GRAPHENE AND ENZYME MODIFIED ELETRODE FOR ULTRASENSITIVE ACETYLCHOLINE DETECTION IN MICROFLUIDIC DEVICE

PIYAWAT PITIKULTHAM

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF ENGINEERING
(BIOMEDICAL ENGINEERING)
FACULTY OF GRADUATE STUDIES
MAHIDO UNIVERSITY
2015**

Thesis
entitled

GRAPHENE AND ENZYME MODIFIED ELETRODE FOR ULTRASENSITIVE ACETYLCHOLINE DETECTION IN MICROFLUIDIC DEVICE

.....

Mr. Piyawat Pitikultham
Candidate

.....

Asst. Prof. Chamras Promptmas,
Ph.D. (Biochemistry)
Major advisor

.....

Assoc. Prof. Norased Nasongkla,
Ph.D. (Polymer Science)
Co-advisor

.....

Prof. Patcharee Lertrit,
M.D., Ph.D. (Biochemistry)
Dean
Faculty of Graduate Studies
Mahidol University

.....

Asst. Prof. Yodchanan Wongsawat,
Ph.D. (Electrical Engineering)
Program Director
Master of Engineering Program in
Biomedical Engineering
Faculty of Engineering,
Mahidol University

Thesis
entitled

GRAPHENE AND ENZYME MODIFIED ELETRODE FOR ULTRASENSITIVE ACETYLCHOLINE DETECTION IN MICROFLUIDIC DEVICE

was submitted to the Faculty of Graduates Studies, Mahidol University
for the degree of Master of Engineering (Biomedical Engineering)
on
November 9,2015

.....
Mr. Piyawat Pitikultham
Candidate

.....
Lect. Benchaporn Lertanantawong,
Ph.D. (Biotechnology)
Chair

.....
Lect. Sanitta Thongpang,
Ph.D. (Biomedical Engineering)
Member

.....
Asst. Prof. Chamras Promptmas,
Ph.D. (Biochemistry)
Member

.....
Assoc. Prof. Norased Nasongkla,
Ph.D. (Polymer Science)
Member

.....
Prof. Patcharee Lertrit,
M.D., Ph.D. (Biochemistry)
Dean
Faculty of Graduate Studies
Mahidol University

.....
Asst. Prof. Jackrit Suthakorn,
Ph.D. (Robotics)
Dean
Faculty of Engineering
Mahidol University

ACKNOWLEDGEMENTS

First of all, I am sincerely grateful to Dr. Pornpimol Sritongkham who is the first major advisor of my work. She always gave me the valuable suggestion. I think she is the kindest woman on earth and the respectful teacher. She took care of me in any circumstances. When I lost inspiration, she encouraged and told me that I should go out for relaxation. I was shocked and deeply saddened to know that she was suffered by cancer. She still smiled to me every time while I visited her. Unfortunately, she passed away. She is in my thoughts for the entire of my life. I will remember that I am one of the lucky ones whose life was touched by this very special woman.

I would like to express sincerely gratitude to Asst. Prof. Dr. Chamras Promptmas who came to help me in thesis revising. He is also a kind person who gave the constructive comments. Moreover, I would like to thank all of the advisory committee, Dr. Sanitta Thongpang, Assoc. Prof. Dr. Norased Nasongkla, Dr. Benchaporn Lertanantawong for the brilliant comments and useful suggestion.

I would like to thank Mr.Chakrit Sriprachuabwong from MEMs Laboratory, National Electronics and Computer Technology Center (NECTEC). He suggested and taught me how to use the instrument that relates with my work.

I would like to say thank you to all of my friends in the Biomedical Engineering, Mahidol University, especially the Biosensor laboratory members.

Finally, I would like to thank my family. My mother and father always encourage and support me in any situation.

Piyawat Pitikultham

GRAPHENE AND ENZYME MODIFIED ELECTRODE FOR ULTRASENSITIVE ACETYLCHOLINE DETECTION IN MICROFLUIDIC DEVICE

PIYAWAT PITIKULTHAM 5537714 EGBE/M

M.Eng. (BIOMEDICAL ENGINEERING)

THESIS ADVISORY COMMITTEE : CHAMRAS PROMPTMAS, Ph.D., NORASED NASONKLA, Ph.D.

ABSTRACT

A promising acetylcholine biosensor based on graphene-PEDOT:PSS (GR-PEDOT:PSS) bienzyme, choline oxidase (ChOx) and acetylcholine esterase (AChE), a modified electrode was successfully fabricated. GR-PEDOT:PSS and MWCNT were selected for electrode modification. The sensitivity of H_2O_2 detection at 0.7V vs Ag/AgCl, based on bare SPE, MWCNT/SPE and GR-PEDOT:PSS/SPE was 6.2 nA/mM, 2.038 µA/mM, and 13.29 µA/mM, respectively. GR-PEDOT:PSS/SPE exhibited the highest sensitivity. ChOx and AChE in the optimized loading ratio were immobilized on GR-PEDOT:PSS/SPE by the 10% glutaraldehyde (GA) vapor cross-linked for 8 minutes. 1% Nafion was coated on the outermost layer of AChE-ChOx/GR-PEDOT:PSS/SPE as a protective film. A new design of electrode, 22 mm in length and 12 mm in width, consisted of a working electrode (WE), a counter electrode (CE) and Ag/AgCl reference electrode (RE) on single piece. It was offered to integrate with the microchannel which was made of transparent polypropylene (PP) plastic sheet. In order to fabricate microfluidic chip, the PP channel was adjoined on the electrode surface by adhesive tape. These microfluidic biosensor chip was cost-effective and easy to use. Moreover, the fabrication procedure was easy and simple. The acetylcholine biosensor modified microfluidic chip platform showed the limit of detection, linearity and sensitivity as 10 µM, 10 µM to 750 µM (R^2 = 0.989), and 430.76 nA/mM, respectively.

KEY WORDS: ACETYLCHOLINE/ MICROFLUIDIC CHIP/ BIOSENSOR/ GRAPHENE/ AMPEROMETRY

69 pages

การปรับปรุงพื้นผิวอิเล็กโทรดด้วยกราฟีนและเอนไซม์สำหรับตรวจวัดสารอะเซทิลโคลีนในชิประบบของไหลจุลภาค

GRAPHENE AND ENZYME MODIFIED ELECTRODE FOR ULTRASENSITIVE ACETYLCHOLINE DETECTION IN A MICROFLUIDIC DEVICE

ปิยะวัฒน์ ปิติกุลธรรม 5537714 EGBE/M

วศ.ม. (วิศวกรรมชีวการแพทย์)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์ : จำรัส พร้อมมาศ, Ph.D., นรเศรษฐ์ ณ สงขลา, Ph.D.

บทคัดย่อ

Acetylcholine เซ็นเซอร์ชนิดดัดแปลงหน้าผิวอีเล็กโทรดด้วย graphene-PEDOT:PSS กับเอ็นไซม์ Choline oxidase และ Acetylcholine esterase ได้ถูกสร้างขึ้นวัสดุนาโน GR-PEDOT:PSS และ Multi walled carbon nanotube ถูกเลือกมาใช้ในการปรับปรุงผิวหน้าอิเล็กโทรดเพื่อเพิ่มการนำไฟฟ้า จากการทดลองการวัดเปรียบเทียบ Hydrogen peroxide ที่ศักย์ไฟฟ้า 0.7 โวลต์เทียบกับขั้วอ้างอิง Ag/AgCl ด้วยอิเล็กโทรดพิมพ์สกีนทั้ง 3 ชนิด ได้แก่ อิเล็กโทรด อิเล็กโทรดที่ปรับปรุงพื้นผิวด้วย Multi walled carbon nanotube และ อิเล็กโทรดที่ปรับปรุงพื้นผิวด้วย GR-PEDOT:PSS ให้ค่า Sensitivity ในการตรวจวัดเท่ากับ 6.2 nA/mM, 2.038 μA/mM และ 13.29 μA/mM ตามลำดับ สรุปได้ว่า GR-PEDOT:PSS ให้ค่าที่ดีที่สุด จึงถูกใช้ในกระบวนการทดลองถัดไป เอ็นไซม์ถูกตรึงบนพื้นผิวอิเล็กโทรดในปริมาณและกระบวนการที่เหมาะสม โดยการใช้ไอระเหยจาก 8% Glutaraldehyde บริเวณพื้นผิวอิเล็กโทรดเป็นเวลา 8 นาที พร้อมทั้งเคลือบผิวด้านนอกสุดด้วย Nafion อิเล็กโทรดพิมพ์สกีนชนิดใหม่ได้ถูกออกแบบให้มีขนาด ยาว 22 มิลลิเมตร กว้าง 12 มิลลิเมตร อิเล็กโทรดถูกนำไปประยุกต์ร่วมกับ Microchannel ที่ทำมาจากแผ่นพลาสติกใส Polypropylene ในการสร้าง Microfluidic chip สามารถทำได้โดยนำ Microchannel มาติดบนอิเล็กโทรดพิมพ์สกีนด้วยกาวสองหน้าชนิดบาง Microfluidic chip ที่พัฒนาขึ้นสามารถตรวจวัดสาร Acetylcholine ความเข้มข้นต่ำสุดที่ 10 μM และสามารถตรวจวัดได้ในช่วงความเข้มข้นตั้งแต่ 10 μM ถึง 750 μM โดยมีความสัมพันธ์เชิงเส้นกับความเข้มข้นของ Acetylcholine ด้วยค่าสหสัมพันธ์ทางสถิติ 0.989 และมีค่า Sensitivity อยู่ที่ 430.76 nA/mM

69 หน้า

CONTENTS

CONTENTS (cont.)

CONTENTS (cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChCl	Acetylcholine chloride
AChE	Acetylcholine esterase
AChEIs	Acetylcholinesterase inhibitors
AD	Alzheimer's disease
APS	Amyloid plaques
BioMEMs	Biomedical microelectromechanical systems
Ch	Choline
ChCl	Choline chloride
ChOx	Choline oxidase
CR-GO	Chemically reduced graphene oxides
CSF	Cerebrospinal fluid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition
FDA	Food and drug administration
GA	Glutaraldehyde
GOx	Glucose oxidase
GR	Graphene
H_2O_2	Hydrogen peroxide
HPLC-ECD	High-performance liquid chromatography with electrochemical detector
HRP	Horseradish peroxidase
LOCs	Lab-on-a-chips
LOD	Limit of detection
M	Molar

LIST OF ABBREVIATIONS (cont.)

ml	Milliliter
mM	Millimolar
MWCNTs	Multi-walled carbon nanotubes
nAN	N-acetylaniline
NFT	Neurofibrillary tangles
polyNIPAM	Poly-N-isopropyl acrylamide
PEG	Poly(ethylene glycol)
POC	Point-of-care
PVA	poly(vinyl alcohol) p
QDs	Quantum dots
RE	Reynold's number
SPE	Screen-printed carbon electrode
SWNT	Single-walled carbon nanotube
V	Volt
VD	Vascular dementia
ZnO	Zinc oxide
μTAS	Micro total analysis systems
μl	Microliter

CHAPTER I
INTRODUCTION

1.1 Background and significance of study

Alzheimer's disease (AD) is one of the neurodegenerative diseases which mostly occur in aging people [(1)]. According to previous research studies, Hebert et al. estimated the prevalence of AD which largely increases in people age 65-year-old, and it exponentially increases in every five years of age [(2)]. According to Alzheimer's Association, Washington, DC, 13% of 65 years or older American population are diagnosed with AD [(3)]. Dr. Alois Alzheimer, a psychiatrist in Germany, is the first one who identified the Alzheimer's disease in 1906 [(4)]. He found a pathological brain tissue which is called amyloid plaques (APS) and neurofibrillary tangles (NFT) by biopsy disoriented patient brain tissues after death [(5)]. APS and NFT is an abnormal aggregation of proteins inside the brain tissues [(1, 6, 7)]. Causes of protein aggregation still not clear, but it affects to neuronal cell function. Cholinergic receptors of brain tissue decrease in AD patients, consequently lead to the deficit of acetylcholine (ACh) [(8)]. Meanwhile, ACh is a principle neurotransmitter for signal transmission in the nervous system, so it induces the cognitive impairment or dementia [(9, 10)]. The previous research showed the lower ACh concentration in the cerebrospinal fluid (CSF) in AD patients compared with healthy people [(11)]. The current treatment options for AD patients use acetylcholinesterase inhibitors (AChEIs) drugs, such as Tacrine, Galantine, Donepezil, and Rivastigmine. All of these drugs are already approved by food and drug administration (FDA) which has the mechanism to cease the cleavages of ACh between the neuron synapses. Results showed an increase in ACh concentration in CSF [(12)]. Therefore, ACh can be used as a biomarker to consider for risk diagnosis of AD.

Conventionally, physician defines dementia by using Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) [(13)]. The initial symptoms are memory decline, loss of the language coherence in speaking and

writing, and malfunction of sensory or motor [14]. Pre-dementia stage is an asymptomatic stage, so it is hard to diagnose from behavior. The using of biomarker is considered for helping an early diagnosis. Many biomarkers of AD were reported, such as, high total tau protein, high phosphor-Tau level, low amyloid beta-42(Aβ42), and low ACh concentration in CSF [15-17].

Indeed, the significant changes in ACh concentration can indicate the risk of AD. According to, Jia et al. (2004), the ACh concentration in the CSF is 34.5±9.0 and 10.7±5.1 nmol/L in healthy people and AD patients, respectively [11]. Therefore, ACh biosensor is considered to use as the primary tool for screening the risk of AD. The necessary is the sensitivity of the biosensor. The requirement is a biosensor which can measure ACh concentration at the nanomolar scale. The consequence is the using of the nanomaterial, and modification with microfluidic chip or lab-on-a-chips (LOCs) technology to amplify the sensitivity of the biosensor.

The first biosensor was fabricated by Clark and Lyons. The glucose biosensor was fabricated based on glucose oxidase (GOx) [18, 19]. GOx can induce the hydrolysis of glucose into hydrogen peroxide (H_2O_2). The current was measured by an electrochemical oxidation of H_2O_2, after applied constant potential, which proportional to the glucose concentration. Many electrochemical biosensors have been reported for sensing biological substances, for example, cholesterol, uric acid, and glutamate. [20-22]. Therefore, ACh biosensor is based on the enzymatic reaction by using AChE and ChOx, respectively. The achieving of ACh reaction is also hydrogen peroxide, which is an electroactive substance [23].

The principle factors that effect to the efficiency of the enzymatic biosensor are the enzyme immobilization methods, such as entrapment, adsorption, covalent bonding, cross-linking, and affinity [24, 25]. Each method has the different advantages and drawbacks which require judiciously in choosing. Therefore, the appropriate method of enzyme immobilization can facilitate the performance of the biosensor. Many research used glutaraldehyde (GA) as a crosslinking agents because it is an easy method and uncomplicated. GA can induce the covalent bonding between the electrode surface and the amine groups of the enzymes. The strengthened adhesion of the enzymes on the electrode surface can prevent the elution of the enzymes while biosensor is functioning, especially in the flowing system [26]. In the other hand, the

using of GA also has the drawbacks, which can distort the active side of the enzyme, and consequently lost enzymatic activity [24, 27].

Graphene (GR), a carbon nanomaterials with a 2-dimensions structure, is considered to facilitate biosensor capability [28]. It was discovered in 2004 by K.S. Novoselov, et al. [29]. Because GR carry the outstanding characteristics, such as high electric conductivity, high thermal conductivity, high surface to volume ratio, and robust structure [30]. Varied application of GR is used in the biomedical field, for example, the nanocarrier for drug delivery system, the intrinsic fluorescence for bioimaging, the enhancement conductivity of electrochemical biosensors [31, 32]. The high surface area of graphene (2630 m^2g^{-1}) lead to the increasing of surface for immobilizing biorecognition elements [33]. Therefore, the using of GR will be enhanced the sensitivity of the electrochemical biosensors.

Microfluidic chip or Lab-on-a-chips (LOCs) technology is another application to synergist biosensor properties. The phenomena changing of the microchannel systems are unique and promise, such as, the rapid heat exchange, mass transfer, and diffusion. The miniaturized platforms can decrease analysis time and reagent consumption, and including of the sample volume. These leads to the low-cost manufacturing of the chips but perform a high sensitivity. Because of a small size, so LOCs can perform the fast screening for the simple diagnosis at the point-of-care [37]. Hence, the LOCs are an attractive platform to promote a capability of chemical analysis.

According to the previous study, many applications were developed for lower LOD. S. Sen and his team achieved in fabrication ACh biosensor which had LOD 1.0 µM by using Pt electrode modified surface with polyvinyl ferocenium perchlorate [38]. The development of the thin-film transistor which was made from the self-assemble single-walled carbon nanotube (SWNT) and was deposited the co-enzyme, ACh and ChOx, on the surface, and reported a measured detection limit was 10 nM [39]. Robert J. et al., reported the high sensitivity detection of the biological toxin was 10 pM by using the immunoassay based on the microfluidic chip [40]. The surface plasmon in the microfluidic chip for the detection of the antibody in the saliva was fabricated and can detect in the picomolar scale [41].

In conclusion, this work focuses on the development of the amperometric ACh biosensor by study an enzyme immobilization method, the use of graphene nanomaterials and applied to low-cost microfluidic chip system which is made from the plastic.

1.2 Problem statement

The incidence values of the AD patient tend to increase with the age. The efficient treatment responses better in a pre-symptomatic stage, but does not express in behavior, which is difficult to diagnosis. ACh can serve as a primary AD biomarker for screening. Meanwhile**,** the ACh concentration of normal people and AD patients were presented around 10.7 ± 5.1 to 34.5 ± 9.0 nmol/L [(11)]. Because of the ACh concentration is in a nanomolar scale, so this work particularly focuses on the sensitivity improvement, and the addition is the low-cost fabrication, easily used and disposable microfluidic chip biosensor.

1.3 Objective

1. To fabricate a high sensitivity ACh biosensor for ACh determination
2. To study the enzyme immolibization method for both ChOx and AChE.
3. To enhance biosensor efficacy by using nanomaterials
4. To fabricate the disposable and low-cost microfluidic chip.

.

