurally and relatively low price making it is an important material in the field of advanced sensor technology.

Carbon nanotubes (CNTs) particularly multi-walled carbon nanotubes (MWCNTs) that have gained high interest as part of the nanoscale electronic devices and sensors. Due to the great properties of MWCNTs including excellent electrical conductivity, firm adsorptive capability, high thermal capacity and high chemical stability (Lu *et al.*, 2007; Tsai *et al.*, 2009), the hollow structure of MWCNTs is good for enzyme adsorption. Gold nanoparticles (AuNPs) are a type of metal nanoparticles which has aroused growing attention in the recent years. The unique optical and physical characteristics of AuNPs have provided great advantages in various fields such as electrochemical sensing field (Das *et al.*, 2011). The catalytic role of AuNPs in the electrochemical applications and its high surface area has improved the detection signal in enzyme based biosensor. It creates a good microenvironment for the immobilization of enzyme and aid in retaining its bioactivity.



Immobilization of enzyme material is the most crucial step in the developing of enzyme based biosensor. In this study, AChE enzyme was immobilized via cross-linking method using glutaraldehyde (GA) as a cross-linker. AChE possess amino group that can bind at carbonyl group of GA. The nanomaterials can enhance the immobilization of AChE on the surface of electrode. The unique electrical, chemical and physical properties of nanomaterials can be very beneficial for fabricating electrochemical biosensing interface. Nanotechnology could be an effective way to develop a biosensor with much desired success based on sensitivity and stability. Figure 1.2 displays the schematic drawing of sensing processes based on modification GCE to determine carbaryl.

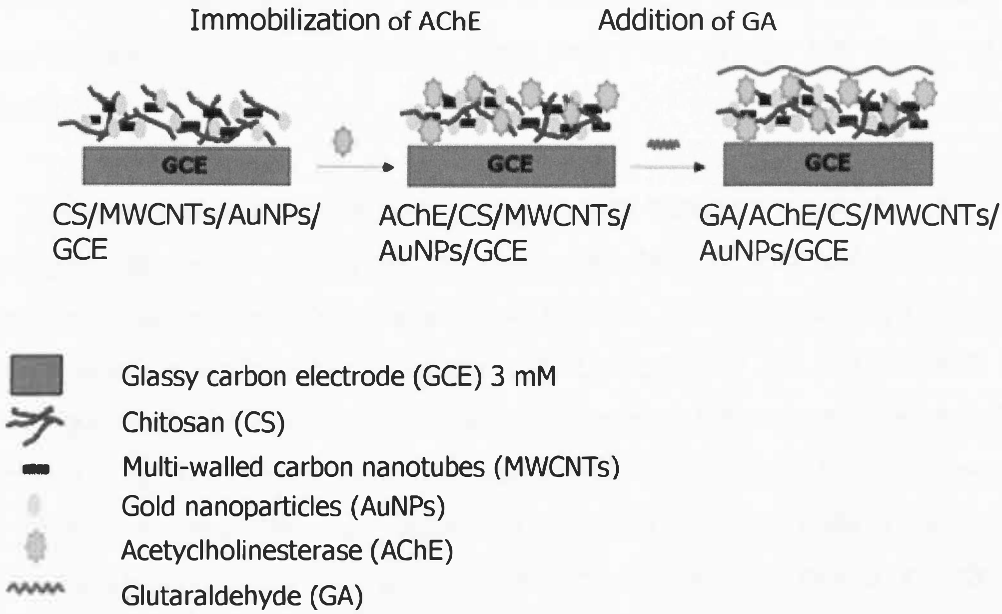


**Figure 1.2 : Schematic representationof the sensing processes for deteremination of carbaryl.**



## 1.2 Problem Statement

Overuses of pesticides can negatively affect the living organisms and environment including severe problems to human health. The excessive use can lead to its bioaccumulation in various matrices including foodstuff (fruits and vegetables), soil and water. Nausea, vomiting, coma, respiratory failure, neurological diseases are some of the consequences that may arise following exposure to these food containing pesticides that could eventually lead to death (Dong *et al.*, 2009).

Carbaryl which belongs to carbamates group has been extensively used as insecticides due to their broad biological activity (Nogueira *et al.*, 2003; Song *et al.*, 2013), low mammalian toxicities and low persistence properties (Zhang and Lee, 2006). However, carbamates (carbaryl) are known as inhibitors of acetylcholinesterase (AChE), a key enzyme in mammal nervous system (Msagati and Mamba, 2012; Shi *et al.*, 2014). Inhibition of this enzyme could affect nerve impulse transmission, inducing neurologic disorder, possibly failure function of the immune, reproductive and endocrine systems or cancer and many others. Because of the high possibility to incur risk of their acute toxicity and their bioaccumulation property which eventually cause long-term damage to human, the detection of carbaryl traces in the environment, food and living things has been rapidly increased.