CHAPTER II
LITERATURE REVIEWS

2.1 Alzheimer's disease

Alzheimer's disease (AD), a neurodegenerative disease, is the one of dementia symptoms which commonly finds in an elderly people. The prevalence tends to increase with age, especially in more than 65-year-old is exponentially increased. There is 13% of 65-year-old people have an Alzheimer's disease and 45% of 85-year-old. Meanwhile, the incidences of AD patients were calculated that it will dramatically elevate in the future by Hebert et al [2]. The prediction is about 5.1, 5.7 and 7.7 million people in 2010, 2020 and 2030, respectively [13]. Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) is the questionnaires for AD diagnosis. The patient who is suspected the dementia must do these questionnaires, but it takes a very long period of time or more than a year to confirm the progress of dementia. The informant questionnaire point will pinpoint a dementia progress [14]. Hence, the biosensor device which has a capability to detect the biomarker of AD is necessary for early diagnosis.

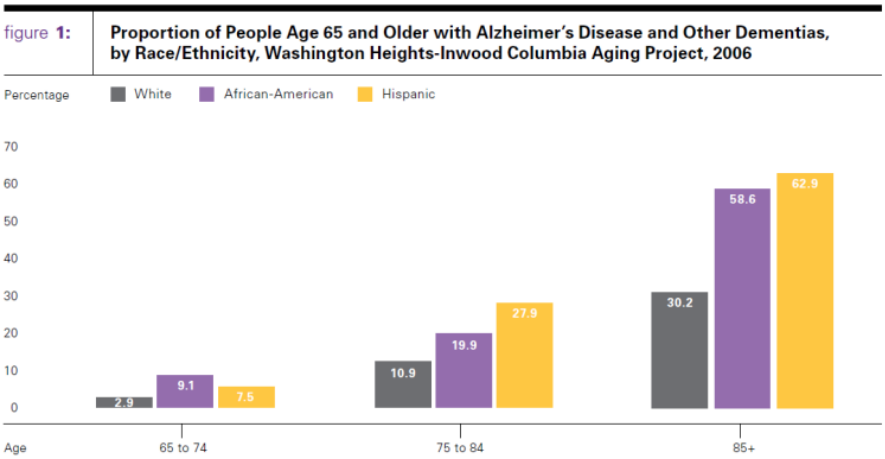

Figure 2.1 The estimation of Alzheimer's disease risks by age and gender [13]

2.1.1 Symptoms of Alzheimer's disease

Because of AD is a neurodegenerative disease. AD patient's symptom will gradually develop. Generally, the initial symptom relates to memory abnormality or defection. For example, hard to remember a new thing and progressively to forget the past experiences. Over the time, the symptoms will more severe, and it is usually irreversible. The disruption of the memory will affect the patient daily life, such as the solving of problems, the disorientation, unable to speak properly, and forget their family members. Meanwhile, the worst cases, advanced Alzheimer's patients, are evitable a caregiver because they are unable to maintain a living basic activity [3, 4, 13].

2.1.2 The causes of Alzheimer's disease

Since 1906, Dr. Alois Alzheimer is a psychiatrist in Germany. He is the first scientist who identified a pathological brain tissue of Alzheimer's disease [42]. One of his patients is 51-year-old women who suffered from memory impairment. Her symptoms are gradually more severe and died after 4 years. He found the unique plaques of protein accumulation in the brain which is called amyloid plagues (APS) and neurofibrillary tangles (NFT). The causes of protein aggregation are still unknown [8], the aggregation of this protein defects to the brain function. The advancing age is exhibited the main factor which is correlated to the accumulation of protein outside the neurons [13]. Well-known neurochemical biomarkers of AD are amyloid beta (Aβ) peptide, total tau protein and acetylcholine [7, 11, 16]. Hence, much research is focused on finding a biomarker, which can be indicated the progressive of AD, especially biological substance which present in CSF.

Much research focuses on finding a biomarker, which can be indicated the progressive of AD. Aβ, a senile plagues component, can be found in extracellular fluid of brain or CSF. The significantly changing of Aβ42, a 42 amino acid in length, was observed. Tremendous research showed the lower concentration of Aβ42 in AD's patients. Aβ42 decreases about 50% when was compared with a healthy people. The cause of Aβ42 reduction remains unknown while the hypothesis is the deposition of Aβ42 in senile plagues. Moreover, the autopsy study illustrated that Aβ42 reduction correlated to the increasing of neocortex and hippocampus plaques. Nevertheless, the Aβ42 reduction is also founded in another neurodegenerative as well.

Tau protein present in the neuronal cell which helps in stabilized the network of neuronal microtubules. The previous study was confirmed the increasing of total tau protein in AD patients. In AD, t-tau concentration increases approximately 300% compared with a control subjects. In the other hand, some report was found increase of t-tau also occurred in another type neurodegenerative diseases [15, 43, 44].

2.1.3 Deficit of acetylcholine in Alzheimer's disease

Acetylcholine is a majority neurotransmitter in central nervous system. ACh significantly decreases in the AD patients. The hypothesis is the accumulation of Aβ42 that lead to the blocking of neuronal cell connection. Generally, ACh is secreted at the terminal of axon for the transmission of the signal to the neighboring neuron. Because of Aβ42 accumulates outside the neuronal cells, consequently the signal transmission is blocked [1, 13]. Jia Jian Ping et al., studied about the differentiation of vascular dementia and Alzheimer's disease by using high-performance liquid chromatography with electrochemical detector (HPLC-ECD) for measurement the ACh and choline concentration in CSF. The result showed that the decrease ACh concentration in CSF remarkable correlates with AD and vascular dementia (VD) patients. The value of ACh concentration is 34.5±9.0, 16.8±7.4, and 10.7±5.1 in normal people, VD, and AD, respectively, which is shown in Table 1. They compared the result with Mini-Mental State Examination (MMSE) score which can indicate the memory impairment. The increasing of MMSE score significantly coincides with the ACh concentration decreasing in CSF. Therefore, ACh concentration decreasing relates with memory impairment [11]. In addition, H. Tohgi et al., also studied in the

Table 2.1 The comparison of MMSE scores with ACh and Choline (Ch) concentration in AD patients and controls or healthy people [11].

Group	Number of sample	ACh (nmol/L)	Ch (nmol/L)	MMSE Scores
AD	22	10.7 ± 5.1	627.6 ± 145.1	15.6 ± 2.9
Controls	20	34.5 ± 9.0	716.0 ± 159.4	28.5 ± 1.2

comparison of MMSE score between AD patients and healthy people, and the results showed in the same way [45].

2.2 Biosensor in medical application

The biosensor technology is developed widely in the recent decades, which a capability to convert a different input, heat, chemical and so on into a measurable signal. Since 1962, the first glucose biosensor was fabricated by Clark and Lyons [18, 19]. Glucose oxidase (GOx) was immobilized on the electrode surface with an oxygen semipermeable membrane, allowing directly measured concentration of the glucose in sample. Normally, biosensor is classified into two main categories: bioreceptor and transducer. Bioreceptor can provide a specific response to each analyze by the recognition element, such as enzyme, DNA probe, and antibody. Meanwhile, the signal generates from the interaction of the bioreceptor and the analyze sample, the transducer performs as a signal conduction part to the signal processing part. Therefore, the judicious development of the transducer will amplify the signal collection of biosensor [46, 47]. Much research focuses on using biosensor for the chemical analysis in diverse fields, such as, clinical diagnosis, environment, and food. The evaluation of the biosensor performance is commonly determined by sensitivity, specificity, reproducibility, stability and response time.

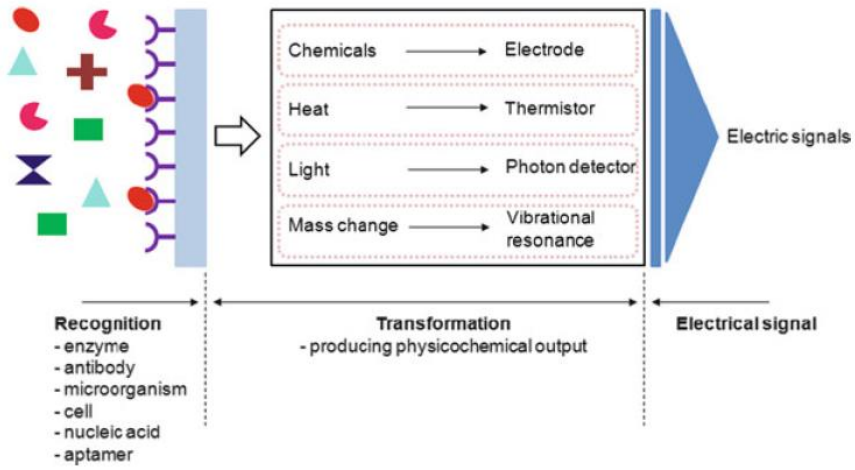

Figure 2.2 The principle components of biosensor [34].

The development of the diagnostic biosensor certainly prospers the medical field, particularly in order to help the early diagnosis of diseases. The various types of the biosensor were reported for the cancer biomarker detection, the DNA analysis, autoimmune diseases, infection diseases, cardiac biomarkers, and saliva diagnosis [48]. Moreover, the usefulness of biosensor is the avoiding of sample preparations and complicated techniques, which require specialist to operate.

2.2.1 Electrochemical biosensor

In this project, the electrochemical biosensor is fabricated for ACh detection by using the amperometric detection technique. Commonly, the electrochemical cell consists of working electrode, reference electrode, and auxiliary or counter electrode. The working electrode serves as a platform for electrochemical system. Following the biocatalytic reaction between the substrate and biological receptor, the electroactive species is produced. By using the amperometric detection, the constant potential is applied to the working electrode versus a reference electrode (Ag/AgCl), subsequently, the redox reaction that generates the measurable current. The auxiliary electrode always uses the inert material: gold, platinum or carbon. It can alter as a cathode or anode which depends on the operation of working electrode, and it helps for the reaction balancing. When the oxidation occurs at the working electrode, current is transported passing in the transducer. The response of detection can measure as a peak of the current which relates to the electroactive species concentration in the system.

. Acetylcholine, a principle neurotransmitter in CNS, is not the electroactive substance. The enzymatic reaction is shown in fig.3. AChE performs as a biological recognition element for providing ACh binding site. Because of the enzymatic reaction, ACh is hydrolyzed into a choline and acetate. Because of the presence of ChOx, the hydrolysis of choline is followed, and H_2O_2 is formed as the product.

$$\text{acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{choline} + \text{acetate}$$

$$\text{choline} + H_2O + 2O_2 \xrightarrow{\text{ChOx}} \text{betaine} + 2H_2O_2$$

Figure 2.3 Enzymatic reaction of AChE and ChOx [23]

Table 2.2 Conclusion of some applications for ACh detection

Method	Material	Enzyme	Immobilization Method	Linearity	LOD	Reference
Amperometric Biosensor	Poly(SNS-NH_2) Graphite electrode	AChE, ChOx	Covalent by GA	0.12 – 10 mM	111 uM	55
Amperometric Biosensor	PVA Pt. electrode	AChE ChOx	Adsorption in PEG	5 – 100 uM	2 uM	56
Amperometric Biosensor	AuNP-AChE /MWCNTs/ChOx Pt.electrode	AChE ChOx	Sol-gel and self assembly	5 – 400 uM	1 uM	23
Amperometric Biosensor	Lichen-like nickel oxide Carbon paste electrode	-	-	0.25 – 5.88 mM	26.7 uM	59
Optical fluorescence	H_2O_2-sensitive Quantum dots	AChE ChOx	-	10 – 5000 uM	10 uM	58
Liquid Chromatography – Electrochemistry	Microdialysis Horseradish peroxidase Redox polymer-coated Glassy carbon electrode	AChE ChOx	-	0.3 – 10 nM	0.3 nM	60
Liquid Chromatography – Electrochemistry	Osmium-peroxide redox polymer Au-ring disc electrode	AChE ChOx	Covalent by GA	-	10 fmol	61

Constant applied potential will induce the redox reaction which generates the measurable current [23].

2.2.2 Biosensor applications for acetylcholine detection

Many applications were developed for ACh detection. Because of the correlation between ACh concentration and the neurodegenerative disease, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, were reported [1]. Much research focuses on the application to measure *in vitro* and *in vivo* ACh concentration [49]. There are many methods for ACh measurement such as, the electrochemical measurement, the microdialysis technique [50], and the high-performance liquid chromatography (HPLC) [51]. Each measurement method has the different advantages and drawbacks, such as microdialysis and HPLC can perform a high accuracy and very low LOD. On the contrary, it requires the complication methods that must operate by specialist and specific device in the laboratory [52]. Therefore, the fabrication of acetylcholine biosensor is necessary because tremendous advantages of the amperometric biosensor, such as the low-cost fabrication, easy to use, and rapid response. Meanwhile, principle of amperometric measurement technique is the current detection, so the working electrodes were modified with conductive polymer or conductive material to increase biosensor sensitivity and decrease the detection time [53, 54].

Fulya E.K. et al. fabricated the ACh amperometric biosensor by coating a conducting polymer 4-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)benzenamine (SNS-NH2) on a graphite electrode. SNS-NH2 can promote the electron transfers, and the amine functional group of polymer can induce the covalent bond with the enzymes [55]. The sponge-liked structure which was made from poly(ethylene glycol) (PEG) and poly(vinyl alcohol) (PVA) by Lucio D. el at. As the result, the polymeric matrix can act as the enzyme containers [56]. Another strategy for increasing the biosensor sensitivity is the using of nanomaterials. Shihuo H. and teams modified working electrode (platinum electrode) with gold-nanoparticles and multi-wall carbon nanotube, which is high conductivity and large surface to volume ratio. All of the modified materials are immobilized on the silica sol, support binding or carrier, to prevent the elution of the nanomaterials in the sample solution [23]. The using zinc

oxide (ZnO) as a support binding was also reported by Minghui Y. et at. Moreover, they used N-acetylaniline (nAN) as a permselective membrane to prevent the interference from another the electroactive substances. This membrane permeates only H_2O_2, so it can completely prevent the unwanted electroactive oxidation, such as ascorbic acid and uric acid [57]. Zhenzhen C. et al., developed the optical acetylcholine biosensor by using Cd-Te quantum dots (QDs). QDs quenching changed because of the presence of H_2O_2. Moreover, the quenching degree relates with ACh, AChE and ChOx concentration [58].

2.3 Amperometric Biosensor based on nanomaterial modification

The principle of the amperometry is the current detection which generate from the redox reaction [54]. Therefore, much research has a trending to modify the working electrode surface in many different ways. The using of the nanomaterials, such as graphene, carbon nanotubes, and gold nanoparticles, can increase surface to volume ratio which is essential for enzyme immobilization [63, 64].

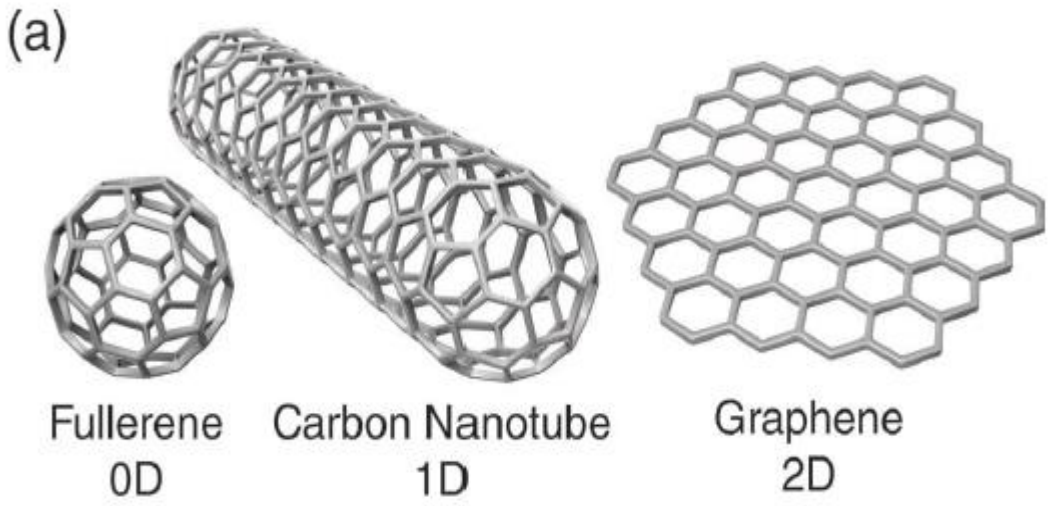

Figure 2.4 Different types of carbon allotropes [65]

2.3.1 Graphene

Graphene, a monolayer of carbon atom consisting of sp^2-bonded carbon, is contained the unique properties [28, 66], which can improve the performance of the electronic devices, such as high electric conductivity, high thermal conductivity, high surface to volume ratio, and robust structure [30]. The combination of graphene with another material: polymer, metal, and oxide, were used to enhance the thermal conductivity, electrical conductivity and flexibility [65]. Graphene exhibits the very high surface area of 2630 m^2 g^{-1}, compared with graphite and single-walled carbon nanotubes (SWCNT) is only 10 m^2 g^{-1} and 1315 m^2 g^{-1}, respectively [30, 33]. S. Alwarappan et al. reported the conductivity of graphene (64 mS cm^{-1}) is 60-fold higher than SWCNT [67]. The graphene modified glassy carbon electrode for detection paracetamol was fabricated by X. Kang et al. The experiment showed the increasing of oxidation and reduction peak of cyclic voltammetry when compared with bare glassy carbon electrode [68]. Ming Z. et al., modified electrodes with chemically reduced graphene oxides (CR-GO). The results showed that CR-GO definitely increase catalytic activity of H_2O_2 in the electrochemical measurement [69]. Changsheng S et al., used polyvinylpyrrolidone-protected graphene modified glassy carbon electrode and polyethylenimine-functionalized ionic liquid for fabrication of glucose biosensor. The linearity is 2 to 14 mM, which is adequate for determination of blood glucose in practical application [70].

Graphene-PEDOT:PSS (GR-PEDOT:PSS) is the nanocomposites which enhance the performance of electroanalytical chemistry, such as reduces the detection potential, improves the amperometric current response, and offers good stability. the acetylcholine biosensor based on GR-PEDOT:PSS modified electrode exhibits the good sensitivity and high current response. Furthermore, it provides the high surface area which is effective enzyme loading.

2.3.2 Carbon nanotubes (CNTs)

CNTs is another type of a carbon allotrope, which have a special exclusive structure, a cylindrical shaped. There are two types of CNTs (1) Multi-walled carbon nanotubes (MWCNTs) and (2) Single-walled carbon nanotubes (SWCNTs). The numerous porous of CNTs structure is very appropriate to anchor the biological

molecule, such as enzyme, DNA, and antibody. Furthermore, CNTs also have a notable electrical conductivity and biocompatible [71].

Since 1996, the first CNTs modified biosensor for the dopamine detection was fabricated by Britto et al [72]. Much research attempts to use CNTs with another electrochemical biosensor. For comparison between CNTs electrodes and the conventional carbon electrodes, CNTs definitely surpasses the conventional carbon electrodes, such as increase sensitivity, less time response, and decrease LOD. M. Musameh et al., reported a glassy carbon electrode modified with CNTs provided a lower constant potential in NADH detection [73]. However, the restriction of CNTs is the hydrophobic properties, which cannot dissolve in the aqueous solution, unless in non-polar organic solution or oxidative acid process. For example, sonicated CNTs with nitric acid which defects CNTs surface for producing carboxylated sites, consequently the dissolution of CNTs [62].

2.4 Enzyme-based electrochemical biosensor

The enzyme-based electrode, a coating of the enzymes on an electrode surface, is extensively popular fabrication for the electrochemical biosensor. Enzyme is the protein biological substance, which carry a characteristic binding site [53]. The property of the binding site handles a high specificity to catalyze a specific substance, and produces a detectable electroactive product such as hydrogen peroxide (H_2O_2) [53]. The benefits of the enzymes are the rapid catalysis the substance into the product, consequently is the fast response which can show the results immediately. This is suitable for the point-of-care. The monitoring to prevent loss activity of the enzymes must be considered prudently because enzyme is the protein which is sensitive to denature by the inappropriate environment. Therefore, the enzyme immobilization method must be friendly for maintenance enzyme activity and the stability of the enzyme which relates to the lifespan of biosensor [24, 74]. Generally, the enzyme immobilization method can be classified into three methods: adsorption on a supporter, cross-linking, and encapsulation with carrier.

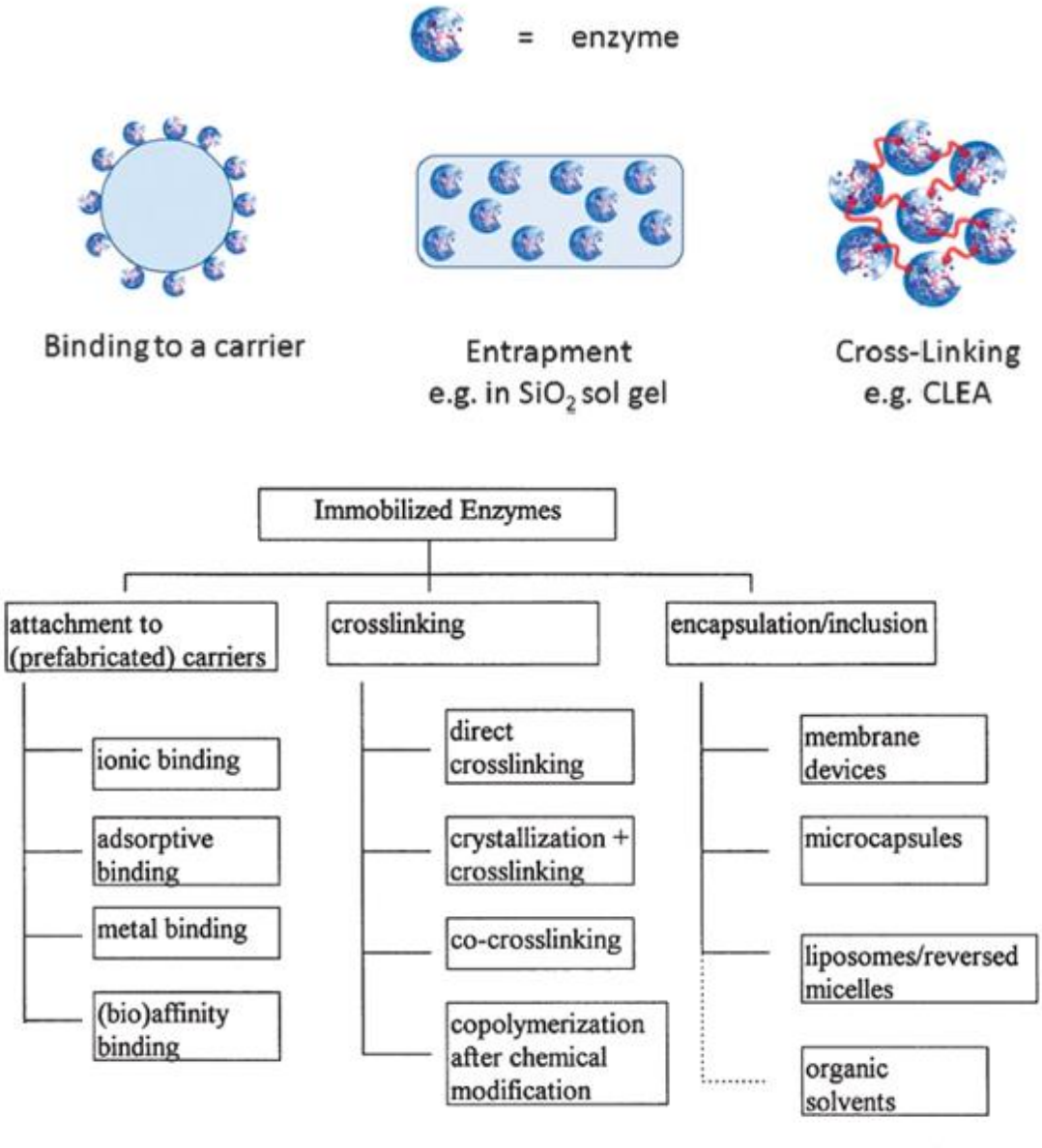

Figure 2.5 Typical methods for enzyme immobilization [25, 75]

2.4.1 Adsorption

The adsorption is the simplest way for the enzyme immobilization. Enzyme is deposited by dropping onto the working electrode surface, and it is bound by van der Waals force, electrostatic or hydrophobic interaction. Nevertheless, the physical adsorption is fragile, very easy to elute out. Consequence is the using of the carriers or binding supporters for strengthening the enzyme immobilization. The emphasis of the enzymes ability is the conformation of the protein folding, so the

carrier biocompatible is essential. Normally, the carriers are usually made from a natural, organic or inorganic polymer, such as a polysaccharide such as chitosan and agarose, a synthetic organic polymer such as mesoporous silicas, and the biocompatible polymer such as, PEG, PVA, and poly-N-isopropyl acrylamide (polyNIPAM). Occasionally, the carrier is called as the prefabricated supports, there is no presence of the enzymes while the carrier process, the immobilized enzyme will be attached on the outermost of the carrier platform [25].

2.4.2 Crosslinking

Cross-linking is the method to induce the covalent bonds between each amino group of enzyme molecules by using the bi-functional compound or cross-linking agent, such as GA. The covalent bond performs more robust than the adsorption method. On the contrary, the covalent bonds can alter the conformation of the enzyme which lead to the changing of enzyme binding side. Y-G. Li et al., studied the AChE immobilization on the SPE by glutaraldehyde vapor. The results showed that the covalent bonds successfully prevent an enzyme dissolving in the flow system, but vapor prolongation significantly influences the loss of enzyme activity [26].

2.4.3 Entrapment

The entrapment is the fabrication of platforms which entraps the enzyme inside. The enzyme is presence while the platform fabrication process. The lattice structures are prohibited the leaking of the enzyme, but allows the small substance to diffuse through. Sol-gel is widely used for amperometric biosensor. For example, the silica sol-gel for AChE and horseradish peroxidase (HRP) immobilization [76, 77], the carbon nanotube aqueous sol-gel which is enzyme-friendly environment [78]. The importance is a sol-gel formation process, which must be judiciously controlled, such as, thermal, chemical and pH, for preventing enzyme denature. Moreover, the porosity of the sol-gel matrix is another factor. If, the pore is too large, the enzyme can leak out easily [75, 79].

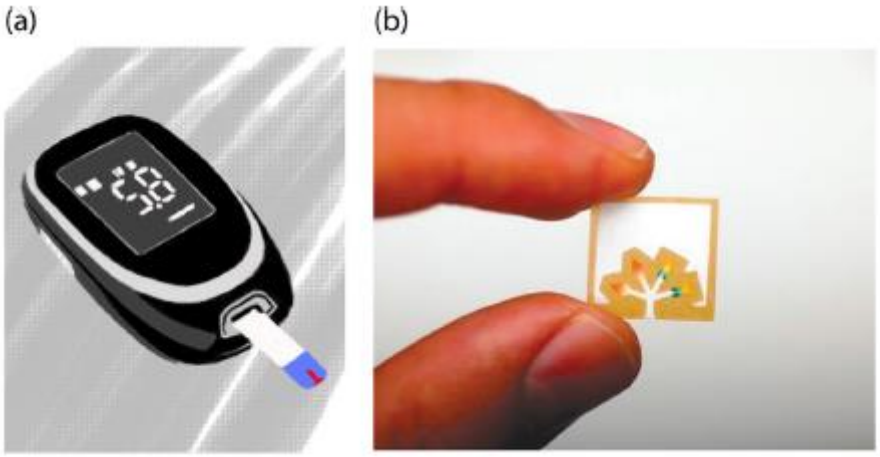

Figure 2.6 (a) A small size glucose biosensor, (b) a paper-based microfluidic chip [80].

2.5 Microfluidic chip technology

Microfluidic chips or Lab-On-a-Chip technology (LOC) is a small size chip which containing a laboratory process inside. Normally, LOC technology usually integrates with the micro total analysis systems (μTAS) and the biomedical microelectromechanical systems (BioMEMs). Most of LOC were developed for the medical diagnosis, especially in the molecular analysis, the biodefense, the molecular biology, and the microelectronics [81]. The principle of LOC is a miniaturizing into a micro size or lower, consequently the special phenomena changing which cannot achieve at a normal scale, such as, the laminar flow or continuous flow in the microfluidic channel. The microchannel inside the LOC system exhibits the high diffusion rate, mass transfer, and high surface to volume ratio. Herein, it can carry the high efficiency of chemical reaction and sensitivity increase in the electrochemical field, especially simultaneous diagnosis at the point-of-care (POC). Moreover, the small size of LOC can decrease the fabrication cost, so it is the disposable platform immediately after use [82, 83].

2.5.1 Microfluidic chip in biosensor applications

The outstanding characteristic of the microfluidic chip is the new aspect. In recent decade, the study of the microfluidic chip increases exponentially because it has a potential to improve more complicated in experiment. Jinseok H. and Richard M.C. were fabricated the optical biosensor which bases on the array microfluidic chip

for glucose, galactose and paraoxon detection. The enzymes were immobilized in the hydrogel micro patch, and placed the hydrogel patch inside the microchannel. H_20_2, the product from enzymatic catalysis, can react with a presence of non-fluorescent Amplex Red, so it generates the different fluorescence intensity in the different concentration of glucose. They suggested that this technique certainly can use in the electrochemical biosensor as well [(84)]. Microfluidic biosensor based on optical fluorescence for DNA detection was also reported by Jun W. et al. [(85)]. The microfluidic chips which have a capability to quantify *Escherichia coli* bacteria were reported by M. Safavieh et al. The microchannel is made of poly-dimethyl siloxane (PDMS) by soft-lithography. There are two parallels of the microchannel which is positive and negative control sample. Meanwhile, each microchannel consists of loop-mediated isothermal amplification and detection chamber, for amplification DNA target and electrochemical measurement, respectively. The DNA existences will attract with the redox molecule, subsequently the decrease of the anodic oxidation peak [(86)].

Furthermore, the varieties of the microfluidic chips were reported for the biomarkers of the medical diagnosis, such as, cholesterol, creatinine, glucose, and L-glutamate [(87-90)]. Chu-Ya Y. et al., discovered the notable interleukin-8 biosensor which using the surface plasmon technique integrated microfluidic channel. The exclusivity is a small amount of sample, 100 microliter , and the remarkable LOD, 186 pM, within 13 mins [(41).]

The principle of electrochemical biosensor is biological analysis, which usually involves in chemical reaction. While the features of microfluidic chips are the phenomenon changing in microchannel, which is able to stimulate the chemical reaction, such as, a fast diffusion, rapid mass transfer, high surface to volume ratio, and etc. Moreover, the using of the nanomaterials to modify the working electrode is considered. It certainly increases conductivity, and surface area for the biorecognition element immobilizatiton. The enzyme immobilization strategy is also judiciously considered, which must have the robustness and maintaining enzyme activity. Therefore, the heart of development is not to find a new mechanism for ACh detection, but for developing the better system which is easy to use, higher efficient, more convenience, and low-cost fabrication.

CHAPTER III
MATERIALS AND METHODS

This chapter introduces and explains the materials and methods that were used in the experiment. First topic describes about the chemical reagents and the instrument. Second topic describes about the experiment plans and processes.

3.1 Materials

3.1.1 Chemical reagents

Table 3.1 List of chemical reagents and the source company

Chemical Name	Company
Acetylcholinesterase from *Electrophorus electricus* (electric eel)	Sigma
Choline Oxidase from *Arthrobacter globiformis*	Sigma
25% Glutaraldehyde solution	Sigma
75-85% deacetylated chitosan	Sigma
Acetic acid (CH_3COOH)	Sigma
Functionalized - multi-walled carbon nanotubes (MWCNT)	Sigma
Functionalized - multi-walled carbon nanotubes (MWCNT)	Sigma
Graphene-PEDOT:PSS conductive ink	Innophene

Table 3.1 List of chemical reagents and the source company (cont.)

Chemical name	Company
Sodium phosphate monobasic (NaH_2PO_4)	Sigma
Sodium phosphate dibasic (Na_2HPO_4)	Sigma
Sodium chloride (NaCl)	Sigma
Choline chloride	Sigma
HEPES buffer solution	Sigma
Potassium chloride (KCl)	Sigma

3.1.2 Instruments

- Mini Potentiostat (910 PSTAT mini, Metrohm)
- Analytical Balance (OHAUS, Pioneer)
- Centrifuge Mixer (CM-50, Fugamix)
- Ultrasonic cleaner GT-1730QTS, Comcube company)
- Automatic pipette (Gilson, Inc.)
- Digital hotplate magnetic stirrer (AMTAST-PRO)
- Oven (Fuzzy control system, Wiseven)
- Dimatrix material printer (Fujifilm)
- Craft Robo-Pros (CE-5000-40-CRP)
- SEM LV Mode (Low vacuum)

3.1.3 Reagent preparation

- **0.01 M HEPES buffer solution**

100 µl of 1 M HEPES buffer was added into 9.9 ml deionized water for achieving 0.01 M HEPES buffer solution.

- 0.1 M phosphate buffer solution, pH 7.4

Firstly, 6 g sodium phosphate monobasic (NaH_2PO_4, MW = 119.98) was dissolved in 500 ml deionized water to achieve 0.1 M NaH_2PO_4 solution. Next, 7.15 g sodium phosphate dibasic (NA_2HPO_4, MW = 141.96) was dissolved in 500 ml deionized water to achieve 0.1 M NA_2HPO_4 solution. 0.1 M NA_2HPO_4 solution is the alkaline solution. pH was measured by the sensitive pH meter. pH will gradually decrease because of the addition of 0.1 M NaH_2PO_4 solution. We added 0.1 M NaH_2PO_4 solution until pH 7.4

- Enzyme solution

Choline oxidase solution

Choline oxidase dry powder (500U/bottle) was purchased from Sigma Aldrich, Singapore. ChOx powder, containing 500U, was added in 1.5 ml of 0.01 M HEPES buffer. 3 µl/U of ChOx enzyme solution was achieved. The enzyme solution was stored in - 4 C°.

Acetylcholine esterase solution

Acetylcholine esterase dry powder (2000 U/bottle) was purchased from Sigma Aldrich, Singapore. AChE powder, containing 2000 U, was added in 10 ml of 0.01 HEPES buffer. 5 µl/U of AChE enzyme solution was achieved. The enzyme solution was stored in - 4 C°

- MWCNT dispersion in chitosan

1 mg/ml MWCNT-CS was prepared by the following step. Firstly, chitosan 5 mg was dissolved in 1 ml of 1% acetic acid. This mixture was stirred by magnetic stirrer for 15 minutes. The homogeneous 0.5% chitosan solution is achieved. Then added 1 mg MWCNT powder in 1 ml of 0.5% chitosan solution, and sonicated for 2 hours. The homogeneous colloid suspension of MWCNT-CS was achieved.

- 1% nafion solution

In order to prepare 1% nafion solution, 200 µl of 5% nafion solution was diluted 800 µl of ethanol.

- Acetylcholine chloride solution

Acetylcholine chloride (MW = 181.66) solution was prepared by dissolved in pH 7.4 phosphate buffer solution. 0.018 g AChCl was added into 10 ml of pH 7.4 PBS, consequently achieving 1 mM AChCl solution. For another lower concentration of AChCl solution, they were gotten from series dilution of this solution.

- Choline chloride solution

Choline chloride (MW = 139.62) solution was prepared by dissolved in pH 7.4 PBS. 0.014 g ChCl was added into 10 ml of pH 7.4 PBS, consequently achieving 1 mM ChCl solution. For another lower concentration of ChCl, they were gotten from series dilution of this solution

- Glutaraldehyde solution

2 ml of 50% glutaraldehyde solution was mixed with 8 ml of deionized water to achieve 10 ml of 10% glutaraldehyde.