Conventional analytical techniques particularly based on chromatography and electrophoresis are providing an excellent detection at low concentration of pesticides in various matrices (Tegeler and El Rassi, 2001). Moreover, pairing the chromatographic methods with selective detection systems are more reliable and found high efficiency. However, the above techniques have several drawbacks that related to its complexity and time-consuming procedures, requiring sample extraction and consumption high volume of organic solvents. In addition, expensive sophisticated instruments and skilled operators are mostly required in order to conduct the analysis (Alder *et al.*, 2006; Narakathu *et al.*, 2011).

Over the years, the researcher depends on conventional methods including HPLC, GC and other chromatographic methods for the pesticide detection present in the environment. The complexity and complicated handling of such methods has limited their practical use. Enzyme inhibition based biosensor is the most widely applied to detect the pesticides residues that accumulated in different sorts of matrices such as foods, soils, ground and surface waters. The ability of the pesticides has inhibited various types of enzymes found within insects and other pests lead to the insertion of several types of enzymes into biosensors in which developed for pesticide detection (Van Dyk and Pletschke, 2011). The use of the enzyme could provide better detection in terms of sensitivity and selectivity for the targeted compound. Among enzymes, AChE is emerged as a preferable biological element and selective method for monitoring toxic analytes such as pesticides.



## 1.3 Significances of Study

Pesticides have been acknowledged to represent a major hazardous environmental pollutant. It is one of the main substances used worldwide for the high quality of agricultural products. Unfortunately, excessive use of this pesticide adversely affected both human lives and environment. The presence of carbaryl residues in crops even though very low concentration is harmful to the consumers due to the bioaccumulation effects caused by the pesticide. Besides, some amounts of this residue tend to reach water sources through agricultural runoffs. Mutagenesis, endocrine disruption, and altered immune system of humans are few negative impacts resulted by the carbaryl exposure. Worst, instead of the targeted insects, carbaryl can also show toxicity to various non-targeted species (birds and aquatic organisms).

This study is important as to determine the pesticide residue present in vegetables. The developed method is a good approach to be implemented for rapid detection of pesticide residues in vegetables products at least faster than the conventional methods. This rapid monitoring of pesticides can help to keep the safety of consumers. It can replace the existing conventional methods in which more laborious and time-consuming. The process of detection is more simple, cost-effective and easy to conduct besides produces the satisfactory results. This method is newly developed and potentially applied in agriculture field as a convenient approach for monitoring pesticide in vegetables.



## 1.4 Research Objectives

The objectives in this study are:

1. To characterize and optimize the developed AChE biosensor based on CS/ MWCNTs/AuNPs/GCE.
2. To evaluate the performance of the developed AChE biosensor such as the repeatability, reproducibility, interference, storage stability and difference concentrations.
3. To analyze the presence of carbaryl pesticide in vegetables samples, namely: *Brassica oleracea* Var. L. *capitata* (cabbage) and *Brassica oleracea* Var. L. *italica* (broccoli).

# CHAPTER 2

# LITERATURE REVIEW

This chapter cover the explanation for different classes of pesticides available and the selected model of compound used in the current study. The existing conventional and advanced detection methods for the analyte are described for comparison. Furthermore, the nanomaterials employed and the principle of electrochemical technique for the developed AChE biosensor is explained with more details in this chapter.



## 2.1 Pesticide

The word pesticide is generally a broader term that used to eradicate pests. Pesticides are depicted as any substance or chemical designated for destroying, combating, controlling or mitigating any pest including insect, rodent, fungus, weeds and nematodes, aquatic plant or animal life bacteria or other microorganisms. Pesticides can exist in the form of inorganic, synthetic or biological (bio-pesticides). During the past decades, many pesticides have been used widely in agriculture to decline crop losses thus increasing the yield productivity in agricultural (Cooper and Dobson, 2007; Popp *et al.*, 2013; Verger and Boobis, 2013). For instance, insecticide is to kill insects, herbicide to destroy unwanted plants and fungicides acting on fungal. Additionally, weedicide is specifically targeting the weeds and rodenticide is for rodent animals.

Four main classes of insecticides have been widely used namely carbamates, organophosphorus, organochlorines and pyrethroids. Carbamate and organophosphorus insecticides are widely used in modern agriculture. However,