3.2 Methods

3.2.1 Electrochemical measurements

All of the electrochemical measurements were operated by using Mini Potentiostat (910 PSTAT mini, Metrohm, Thailand). The amperometric detections were performed in the suitable constant potential. Electrochemical system consists of 3 types of electrodes, the working electrode (screen-printed electrode, Quasense, Thailand), the Ag/AgCl reference electrode, and the platinum counter electrode. The buffer solution that was used in all experiments is the 0.1 M phosphate buffer solution, pH 7.4

In this study, there are 2 types of electrodes

- The screen-printed electrode that contains only the working electrode (Figure 3.1 A), and was used in the conventional electrochemical system. In this system, it has conventional platinum rod performing as the counter electrode, and conventional Ag/AgCl performing as the reference electrode.

- This is the new designed screen printed electrode (Figure 3.1 B) for the microfluidic chip platform. Three types of electrodes, working, reference, and counter electrode, were printed on the single substrate. This newly designed electrode is easy to use, because we can directly drop the sample on the electrode for evaluation.

3.2.2 Preparation of nanomaterials modified electrode

The nanomaterials that was used to modify the electrodes were consisted of two kind nanomaterials, GR-PEDOT:PSS and MWCNT-CS. 2 µl of GR-PEDOT:PSS was directly deposited onto working electrode surface of SPE and allowed to dry for 1 hour at 37 °C. GR-PEDOT:PSS modified screen-printed electrode (GR-PEDOT:PSS/SPE) was achieved. MWNCT-CS modified screen-printed electrode (MWCNT-CS/SPE) was prepared by the same process, but used the 2 µl MWCNT-CS instead of GR-PEDOT:PSS solution.

3.2.3 Assessment of nanomaterial modified electrode

In order to assess the enhancement of electrode conductivity by nanomaterial, three types of electrodes, SPE, MWCNT-CS/SPE, and GR-PEDOT:PSS/SPE were used in this experiment. All of them were used to detect the hydrogen peroxide (H_2O_2) by amperometric technique. This technique controls the constant applied potential of system. Constant applied potential induces the redox reaction of H_2O_2. H_2O_2 was varied into several concentrations, 1, 5, 2.5, 1.25, and 0.625 mM.

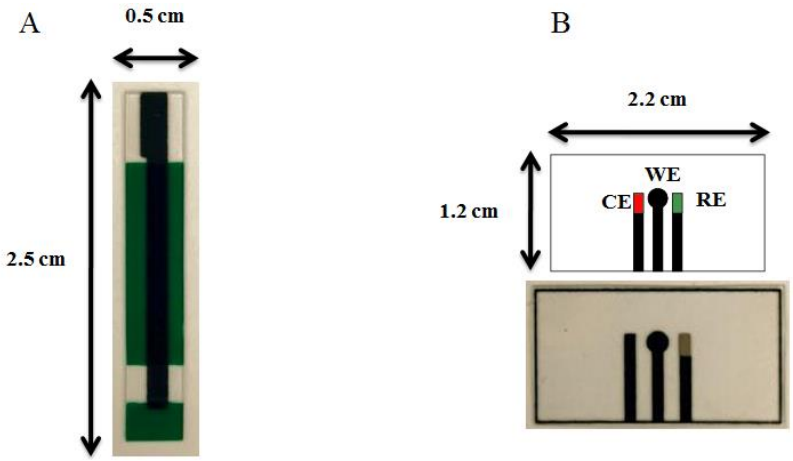

Figure 3.1 A: Carbon based screen printed electrode, B: Carbon based screen printed electrode for microfluidic chip platform

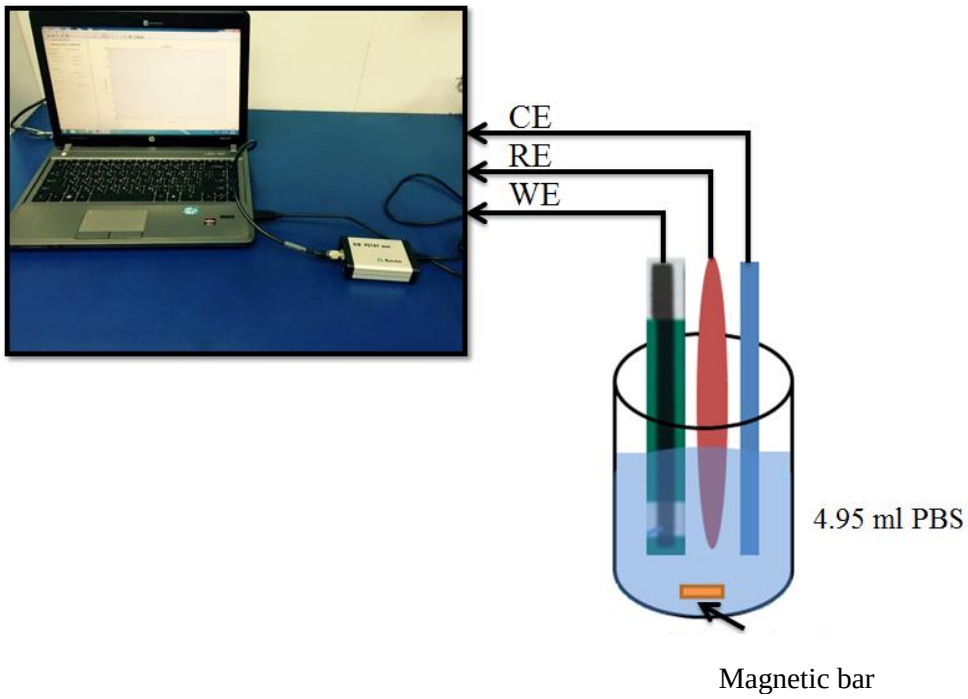

Figure 3.2 Conventional electrochemical system of the amperometric detection

3.2.3 Assessment of nanomaterial modified electrode

In order to assess the enhancement of electrode conductivity by nanomaterial, three types of electrodes, SPE, MWCNT-CS/SPE, and GR-PEDOT:PSS/SPE were used in this experiment. All of them were used to detect the hydrogen peroxide (H_2O_2) by amperometric technique. This technique controls the constant applied potential of system. Constant applied potential induces the redox reaction of H_2O_2. H_2O_2 was varied into several concentrations, 1, 5, 2.5, 1.25, and 0.625 mM.

Working electrode surface was immersed into the solution and the measurement was started. The electrochemical system containing 4.95 ml of pH 7.4 PBS with constant applied potential, 700 mV versus Ag/AgCl reference electrode (Figure 3.2). After the signal reaches to the stable baseline, 50 µl of H_2O_2 was added into the system.

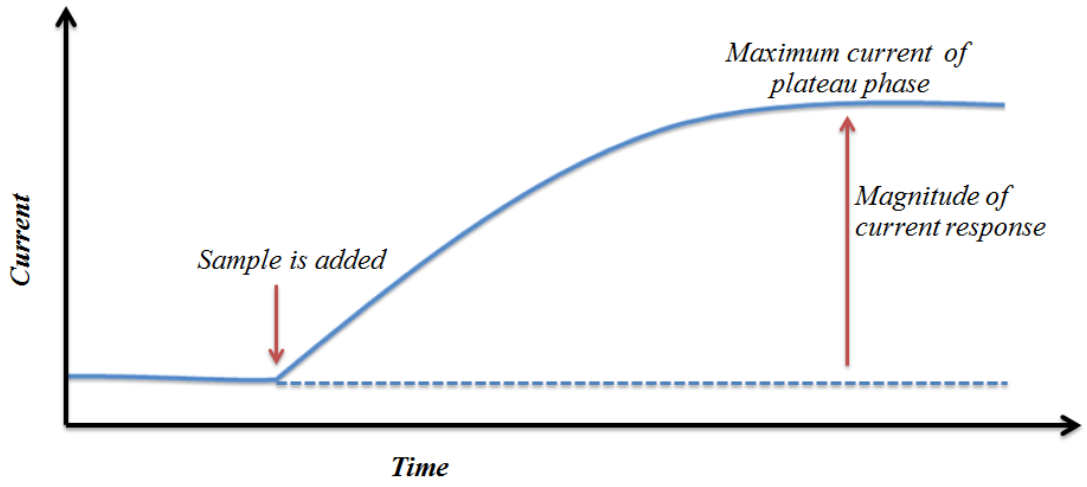

Figure 3.3 Illustration of experimental step for evaluation of the current response by amperometry

After substrate was added, the peak of current response that proportionally relates with the substrate concentration will occur (Figure 3.2). Current response is calculated from the magnitude of current response.

The assessment comes from the sensitivity comparison between 3 types of electrode, SPE, MWCNT-CS/SPE, and GR-PEDOT:PSS/SPE. The sensitivity is calculated from the slope of standard curve that acquires from the determination of various H_2O_2 concentrations, 1, 5, 2.5, 1.25, and 0.625 mM.

3.2.4 Optimization of constant applied potential

Amperometry was selected in this experiment. In order to choose the appropriate constant applied potential, we varied the constant applied potential in the range of 0.0 to -0.4 volt. ChOx was immobilized by the physical adsorption on the working area of screen-printed electrode which is called ChOx/GR-PEDOT:PSS/SPE. Enzyme modified electrodes were compared with non-enzyme modified electrodes. This is to confirm that the current response arise from the enzymatic reaction.

3.2.5 Morphology study

The morphology of electrode surface was observed by using the scanning electron microscope (SEM LV Mode, low vacuum). Bare SPE surface was compared with GR-PEDOT:PSS/SPE.

3.2.6 Enzyme immobilization

In this study, ChOx was immobilized on the working electrode surface. Enzyme immobilization was performed by four different methods, (1) ChOx/GR-PEDOT:PSS/SPE, (2) ChOx/GR-PEDOT:PSS/SPE with GA vaporization, (3) Nafion/ChOx/GR-PEDOT:PSS/SPE, and (4) Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vaporization. All of the immobilization methods used the same amount of enzyme concentration which is 1 U ChOx.

The first enzyme immobilization method is the physical absorption. The 3 µl ChOx solution (1 unit ChOx) was deposited on the GR-PEDOT:PSS/SPE working surface, and allowed to dry at room temperature (25 °C) for 1 hour. ChOx/GR-PEDOT:PSS/SPE was acquired. In order to remove the free enzyme, the electrode was washed by using automatic pipette injection of 1 ml PBS for 3 times.

The second method is the immobilization of enzyme by chemical cross-linked. Glutaraldehyde, a bi-functional group, was selected as a cross-linked agent. ChOx solution was deposited on the GR-PEDOT:PSS/SPE working surface, and allowed to dry at room temperature (25 °C) for 1 hour. Enzyme modified electrode was brought to place in the beaker volume 250 ml which contains 5 ml of 10% glutaraldehyde for 8 mins. The beaker was closed by the parafilm. The vaporization of glutaraldehyde inside the close system will induce the cross-linked of enzyme. In order to remove the free enzyme, the electrode was washed by using automatic pipette injection of 1 ml PBS for 3 times. Finally, ChOx/GR-PEDOT:PSS/SPE with GA vaporization was complete.

The third method is the immobilization of enzyme by using the thin film covering. Nafion, a conducting polymer, was used in this procedure. ChOx/GR-PEDOT:PSS/SPE was prepared as the previous method. After washing for removing the free enzyme, then 3 microliter of 1% nafion solution was deposited on the enzyme layer, and allowed to dry at room temperature (25 °C) for 1 hour. Finally, Nafion/ChOx/GR-PEDOT:PSS/SPE was complete.

The last method is the immobilization of enzyme by using both chemical cross-linked and the thin film covering. For the cross-linked procedure, the electrode was processed as same as the second method, ChOx/GR-PEDOT:PSS/SPE with GA vaporization. After washing for removing the free enzyme, then 3 microliter of 1% nafion solution was deposited on the enzyme layer, and allowed to dry at room temperature (25 °C) for 1 hour. Finally, Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vaporization was complete.

3.2.7 Assessment of enzyme immobilization

The assessment of enzyme immobilization was performed by using the amperometry. All of the enzyme immobilization methods were evaluated in the same condition for determination of 1 mM choline chloride, -0.2 volt versus Ag/AgCl. The current response was compared and evaluated.

3.2.8 Optimization of enzyme concentration

This study has 2 steps: Optimization of ChOx concentration, and Optimization of AChE concentration. In order to evaluate the optimal concentration, enzyme was immobilized on the working electrode surface in the different concentrations, 0.5 unit to 3.0 unit. All of them were used for the determination of substrate in the same condition by amperometry. The current response was recorded and observed to optimize the suitable concentration.

$$\text{acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{choline} + \text{acetate}$$

$$\text{choline} + H_2O + 2O_2 \xrightarrow{\text{ChOx}} \text{betaine} + 2H_2O_2$$

Figure 3.4 Hydrolysis of enzymatic reaction for acetylcholine detection

3.2.9 Assessment of calibration curve

This step of experiment is the procedure for indicating the range of acetylcholine chloride measurement. In order to generate the calibration curve, the known acetylcholine concentration, 0.01 mM to 2.0 mM, was detected by acetylcholine sensor with the amperometric method. The current response were recorded and plotted to find the linearity range and sensitivity of our acetylcholine sensor.

3.2.10 Design and fabrication of microfluidic chip

The microfluidic chip is contained three layers, screen printed carbon electrode (SPE), adhesive layer, and microfluidic channel. They were designed by Adobe illustrator CS6 software. The electrode design was manufactured by the Quasense Company, SPCE manufacturer in Thailand. SPEs consist of a working electrode (WE), a counter electrode (CE), and Ag/AgCl reference electrode (RE) (Figure 3.5). SPCE size is 22 mm in length and 12 mm in width. The inkjet printer (Fujifilm Dimatix Materials Printer) (Figure 3.7 (a)) was employed for printing of GR-PEDOT:PSS layers on the working electrode of SPEs. Adhesive layer is made of double side ethylene vinyl acetate (EVA) or a commercial adhesive tape 0.1 mm in thickness. The microfluidic channel is made of transparent polypropylene (PP) sheet 0.1 mm in thickness. Both of adhesive layer and microfluidic channel was cut into the same pattern by Craft ROBO-Pros (CE5000-40-CRP) (Figure 3.6 (b)). The microfluidic channel is 11 mm in length and 1 mm in width. At the both sides of terminal channel have the inlet and outlet which has a diameter 2 mm and 3 mm, respectively. The PP channel was adjoined with the adhesive layer meanwhile the other side of adhesive layer was adjoined to SPCE. The microfluidic chip fabrication was completed.

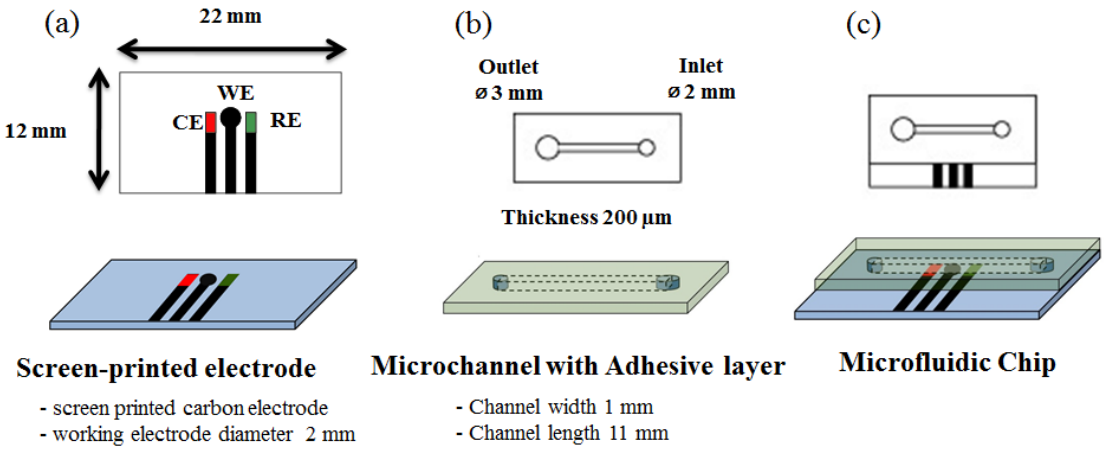

Figure 3.5 Schematic of microfluidic chip assembly including of (a) SPE and (b) microchannel with adhesive layer. These components will be connected together in the final step of microfluidic chip fabrication.

3.2.11 Assessment of the laminar flow in microfluidic chip

A laminar flow or the streamline flow is the phenomena that occur inside the small microfluidic channel. The fluid can flow with parallel line behavior. The fluid will flow consecutively without mixing. Actually, the factor that relates with the laminar flow is the Reynold's number (RE). It can calculate by the following equation (Figure 3.7). If RE is less than 2000, the fluid inside will have the laminar flow behavior. The laminar flow can be tested by using two solutions which has different color, green and red. Firstly, the red solution was added into the microfluidic chip via inlet channel. Then, the green solution was added subsequently. If our microfluidic chip has a laminar flow behavior, the green solution will push the red solution to the outlet without mixing.

3.2.12 Electrochemical measurements in microfluidic chip platform

The electrochemical sensing in the microfluidic chip platform was also performed by the amperometry at the appropriate constant potential. The microfluidic channel modified electrode was connected with the connector and Potentiostat (910 PSTAT mini). The calculation of the volume inside the chip channel is approximately 11 µl. Therefore, 6 µl PBS was firstly added into the inlet channel. PBS gradually flows to the working area and outlet. Subsequently, we started the amperometric measurement. After the signal reached to the stable baseline, 5 µl of substrate solution

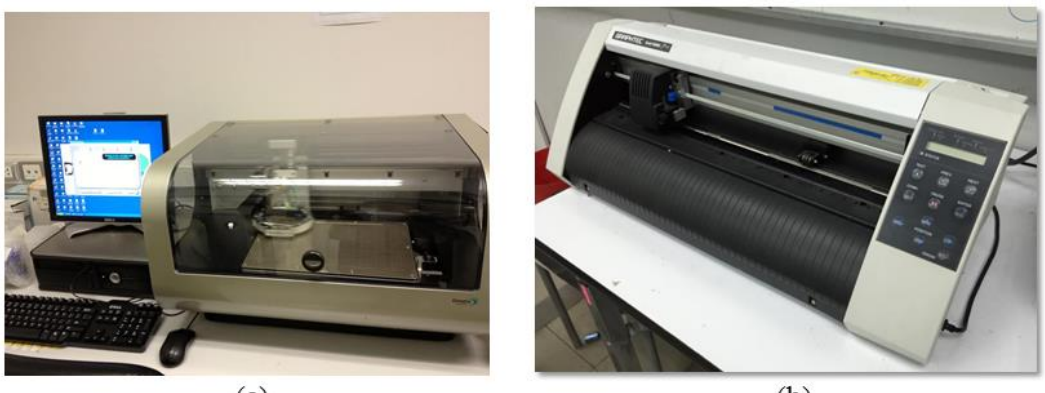

Figure 3.6 Microfluidic chip fabrication equipment: (a) Fujifilm Dimatix Materials Printer and (b) Craft ROBO-Pros (CE5000-40-CRP)

was added into the inlet channel. Because of the laminar flow, the substrate will push PBS to the outlet without mixing. The current response which comes from enzymatic reaction was observed and evaluated

$$Re = \frac{\rho V D}{\mu}$$

where

Re is Reynold's number
ρ is the density of the fluid (kg/m^3)
V is the velocity of the fluid (m/s^2)
D_H is hydraulic diameter (m)
μ is viscosity of the fluid
A is cross sectional area
P is wetted perimeter

For rectangular shape diameter is calculated from Hydraulic diameter

$$D = D_H = \frac{4A}{P}$$

Figure 3.7 The formula for the calculation of the Reynold's number

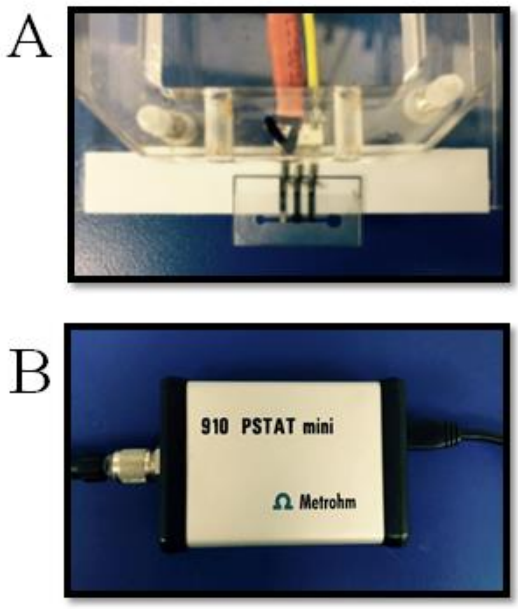

Figure 3.8 Microfluidic chip (A) connects with the Potentiostat for the electrochemical measurement (B)

.

CHAPTER IV
RESULTS AND DISCUSSION

This chapter is to demonstrate the results of study. The study is classified into two major parts. 1) The optimization of nanomaterial modified SPE, enzyme immobilization method, and the acetylcholine measurement by the screen-printed modified enzyme and nanomaterials, 2) the enzyme modified new design screen-printed electrode for the microfluidic chip platform, acetylcholine measurement by microfluidic chip platform

4.1 Acetylcholine biosensor based on screen-printed electrode

4.1.1 The nanomaterial modified electrode

In this part, we demonstrate the method for assessment of the experiments. In order to select the nanomaterial for modified electrode surface, we ought to compare the efficacy of each nanomaterial by measurement of H_2O_2. Then the sensitivity of each nanomaterial modified electrode was compared.

In this study, we used the nanomaterials to modify the electrodes. The nanomaterial modified electrodes consisted of two different nanomaterials, GR-PEDOT: PSS and MWCNT-CS, therefore, there are 3 types of the electrodes, bare SPE, MWCNT-CS/SPE, and GR-PEDOT:PSS/SPE. In order to compare the efficacy of all electrodes, we used them to measure the amperometry of H_2O_2. The electrochemical system containing 4.95 ml of pH 7.4 PBS with constant applied potential, 700 mV versus Ag/AgCl reference electrode. Working electrode surface was immersed into the solution and the measurement was started. After acquired the stabilized baseline, 50 µl of H_2O_2 substrate was added to achieve the final concentration concentrations, 1, 5, 2.5, 1.25, and 0.625 mM H_2O_2. Three different

types of electrode modification were performed by these procedures in the different H_2O_2.

Firstly, the current response of bare SPE is shown in figure 4.1 which shows that the current response, 0.6 nA to 58.42 nA, proportionally increasing with H_2O_2 concentration. However, we cannot measure any signal at 0.0625 mM H_2O_2. The sensitivity of bare SPE was calculated from the slope (y = 6.1961x - 0.261, R^2 = 0.996) which is 6.2 nA/mM

Secondly, the current response of MWCNT-CS/SPE is shown in figure 4.2 which shows the current response, 0.1764 µA to 2.139 µA, proportionally increasing with H_2O_2 concentration. The sensitivity of MWCNT-CS/SPE was calculated from the slope (y = 2.038x – 0.221, R^2 = 0.959) which is 2.038 µA/mM.

Thirdly, the current response of GR-PEDOT:PSS/SPE is shown in figure 4.3 which shows the current response, 0.4036 µA to 6.291 µA, proportionally increasing with H_2O_2 concentration. The sensitivity of GR-PEDOT:PSS/SPE was calculated from the slope (y = 13.286x – 0.447, R^2 = 0.994) which is 13.29 µA/mM.

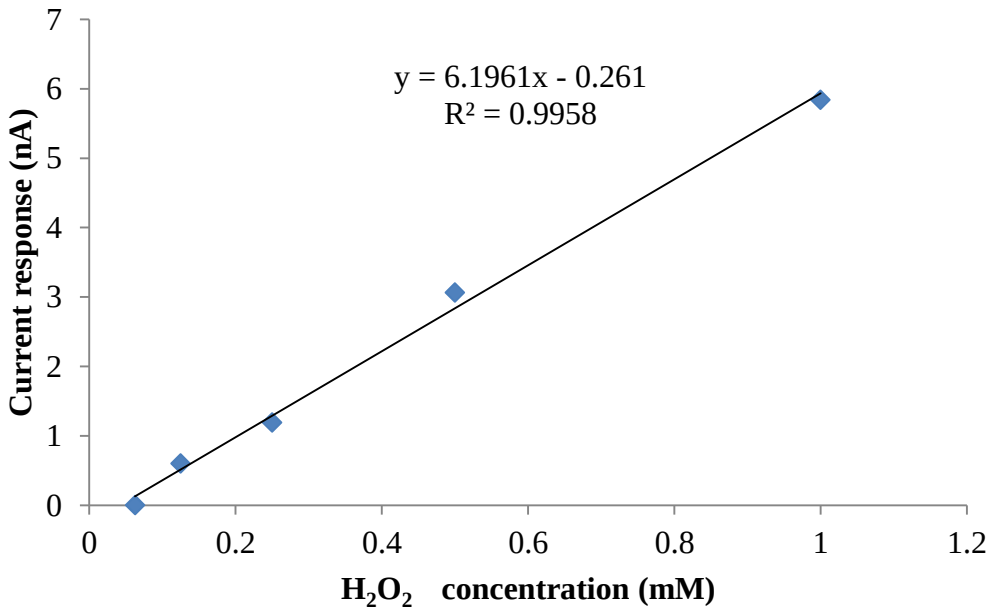

Figure 4.1 Current response of bare SPE with presence of H_2O_2 in the different concentrations at 700 mV vs Ag/AgCl reference electrode

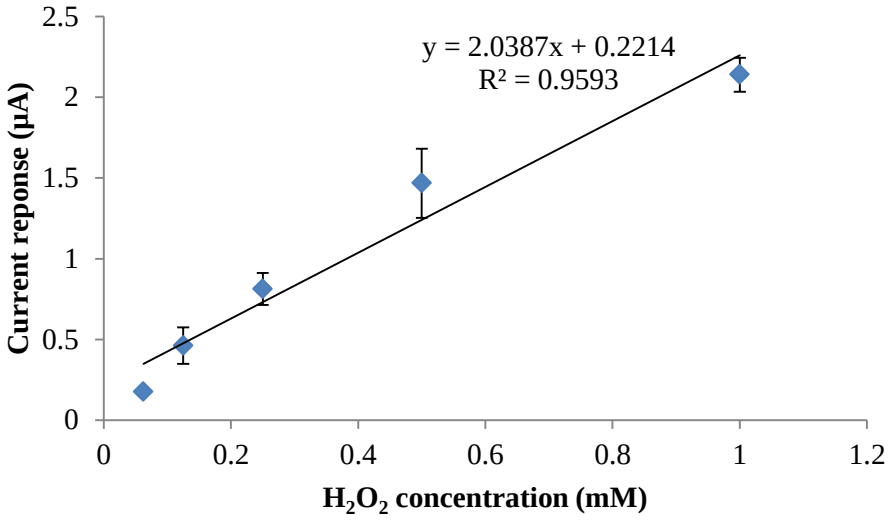

Figure 4.2 Current response of MWCNT/SPE with presence of H_2O_2 in the different concentrations at 700 mV vs Ag/AgCl reference electrode

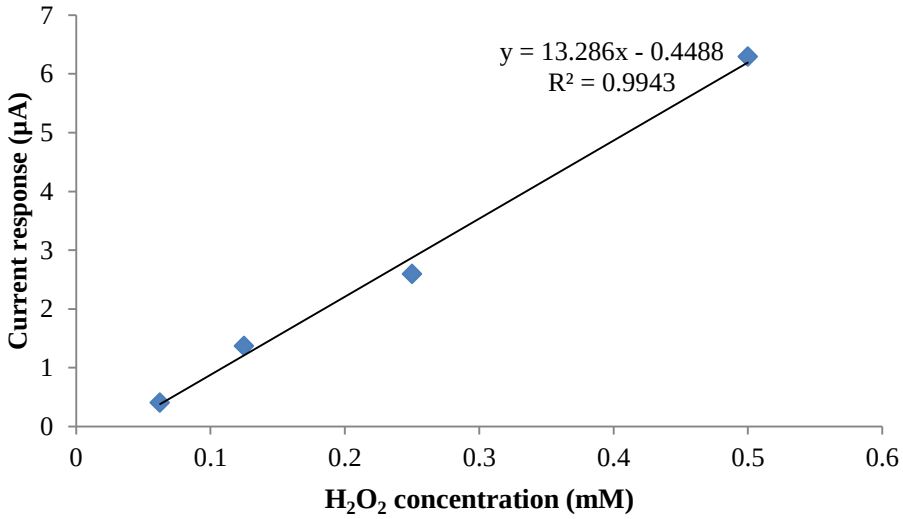

Figure 4.3 Current response of GR-PEDOT:PSS/SPE with presence of H_2O_2 in different concentration at 700 mV vs Ag/AgCl reference electrode

From all the above, the comparison of the current response values is shown in table 4.1. The sensitivity of H_2O_2 detection, at 0.7V vs Ag/AgCl, based on bare SPE, MWCNT/SPE, and GR-PEDOT:PSS/SPE was 6.2 nA/mM, 2.038 µA/mM, and 13.29 µA/mM, respectively (Figure 4.4). For the comparison of the sensitivity between bare/SPE and GR-PEDOT:PSS/SPE, the sensitivity increases approximately 1875 folds. GR-PEDOT:PSS/SPE can definitely amplify the signal response. The increasing of the sensitivity is useful for lowering the limit of the detection in the next step of the experiments. Therefore, GR-PEDOT:PSS was selected for the next step of the experiments.

Table 4.1 Values of the current response by the different electrode modification for determination of 0.0625 to 1 mM H_2O_2 at 700 mV versus Ag/AgCl.

Electrode modification	Current response ± STDEV (µA)				
	H_2O_2 concentration				
	0.0625 mM	0.125 mM	0.25 mM	0.5 mM	1 mM
Bare SPE	N/A	0.0006 ± 0.0000	0.0012 ± 0.0003	0.0031 ± 0.0002	0.0058 ± 0.0007
MWCNT-CS/SPE	0.1764 ± 0.0097	0.4618 ± 0.1125	0.8122 ± 0.0994	1.4673 ± 0.2137	2.1390 ± 0.1057
GR-PEDOT:PSS/SPE	0.4036 ± 0.0429	1.3723 ± 0.0769	2.5937 ± 0.1610	6.2910 ± 0.5851	10.913 ± 0.7842

4.1.2 The optimization of the constant potential

In order to optimize the constant potential for the amperometric detection, the measurement was performed in the electrochemical system containing 1 mM choline chloride. We consider to use the negative potential for acetylcholine detection because we want to prevent the interference signal from the other electroactive species. Therefore, the constant potential was varied in the range of 0.0 to -0.4 volt versus Ag/AgCl. 0.5 unit of choline oxidase was immobilized by physical absorption called ChOx/GR-PEDOT:PSS/SPE. Presence and absence enzyme electrode was compared.

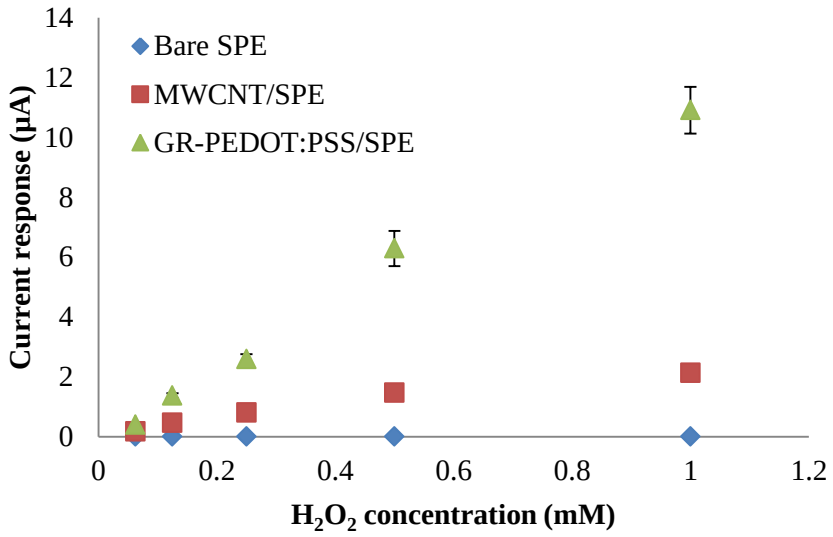

Figure 4.4 Comparison of amperometric detection of different type electrode modification for determination of 0.0625 to 1 mM H_2O_2 at 700 mV versus Ag/AgCl reference electrode

At 0.0 volt and -0.1 volt, there are no current response from ChOx/GR-PEDOT:PSS/SPE and GR-PEDOT:PSS/SPE because too low potential cannot induce the redox reaction. Meanwhile, at -0.2 volt, -0.3 volt, and -0.4 volt, showed the current response between 107.97 ± 24.764 nA, 768.64 ± 115.65 nA, and 1811.3 ± 40.614 nA, respectively. After 1 mM choline chloride adding (at 100 seconds), the current starts to react within 10 seconds, and it reaches to the stable baseline within 50 seconds (Figure 4.5). We selected -0.2 volt versus Ag/AgCl as the optimal constant potential because it shows clearly signal response compared with the higher constant potentials, -0.3 and -0.4 volt. GR-PEDOT:PSS/SPE is don't have enzyme for choline hydrolysis, therefore, it didn't have any current response after adding 1 mM choline chloride.

Table 4.2 Current response of amperometric detection in the various applied constant potential for 1 mM choline chloride

Constant Potential (volt) versus Ag/AgCl	Current Response ± STDEV (nA)	
	ChOx/GR-PEDOT:PSS/SPE	GR-PEDOT:PSS/SPE
0	N/A	N/A
-0.1	N/A	N/A
-0.2	107.97 ± 24.764	N/A
-0.3	768.64 ± 115.65	N/A
-0.4	1811.3 ± 40.614	N/A

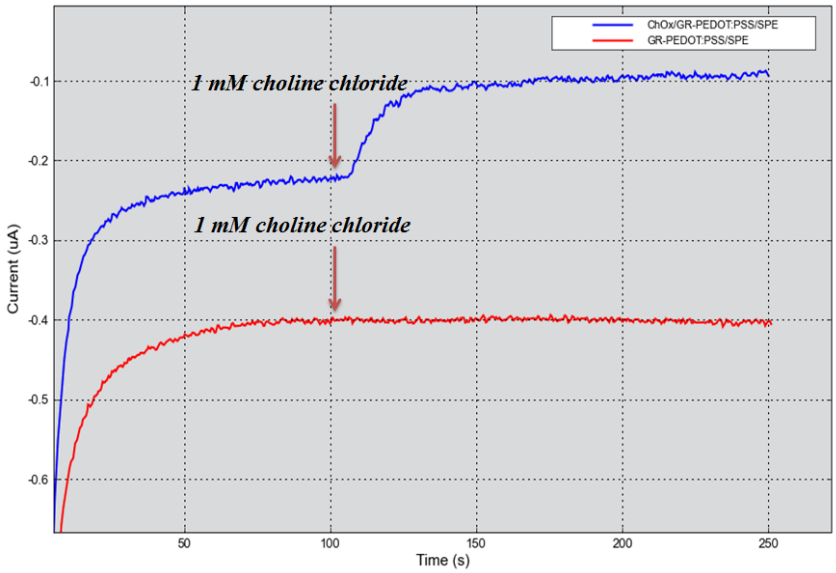

Figure 4.5 Current response of amperometric detection for 1 mM choline chloride determination by ChOx/GR-PEDOT:PSS/SPE (Blue) and GR-PEDOT:PSS/SPE (Red), -0.2 volt versus Ag/AgCl

4.1.3 Characterization of GR-PEDOT:PSS/SPE

In order to observe the surface modification screen-printed electrode by GR-PEDOT:PSS. The morphology study was performed by SEM (JEOL, JSM-5410LV). The surface morphology of bare screen-printed electrode is shown in figure 4.6. The comparison between bare SPE and GR-PEDOT:PSS/SPE is definitely different in the structure of surface. Bare SPE shows the plane surface, while the GR-PEDOT:PSS/SPE definitely increases in 3-dimensional structure. The surface roughness and the different of surface height confirms that GR-PEDOT:PSS/SPE aggregate on the surface, and it increases the surface to volume ratio of working electrode.

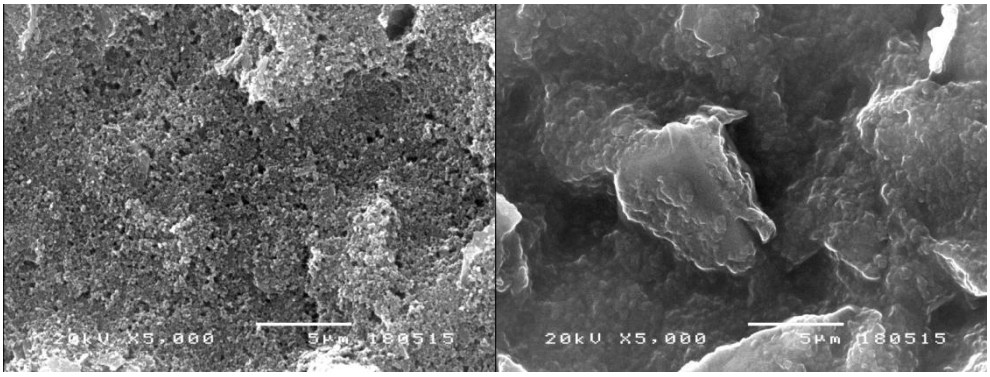

Figure 4.6 SEM image (5000x) of SPE (A), and GR-PEDOT:PSS/SPE (B)

4.1.4 Study of enzyme immobilization

In this study section, we immobilized the ChOx enzyme on the electrode surface by the several different ways. 1 mM choline chloride was measured indirectly via redox reaction of H_2O_2 which hydrolyzes from choline chloride.

Enzyme immobilization was performed by four different methods

1. ChOx/GR-PEDOT:PSS/SPE
2. ChOx/GR-PEDOT:PSS/SPE with GA vapor
3. Nafion/ChOx/GR-PEDOT:PSS/SPE
4. Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vapor

Four types of enzyme modified electrodes were used in this experiment. All of electrode modification contains 1 unit ChOx. The efficacy of enzyme immobilization was analyzed in the electrochemical system containing 1 mM choline

chloride by the amperometry at -200 versus Ag/AgCl reference electrode. Current detection was measured from H_2O_2 hydrolysis of choline chloride by ChOx.

The results were shown in figure 4.7 ChOx/GR-PEDOT:PSS/SPE showed the highest current detection 207.8 ± 45.9 nA, but the highest fluctuation. Due to ChOx was immobilized by the physical absorption. It is very fragile and easy to elute from the working electrode surface which was immersed in electrochemical cell. Actually, we required the sensor that can measure in high accuracy. Therefore, we chose some materials for enhancement the enzyme immobilization.

Next, we used nafion coating on the electrode surface after the enzyme immobilization. It forms as a thin protective film layer, and we called Nafion/ChOx/GR-PEDOT:PSS/SPE. It showed the current response 161.9 ± 18.62 nA. The reduction of standard deviation proved that nafion protective layer is useful for prevention of the enzyme elution. Moreover, we used glutaraldehyde (GA) to enhance the enzyme immobilization efficacy in both with and without nafion coating. We called ChOx/GR-PEDOT:PSS/SPE with GA vapor and Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vapor, and it had the current response, 189.05 ± 11.62and 169.63 ± 11.23, respectively. GA is a bi-functional group or crosslinking agent. GA enhances the enzyme immobilization efficacy by acting as a bridge between amino groups of enzyme and carboxylic groups of GR-PEDOT:PSS, at the same time, it may bind between 2 amine group and forms the matrix structure. Consequently, the enzyme was immobilized inside the matrix formation structure.

From all above, the mean of current response of all methods is not significantly different. The slightly decrease of current response of Nafion/ChOx/GR-PEDOT:PSS/SPE may come from protective film that decreases the diffusion rate of substrate through the working surface. Meanwhile, the using of GA definitely strengthens the enzyme immobilization, but it also defects the enzyme binding site which leads to the decrease of enzyme activity. Furthermore, Nafion is an ionic polymer which owns the property as a cations selective membrane. Therefore, it can prevent the interference from other electroactive species that have a positive charge.

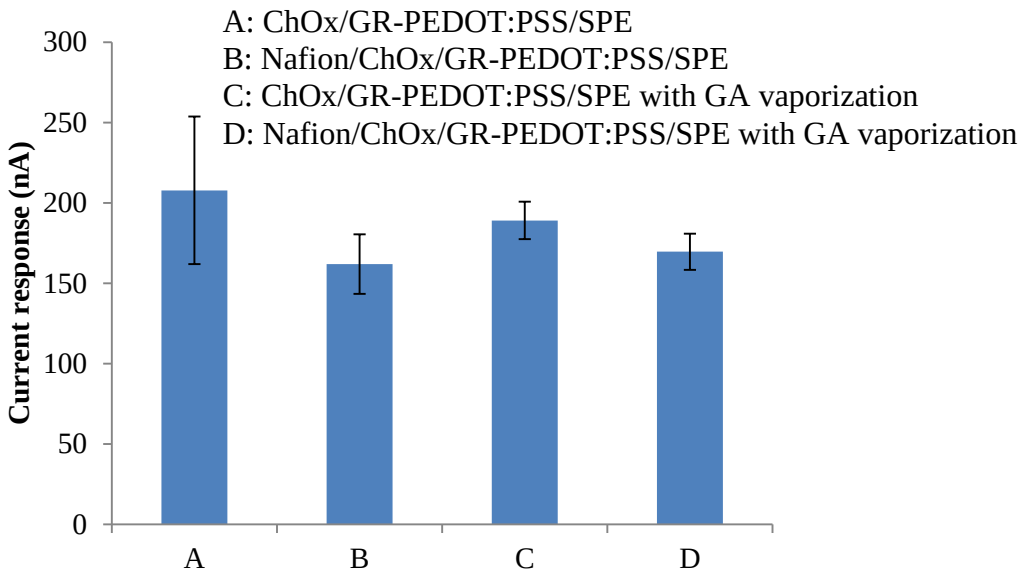

Figure 4.7 Current response of amperometric detection for 1 mM choline chloride determination by different enzyme immobilization methods, -0.2 volt versus Ag/AgCl

In conclusion, we want to fabricate the high accuracy and steady sensor. Therefore, we selected Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vaporiztion for the further study.

4.1.5 The optimization of choline oxidase concentration

Due to ACh detection was achieved by the consecutive reaction of bi-enzyme, AChE and ChOx, respectively. The enzyme concentration ratio must be chosen judiciously. This study has 2 steps: Optimization of ChOx concentration, and Optimization of AChE concentration.

From the previous experiment, the chosen enzyme immobilization method is Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vapor. Therefore, we used this technique to immobilize ChOx in the different concentration between 0.5 unit and 3.0 unit. All modified electrodes were used for 1 mM choline chloride determination by amperometric detection at -0.2 volt versus reference electrode.

The result shows in figure 4.8. In the range of 0.5 to 2.5 unit ChOx, the current response increases proportionally with enzyme concentration. The highest current response, 347.35 ± 4.2658 nA, is at 2.5 unit ChOx. The current response decrease at 3.0 unit ChOx. Because of the cost-effectiveness, we decided to use 1.5 unit ChOx as the optimal concentration for the further experiment.

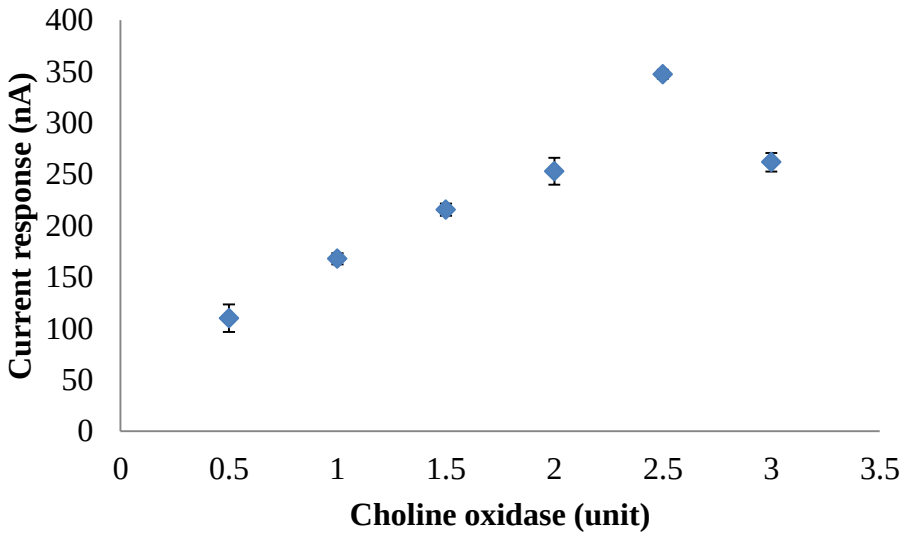

Figure 4.8 Current response of amperometric detection for 1 mM choline chloride determination in various choline oxidase concentration**s**

4.1.6 The optimization of acetylcholine esterase in concentration

In order to determine acetylcholine chloride, we must immobilize both enzymes ChOx and AChE. 1.5 unit ChOx was selected from the previous experiment as the optimized concentration. Therefore, we varied AChE concentration in the range of 0.5 to 3.0 unit, but fixed the ChOx concentration at 1.5 unit. We called Nafion/AChE-ChOx/GR-PEDOT:PSS/SPE with GA vapor.

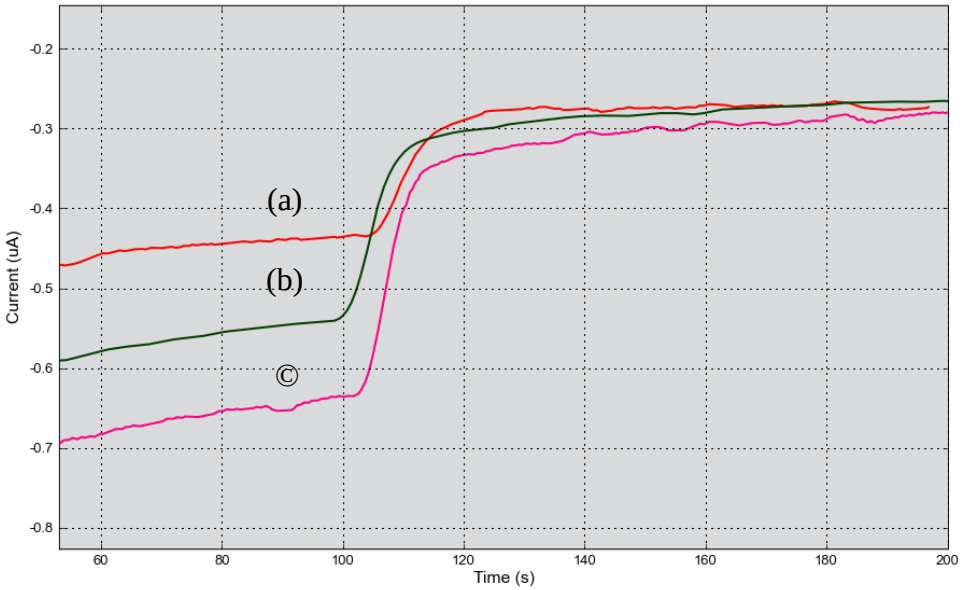

Figure 4.9 Amperometric current response of 1 mM choline chloride determination (a) 1.0 unit ChOx, (b) 1.5 unit ChOx, and (c) 2.5 unit ChOx

The result shows in figure 4.9. In the range of 0.5 to 2.5 unit AChE, the current response increases proportionally with enzyme concentration. The highest current response, 211.59 ± 26.724 nA, is at 2.5 unit AChE. The current values are shown in table 4.4. However, the current response decrease at 3.0 unit AChE.

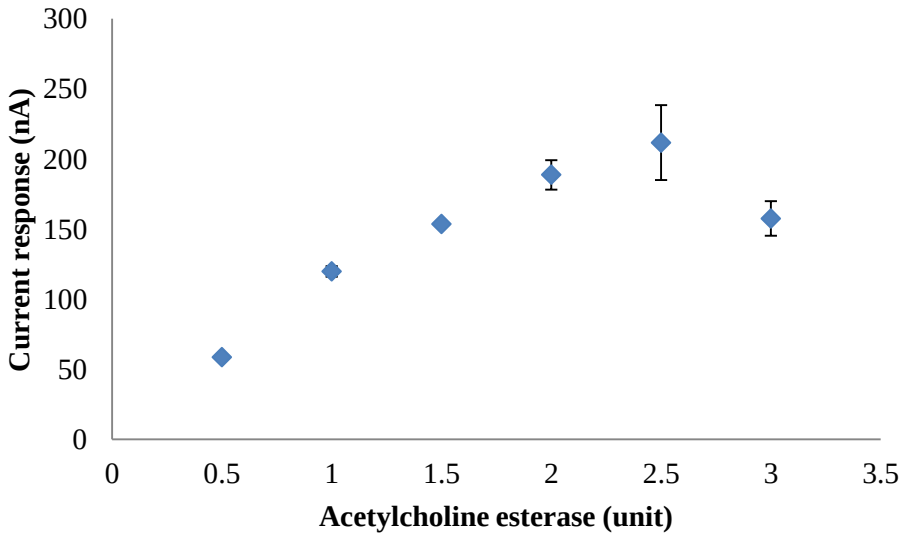

Figure 4.10 Current response of amperometric detection for 1 mM acetylcholine chloride determination in the different acetylcholine esterase concentrations

In conclusion, the Increasing enzyme concentration facilitates the chances of enzyme and substrate binding, consequently the increasing reaction rate and current response. We expect that the graph will show a plateau curve, if we continually increase enzyme concentration. Nevertheless, it decreases at high enzyme concentration. We suppose that the excess enzyme immobilization acts as a barrier which blocks or decrease the diffusion rate and electron transfer rate on an electrode surface, thus lessened in current response. In this experiment, the optimized enzyme concentration ratio is AChE:ChOx is 2.5:1.5 unit

4.1.7 Calibration curve of acetylcholine chloride determination

Nafion/AChE-ChOx/GR-PEDOT:PSS/SPE with GA vapor with the optimized enzyme ratio 2.5U:1.5U (AChE:ChOx) was used to measure acetylcholine chloride concentration. The concentration variation is in the range of 0.50 µM to 1 mM AChCl. The calibration curve was shown in figure 4.9. The range of the current response is 31.347 ± 4.5280 nA to 210.70 ± 26.747 nA with the increase of acetylcholine chloride concentration. The linearity relation of current detection locates in the range of 50 µM to 250 µM (R^2= 0.945). For the concentration higher than 250 µM AChCl, the curve trend is an approximate plateau. This sensor shows the LOD at 0.50 µM, and sensitivity at 486.1 nA/mM.

4.2 Acetylcholine biosensor based in the microfluidic chip platform

4.2.1 Testing of the laminar flow behavior in the microchannel

After the microfluidic chip fabrication, we must test to confirm the properties of our microfluidic chip. Microfluidic chip must show the laminar flow and passive flow without pumping. In order to test this property, we can test by using the solutions which have the different colors, green and red solution. Firstly, 5 µl of green solution was dropped into the microchannel (Figure 4.11 b). The whole part of microchannel was filled with the green solution. Subsequently, 5 µl of red solution was dropped into the same inlet. Because of the laminar flow behavior (Reynold

number < 2300), the fluid flows in the parallel line. Therefore, red solution automatically pushed green solution to the outlet without mixing (Figure 4.11 c).

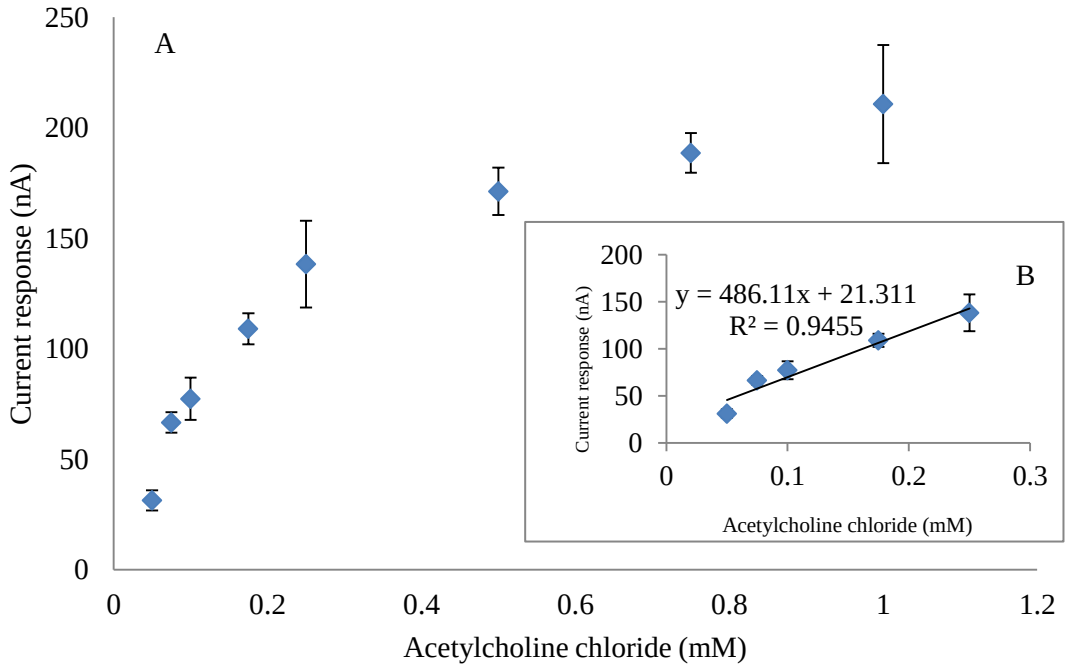

Figure 4.11 A: Amperometric calibration curve of Nafion/AChE-ChOx/GR-PEDOT:PSS/SPE with GA vapor cross-linked, B: Linearity of acetylcholine chloride detection

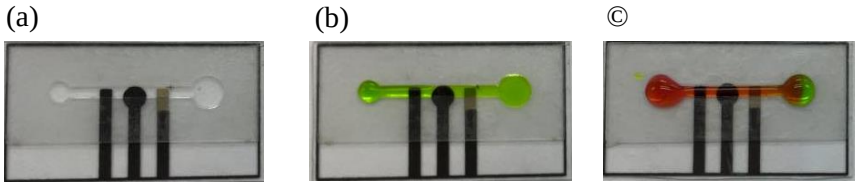

Figure 4.12 Laminar flow in the microfluidic chip (a), the adding of green solution (b) and, the adding of red solution (c), respectively

4.2.2 The optimization of the constant potential

In order to optimize the constant potential for the amperometric detection, the measurement was performed in the microfluidic chip platform for determination of 1 mM choline chloride. All of the electrodes that were modified with the microchannel contained 1 unit ChOx. The constant potential was varied in the range of 0.0 to -0.4 volt versus Ag/AgCl.

Between 0.0 volt to -0.2 volt, there are no current response from ChOx/GR-PEDOT:PSS/SPE, because too low potential cannot induce the redox reaction. At -0.3 volt and -0.4 volt, showed the current response 55.67 ± 9.545 nA and 249.86 ± 30.363 nA, respectively. Meanwhile, GR-PEDOT:PSS/SPE, non-enzyme electrode, didn't show any current response.

Table 4.3 Current response of amperometric detection in the various applied constant potential for 1 mM choline chloride determination

Constant Potential (volt) versus Ag/AgCl	Current Response ± STDEV (nA)	
	ChOx/GR-PEDOT:PSS/SPE	GR-PEDOT:PSS/SPE
0	N/A	N/A
-0.1	N/A	N/A
-0.2	N/A	N/A
-0.3	55.67 ± 9.545	N/A
-0.4	249.86 ± 30.363	N/A

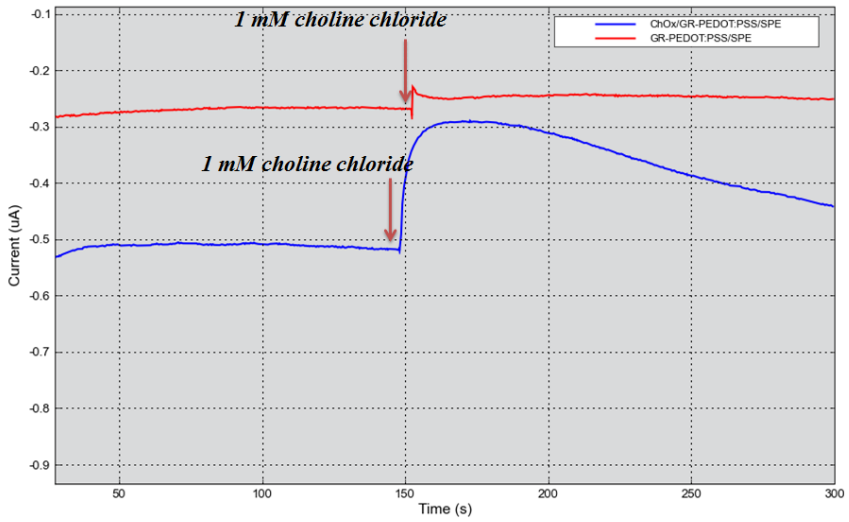

Figure 4.13 Current response of amperometric detection for 1 mM choline chloride determination in microfluidic chip by ChOx/GR-PEDOT:PSS/SPE (Blue) and GR-PEDOT:PSS/SPE (Red), -0.4 volt versus Ag/AgCl

4.2.3 The optimization of choline oxidase concentration

Because we already get the optimization of the enzyme immobilization method from the previous experiment, we continually use glutaraldehyde for generating chemically cross-linked network, and coating with Nafion thin film. It is completely different from the previous screen-printed electrode, such as, containing 3 kinds of electrode: 1.working, 2.reference, and 3.counter electrode on itself, and the changing of working electrode surface area. Therefore, we must optimize the enzyme concentration for using with this microfluidic chip platform.

Choline oxidase was immobilized on the electrode surface by glutaraldehyde vapor and coating with nafion, protective film layer. The amount of choline oxidase was varied between 0.5 to 2.5 unit. We used the amperometric technique, constant applied potential at -0.4 volt, to define the current response. The current response is shown in figure 4.13. 0.5 unit ChOx exhibits the lowest current response. The graph curve shows a plateau curve at 1.0 to 2.5 unit ChOx. The mean of current response is not significantly different, but have a high fluctuation at 2.0 and 2.5 unit ChOx. Because of the cost-effectiveness, we selected 1 unit ChOx as the optimal concentration for enzyme immobilization on the microfluidic chip platform.

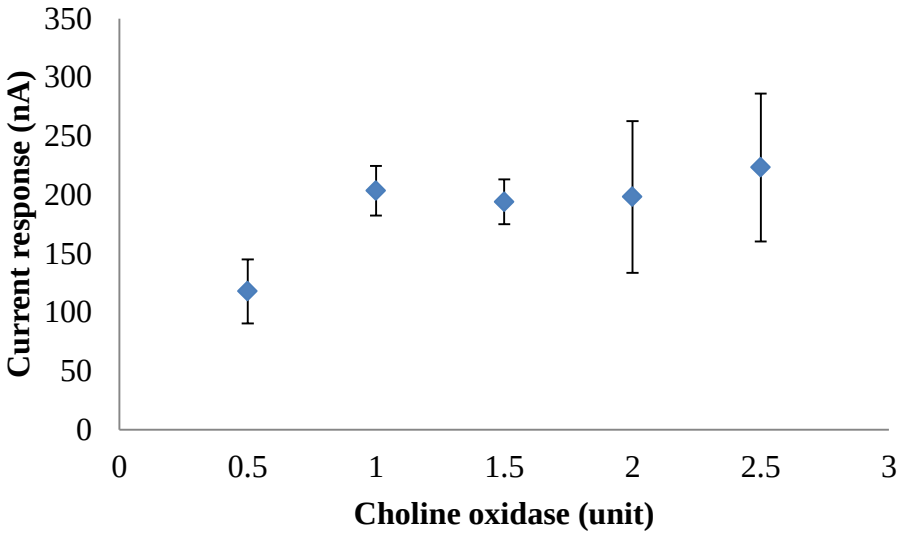

Figure 4.14 Current response of amperometric detection for 1 mM choline chloride determination in the different choline oxidase concentration in the microfluidic chip platform, -0.4 volt versus Ag/AgCl

4.2.4 Optimization of acetylcholine esterase concentration

The different concentrations of acetylcholine esterase were mixed with 1 unit ChOx. AChE was varied into 0.5, 1.0, 1.5, 2.0, and 2.5 unit, and was immobilized on the electrode of microfluidic chip platform by the previously same method. We used them to measure 1 mM acetylcholine chloride in the microfluidic platform. We used the amperometric technique, constant applied potential at -0.4 volt, to define the current response. The current response is shown in figure 4.14. 0.5 unit AChE exhibits the lowest current response. In the range of 0.5 to 1.5 unit, the current response tends to increase with the enzyme concentration. From the current response, we selected the 1.5 unit AChE as the optimal concentration for the further study.

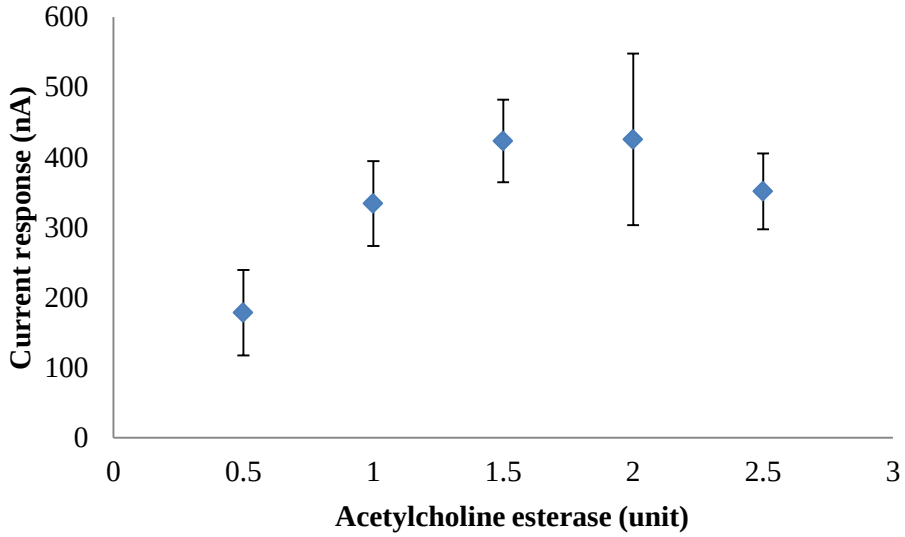

Figure 4.15 Current response of amperometric detection for 1 mM acetylcholine chloride determination in the different acetylcholine esterase concentration in the microfluidic chip

4.2.5 Calibration curve of acetylcholine determination

The optimized enzyme ratio 1.5U:1.0U (AChE:ChOx) was immobilized, chemically cross-linked and thin film coating, on the electrode which is integrated with the microfluidic chip platform. In order to generate the calibration curve, this acetylcholine sensor in the microfluidic chip platform was used to measure current response of the acetylcholine chloride in the different concentrations, 0.01 mM to 2 mM. The result is shown in figure 4.15-A. By the amperometric analysis, the current response is in the range of 116.67 ± 21.548 nA to 467.33 ± 34.819 nA. It increases proportionally with the acetylcholine chloride concentration. The linearity relation of current detection locates in the range of 10 µM to 750 µM (R^2= 0.989, figure 4.15-B). In the range of 750 µM to 2.0 mM, the current response seems to be a plateau curve. The LOD and sensitivity is 10 µM and 430.76 nA/mM, respectively.

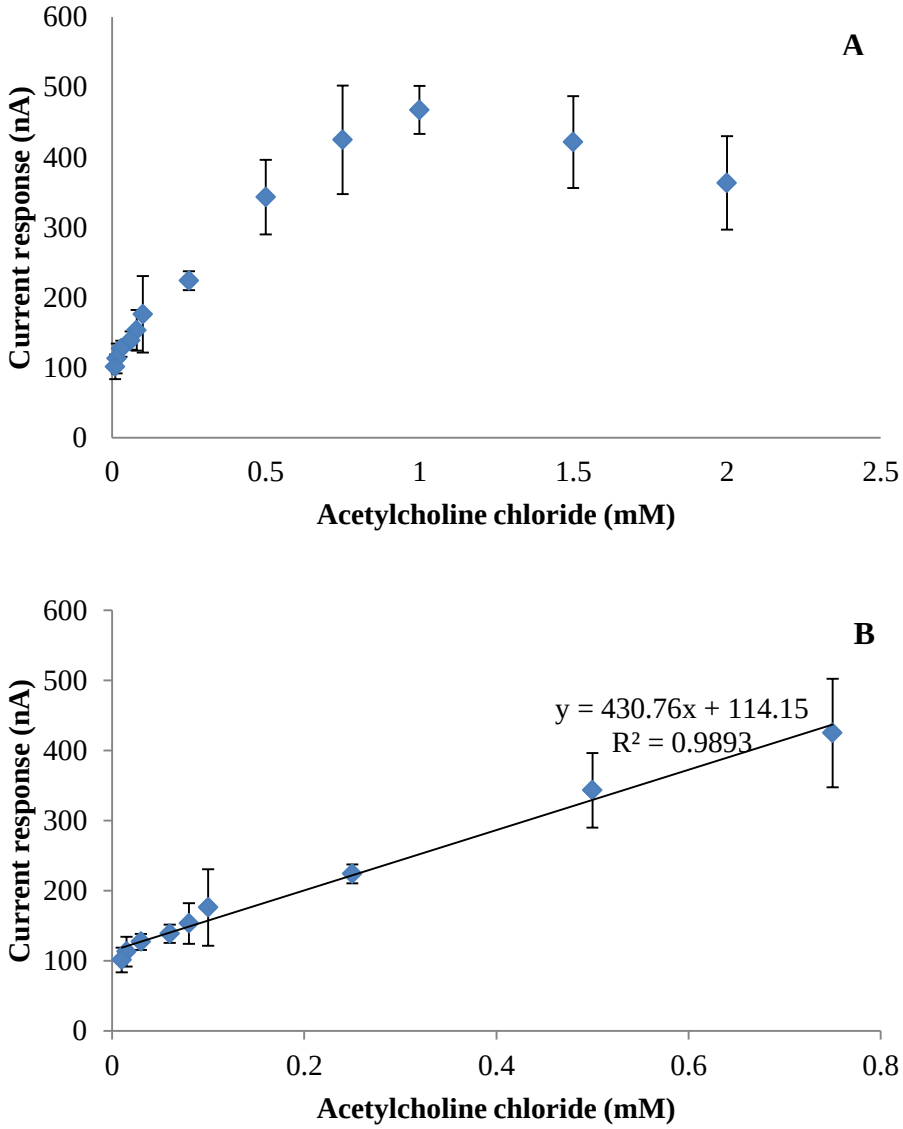

Figure 4.16 A: Calibration curve of amperometric acetylcholine biosensor in the microfluidic chip platform, B: Linearity of acetylcholine chloride detection

CHAPTER V
CONCLUSION

In this study, we aim to fabricate the acetylcholine biosensor which is based on the enzymatic reaction. We focus on using the screen-printed electrode that has a low-cost fabrication.

In the first part, GR-PEDOT:PSS and MWCNT was selected for electrode modification. The sensitivity of H_2O_2 detection, at 0.7V vs Ag/AgCl, based on bare SPE, MWCNT/SPE, and GR-PEDOT:PSS/SPE was 6.196 nA/mM, 2.038 µA/mM, and 13.29 µA/mM, respectively. GR-PEDOT:PSS/SPE exhibited the highest sensitivity, and was selected for the further study. ChOx was immobilized on GR-PEDOT:PSS/SPE by 10% GA vapor cross-linked for 8 minutes and 1% Nafion solution coating (NAFION/ChOx/GR-PEDOT:PSS/SPE). It showed an extremely low fluctuation of current detection when compared with physical absorption. Thus it confirmed that this method can prevent the elution of enzyme from SPE surface. For the amount of enzyme optimization, 0.5 unit to 4.0 unit of ChOx was varied for amperometric detection in pH 7.4 PBS containing 1 mM ChCl at -200 mV versus Ag/AgCl. 2.5 unit of ChOx showed the highest current response. However, we opted to use 1.5 unit of ChOx for the low-cost fabrication. The variation of AChE, 0.5 unit to 3.0 unit, was mixed with 1.5 unit of ChOx. The results showed that 2.5 unit of AChE exhibited the highest current detection of PBS containing 1 mM AChCl. Therefore, the optimization of enzyme ratio is 1.5:2.5 (ChOx:AChE). The calibration curve shows the limit of detection, linearity and sensitivity, 50 µM, 50 to 250 µM and 486.1 nA/mM, respectively.

The second part, we applied acetylcholine biosensor with the microfluidic chip platform. Our microfluidic chip is cost-effectiveness and easy to use. Moreover, the fabrication procedure is easy and simple. Our acetylcholine biosensor modified microfluidic chip platform shows the limit of detection, linearity, and sensitivity, 10 µM, 10 µM to 750 µM, and 430.76 nA/mM, respectively.

However, the limit of detection of this sensor is not enough to use for the Alzheimer's disease screening platform. Acetylcholine in the cerebrospinal fluid of human is in the nanomolar scale. The limit of detection would be improve in the future study.

REFERENCES

1. Sonkusare SK, Kaul CL, Ramarao P. Dementia of Alzheimer's disease and other neurodegenerative disorders--memantine, a new hope. Pharmacological research : the official journal of the Italian Pharmacological Society. 2005 Jan;51(1):1-17. PubMed PMID: 15519530.
2. Hebert LE, Scherr PA, Bienias JL, Bennett DA, Evans DA. Alzheimer Disease in the US Population. Arch Neurol. 2003;60:1119-22.
3.Association As. Early Onset Dementia A National Challenge, A Future Crisis. 2006 June.
4. Berchtold NC, Cotman CW. Evolution in the Conceptualization of Dementia and Alzheimer’s Disease: Greco-Roman Period to the 1960s. Neurobiology of Aging. 1998;19(3):173-89.
5. Hardy J. A hundred years of Alzheimer's disease research. Neuron. 2006 Oct 5;52(1):3-13. PubMed PMID: 17015223.
6. Lasagna-Reeves CA, Castillo-Carranza DL, Sengupta U, Sarmiento J, Troncoso J, Jackson GR, et al. Identification of oligomers at early stages of tau aggregation in Alzheimer's disease. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2012 May;26(5):1946-59. PubMed PMID: 22253473.
7. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science. 2002 Jul 19;297(5580):353-6. PubMed PMID: 12130773.
8. Kihara T, Shimohama S. Alzheimer's disease and acetylcholine receptors. Acta Neurobiol Exp. 2004;64:99-105.
9. Homberg U. Neurotransmitters and Neuropeptides in the Brain of the Locust. MICROSCOPY RESEARCH AND TECHNIQUE. 2002;56:189-209.

10. Croxson PL, F. PG, Browning, Gaffan D, Baxter MG. Acetylcholine facilitates recovery of episodic memory after brain damage. J Neurosci. 2012;32(40):13787-95.
11. Jian-ping J, Jian-min J, Wei-dong Z, Min X, Chang-biao C, Xin Y, et al. Differential acetylcholine and choline concentrations in the cerebrospinal fluid of patients with Alzheimer's disease and vascular dementia. Chinese Medical Journal. 2004;117(8):1161-4.
12. J B. Cholinesterase inhibitors for Alzheimer's disease. Cochrane Database of Systematic Reviews. 2006;1.
13. Fredman L, James BD, Johnson TJ, Scholz KP, Weuve J. Alzheimer's disease facts and figures. Alzheimers's association. 2012.
14. Sperling RA, Karlawish J, Johnson KA. Preclinical Alzheimer disease-the challenges ahead. Nature reviews Neurology. 2013 Jan;9(1):54-8. PubMed PMID: 23183885. Pubmed Central PMCID: 3643203.
15. Hampel H, Burger K, Teipel SJ, Bokde AL, Zetterberg H, Blennow K. Core candidate neurochemical and imaging biomarkers of Alzheimer's disease. Alzheimer's & dementia : the journal of the Alzheimer's Association. 2008 Jan;4(1):38-48. PubMed PMID: 18631949.
16. Andreasen N, Blennow K. Beta-Amyloid protein in cerebrospinal fluid as a biomarker for Alzheimer's disease. Peptides. 2002 May;23:1205-14.
17. Bucha KBrn, Padberga F, Noldea T, Teipela SJ, bnera SS, Haslingera A, et al. Cerebrospinal fluid tau protein shows a better discrimination in young old (,70 years) than in old old patients with Alzheimer's disease compared with controls. Neuroscience letters. 1999;277:21-4.
18. Wang J. Glucose Biosensors: 40 Years of Advances and Challenges. Electroanalysis. 2001;13(12).
19. Heineman WR, Jensen WB. Leland C. Clark Jr. (1918–2005). Biosensors and Bioelectronics. 2006;21(8):1403-4.
20. Cete S, Yasar A, Arslan F. An amperometric biosensor for uric acid determination prepared from uricase immobilized in polypyrrole film. Artificial cells, blood substitutes, and immobilization biotechnology. 2006;34(3):367-80. PubMed PMID: 16809136.

21. Muhammet SM, Cete S, Arslan F, Yasar A. Amperometric cholesterol biosensors based on the electropolymerization of pyrrole and aniline in sulphuric Acid for the determination of cholesterol in serum. Artificial cells, blood substitutes, and immobilization biotechnology. 2009;37(6):273-8. PubMed PMID: 19922167.

22. Batra B, Pundir CS. An amperometric glutamate biosensor based on immobilization of glutamate oxidase onto carboxylated multiwalled carbon nanotubes/gold nanoparticles/chitosan composite film modified Au electrode. Biosensors & bioelectronics. 2013 Sep 15;47:496-501. PubMed PMID: 23628843.

23. Hou S, Ou Z, Chen Q, Wu B. Amperometric acetylcholine biosensor based on self-assembly of gold nanoparticles and acetylcholinesterase on the sol-gel/multi-walled carbon nanotubes/choline oxidase composite-modified platinum electrode. Biosensors & bioelectronics. 2012 Mar 15;33(1):44-9. PubMed PMID: 22230694.

24. Sassolas A, Blum LJ, Leca-Bouvier BD. Immobilization strategies to develop enzymatic biosensors. Biotechnology advances. 2012 May-Jun;30(3):489-511. PubMed PMID: 21951558.

25. Sheldon RA, van Pelt S. Enzyme immobilisation in biocatalysis: why, what and how. Chemical Society reviews. 2013 Jul 8;42(15):6223-35. PubMed PMID: 23532151.

26. Lia Y-G, Zhoua Y-X, Fenga J-L, Jiangb Z-H, Ma L-R. Immobilization of enzyme on screen-printed electrode by exposure to glutaraldehyde vapour for the construction of amperometric acetylcholinesterase electrodes. Analytica Chimica Acta 1999;382:277-82.

27. Migneault I, Dartiguenave C, Bertrand MJ, Waldron KC. Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. BioTechniques. 2004;37:790-802.

28. GEIM AK, NOVOSELOV KS. The rise of graphene. nature materials. 2007;6.

29. Novoselov KS, Geim AK, Morozov SV, Jiang D, Zhang Y, Dubonos SV, et al. Electric Field Effect in Atomically Thin Carbon Films. Science. 2004;306.

30. Chen XM, Wu GH, Jiang YQ, Wang YR, Chen X. Graphene and graphene-based nanomaterials: the promising materials for bright future of electroanalytical chemistry. The Analyst. 2011 Nov 21;136(22):4631-40. PubMed PMID: 21975368.

31. Shen H, Zhang L, Liu M, Zhang Z. Biomedical applications of graphene. Theranostics. 2012;2(3):283-94. PubMed PMID: 22448195. Pubmed Central PMCID: 3311234.

32. Liu K, Zhang J-J, Cheng F-F, Zheng T-T, Wang C, Zhu J-J. Green and facile synthesis of highly biocompatible graphene nanosheets and its application for cellular imaging and drug delivery. Journal of Materials Chemistry. 2011;21(32):12034.

33. Zhu Y, Murali S, Cai W, Li X, Suk JW, Potts JR, et al. Graphene and graphene oxide: synthesis, properties, and applications. Advanced materials. 2010 Sep 15;22(35):3906-24. PubMed PMID: 20706983.

34. Noh J, Kim HC, Chung TD. Biosensors in microfluidic chips. Topics in current chemistry. 2011;304:117-52. PubMed PMID: 21516388.

35. Fiorini GS, Chiu DT. Disposable microfluidic devices: fabrication, function, and application. BioTechniques. 2005;38:429-46.

36. Liu KK, Wu RG, Chuang YJ, Khoo HS, Huang SH, Tseng FG. Microfluidic systems for biosensing. Sensors. 2010;10(7):6623-61. PubMed PMID: 22163570. Pubmed Central PMCID: 3231127.

37. Sia SK, Kricka LJ. Microfluidics and point-of-care testing. Lab on a chip. 2008 Dec;8(12):1982-3. PubMed PMID: 19023459.

38. Sen S, Gulce A, Gulce H. Polyvinylferrocenium modified Pt electrode for the design of amperometric choline and acetylcholine enzyme electrodes. Biosensors & bioelectronics. 2004 May 15;19(10):1261-8. PubMed PMID: 15046758.

39. Xue W, Cui T. A thin-film transistor based acetylcholine sensor using self-assembled carbon nanotubes and SiO2 nanoparticles. Sensors and Actuators B: Chemical. 2008;134(2):981-7.

40. Meagher RJ, Hatch AV, Renzi RF, Singh AK. An integrated microfluidic platform for sensitive and rapid detection of biological toxins. Lab on a chip. 2008 Dec;8(12):2046-53. PubMed PMID: 19023467.

41.Yang CY, Brooks E, Li Y, Denny P, Ho CM, Qi F, et al. Detection of picomolar levels of interleukin-8 in human saliva by SPR. Lab on a chip. 2005 Oct;5(10):1017-23. PubMed PMID: 16175255.

42. Castellani RJ, Rolston RK, Smith MA. Alzheimer disease. Disease-a-month : DM. 2010 Sep;56(9):484-546. PubMed PMID: 20831921. Pubmed Central PMCID: 2941917.

43. Zetterberg H, Blennow K. Biological CSF Markers of Alzheimer's Disease. 2008;89:261-8.

44. Holtzman DM. CSF biomarkers for Alzheimer's disease: current utility and potential future use. Neurobiol Aging. 2011 Dec;32 Suppl 1:S4-9. PubMed PMID: 22078172. Pubmed Central PMCID: 3233690.

45. Tohgi H, Abe T, Kimura M, Saheki M, Takahashi S. Cerebrospinal fluid acetylcholine and choline in vascular dementia of Binswanger and multiple small infarct types as compared with Alzheimer-type dementia. Neural Transmission. 1996;103:1211-20.

46. THEÂVENOT DR, TOTH K, DURST RA, WILSON GS. Electrochemical Biosensor: Recommended Definitions and Classification. Pure Appl Chem. 1999;71:2333-48.

47. Ronkainen NJ, Halsall HB, Heineman WR. Electrochemical Biosensors. Chemical Society reviews. 2010 May;39(5):1747-63. PubMed PMID: 20419217.

48. D'Orazio P. Biosensors in clinical chemistry - 2011 update. Clinica chimica acta; international journal of clinical chemistry. 2011 Sep 18;412(19-20):1749-61. PubMed PMID: 21729694.

49. Perry M, Li Q, Kennedy RT. Review of recent advances in analytical techniques for the determination of neurotransmitters. Anal Chim Acta. 2009 Oct 19;653(1):1-22. PubMed PMID: 19800472. Pubmed Central PMCID: 2759352.

50. Kim B, Song HS, Jin HJ, Park EJ, Lee SH, Lee BY, et al. Highly selective and sensitive detection of neurotransmitters using receptor-modified single-

walled carbon nanotube sensors. Nanotechnology. 2013 Jul 19;24(28):285501. PubMed PMID: 23792421.

51. Uutela P, Reinila R, Piepponen P, Ketola RA, Kostiainen R. Analysis of acetylcholine and choline in microdialysis samples by liquid chromatography/tandem mass spectrometry. Rapid communications in mass spectrometry : RCM. 2005;19(20):2950-6. PubMed PMID: 16180202.
52. Nirogi R, Mudigonda K, Kandikere V, Ponnamaneni R. Quantification of acetylcholine, an essential neurotransmitter, in brain microdialysis samples by liquid chromatography mass spectrometry. Biomedical chromatography : BMC. 2010 Jan;24(1):39-48. PubMed PMID: 19877295.
53. Chambers JP, Arulanandam BP, Matta LL, Weis A, Valdes JJ. Biosensor Recognition Elements. Curr Issues Mol Biol. 2008;10:1-12.
54. Grieshaber D, MacKenzie R, Voros J, Reimhult E. Electrochemical Biosensors - Sensor Principles and Architectures. Sensors. 2008;8:1400-58.
55. Kanik FE, Kolb M, Timur S, Bahadir M, Toppare L. An amperometric acetylcholine biosensor based on a conducting polymer. International journal of biological macromolecules. 2013 Aug;59:111-8. PubMed PMID: 23603072.
56. Dorettia L, Ferraraa D, Lorab S, Schiavonc F, Veronesec FM. Acetylcholine biosensor involving entrapment of acetylcholinesterase and poly(ethylene glycol)-modified choline oxidase in a poly(vinyl alcohol) cryogel membrane. Enzyme and Microbial Technology. 2000;27:279-85.
57. Yang M, Yang Y, Yang Y, Shen G, Yu R. Microbiosensor for acetylcholine and choline based on electropolymerization/sol–gel derived composite membrane. Analytica Chimica Acta. 2005;530(2):205-11.
58. Chen Z, Ren X, Meng X, Chen D, Yan C, Ren J, et al. Optical detection of choline and acetylcholine based on H(2)O(2)-sensitive quantum dots. Biosensors & bioelectronics. 2011 Oct 15;28(1):50-5. PubMed PMID: 21816604.
59. Sattarahmady N, Heli H, Vais RD. An electrochemical acetylcholine sensor based on lichen-like nickel oxide nanostructure. Biosensors & bioelectronics. 2013 Oct 15;48:197-202. PubMed PMID: 23685316.

60. De Bundel D, Sarre S, Van Eeckhaut A, Smolders I, Michotte Y. Critical Evaluation of Acetylcholine Determination in Rat Brain Microdialysates using Ion-Pair Liquid Chromatography with Amperometric Detection. Sensors. 2008;8(8):5171-85.

61. Yamamoto K, Sato K, Chikuma T, Kato T. A highly sensitive and stable detection of acetylcholine by HPLC-osmium-horseradish peroxidase redox polymer electrode coated on a gold radial flow ring disk. Analytica Chimica Acta. 2004;521(2):209-13.

62. Le Goff A, Holzinger M, Cosnier S. Enzymatic biosensors based on SWCNT-conducting polymer electrodes. The Analyst. 2011 Apr 7;136(7):1279-87. PubMed PMID: 21311804.

63. Wei D, Bailey MJ, Andrew P, Ryhanen T. Electrochemical biosensors at the nanoscale. Lab on a chip. 2009 Aug 7;9(15):2123-31. PubMed PMID: 19606287.

64. Putzbach W, Ronkainen NJ. Immobilization techniques in the fabrication of nanomaterial-based electrochemical biosensors: a review. Sensors. 2013;13(4):4811-40. PubMed PMID: 23580051. Pubmed Central PMCID: 3673113.

65. Weiss NO, Zhou H, Liao L, Liu Y, Jiang S, Huang Y, et al. Graphene: an emerging electronic material. Advanced materials. 2012 Nov 14;24(43):5782-825. PubMed PMID: 22930422.

66. Maka KF, Sfeirb MY, Misewichb JA, Heinza TF. The evolution of electronic structure in few-layer graphene revealed by optical spectroscopy. PNAS. 2010;107(34):14999-5004.

67. Alwarappan S, Erdem A, Liu C, Li C-Z. Probing the Electrochemical Properties of Graphene Nanosheets for Biosensing Applications. J Phys Chem. 2009;113:8853-7.

68. Kang X, Wang J, Wu H, Liu J, Aksay IA, Lin Y. A graphene-based electrochemical sensor for sensitive detection of paracetamol. Talanta. 2010 May 15;81(3):754-9. PubMed PMID: 20298849.

69. Zhou M, Zhai Y, Dong* S. Electrochemical Sensing and Biosensing Platform Based on Chemically Reduced Graphene Oxide. Anal Chem. 2009;81:5603–13.

70. Shan C, Yang H, Song J, Han D, Ivaska A, Niu L. Direct Electrochemistry of Glucose Oxidase and Biosensing for Glucose Based on Graphene. Anal Chem. 2009;81:2378–82.

71. Wang J. Carbon-Nanotube Based Electrochemical Biosensors: A Review. Electroanalysis. 2005;17(1):7-14.

72. Britto PJ, Santhanam KSV, Ajayan PM. Carbon nanotube electrode for oxidation of dopamine. Bioelectrochemistry and Bioenergetic. 1996;41:121-5.

73. Musameh M, Wang J, Merkoci A, Lin Y. Low-potential stable NADH detection at carbon-nanotube-modified glassy carbon electrodes. Electrochemistry Communications. 2002;4:743-6.

74. Krenkova J, Foret F. Immobilized microfluidic enzymatic reactors. Electrophoresis. 2004 Nov;25(21-22):3550-63. PubMed PMID: 15565708.

75. Tischer W, Wedekind F. Immobilized Enzymes: Methods and Applications. Topics in current chemistry. 1999;200:95-126.

76. Anitha K, Mohan SV, Reddy SJ. Development of acetylcholinesterase silica sol-gel immobilized biosensor--an application towards oxydemeton methyl detection. Biosensors & bioelectronics. 2004 Nov 1;20(4):848-56. PubMed PMID: 15522601.

77. Li J, Tan SN, Oh JT. Silica sol-gel immobilized amperometric enzyme electrode for peroxide determination in the organic phase. Electroanalytical Chemistry. 1998;448:69-77.

78. Gavalas VG, Law SA, Christopher Ball J, Andrews R, Bachas LG. Carbon nanotube aqueous sol-gel composites: enzyme-friendly platforms for the development of stable biosensors. Analytical biochemistry. 2004 Jun 15;329(2):247-52. PubMed PMID: 15158483.

79. Moreno-Bondi MC, Benito-Peña E. Fundamentals of Enzyme-based Sensors. Optical Chemical Sensors. 2006; 224:323-52.

80. Streets AM, Huang Y. Chip in a lab: Microfluidics for next generation life science research. Biomicrofluidics. 2013 Jan;7(1):11302. PubMed PMID: 23460772. Pubmed Central PMCID: 3574129.

81.Whitesides GM. The origins and the future of microfluidics. Nature. 2006 Jul 27;442(7101):368-73. PubMed PMID: 16871203.

82. Lion N, Reymond F, Girault HH, Rossier JS. Why the move to microfluidics for protein analysis? Current opinion in biotechnology. 2004 Feb;15(1):31-7. PubMed PMID: 15102463.

83. Zimmerman WB. Electrochemical microfluidics. Chemical Engineering Science. 2011;66(7):1412-25.

84. Heo J, Crooks RM. Microfluidic Biosensor Based on an Array of Hydrogel-Entrapped Enzymes. 2005;77:6842-51.

85. Wang J, Aki M, Onoshima D, Arinaga K, Kaji N, Tokeshi M, et al. Microfluidic biosensor for the detection of DNA by fluorescence enhancement and the following streptavidin detection by fluorescence quenching. Biosensors & bioelectronics. 2013 Aug 6;51C:280-5. PubMed PMID: 23974159.

86. Safavieh M, Ahmed MU, Tolba M, Zourob M. Microfluidic electrochemical assay for rapid detection and quantification of Escherichia coli. Biosensors & bioelectronics. 2012 Jan 15;31(1):523-8. PubMed PMID: 22177893.

87. Wisitsoraat A, Sritongkham P, Karuwan C, Phokharatkul D, Maturos T, Tuantranont A. Fast cholesterol detection using flow injection microfluidic device with functionalized carbon nanotubes based electrochemical sensor. Biosensors & bioelectronics. 2010 Dec 15;26(4):1514-20. PubMed PMID: 20727731.

88. Nakamoto K, Kurita R, Niwa O. One-chip biosensor for simultaneous disease marker/calibration substance measurement in human urine by electrochemical surface plasmon resonance method. Biosensors & bioelectronics. 2010 Dec 15;26(4):1536-42. PubMed PMID: 20888212.

89. Hong CC, Wang CY, Peng KT, Chu IM. A microfluidic chip platform with electrochemical carbon nanotube electrodes for pre-clinical evaluation of antibiotics nanocapsules. Biosensors & bioelectronics. 2011 Apr 15;26(8):3620-6. PubMed PMID: 21377860.

90. Xiong M, Gu B, Zhang JD, Xu JJ, Chen HY, Zhong H. Glucose microfluidic biosensors based on reversible enzyme immobilization on photopatterned stimuli-responsive polymer. Biosensors & bioelectronics. 2013 Jun 24;50C:229-34. PubMed PMID: 23867353.

APPENDIX

The 2014 Biomedical Engineering International Conference (BMEiCON-2014)

Amperometric Acetylcholine Biosensor Based on Graphene-PEDOT:PSS Modified Electrode

Piyawat Pitikultham
Department of Biomedical Engineering, Faculty of Engineering, Mahidol University, Nakhon Pathom, Thailand,
piyawat.pit@student.mahidol.ac.th

Chakrit Sriprachuabwong
MEMS Laboratory
National Electronics and Computer Technology Center (NECTEC)
Pathumthani, Thailand,
chakrit.sriprachuabwong@nectec.or.th

Pornpimol Sritongkham
Department of Biomedical Engineering, Faculty of Engineering, Mahidol University, Nakhon Pathom, Thailand,
pornpimol.srt@mahidol.ac.th

***Abstract*—A promising acetylcholine biosensor based on graphene-PEDOT:PSS (GR-PEDOT:PSS) and bienzyme, choline oxidase (ChOx) and acetylcholine esterase (AChE), modified electrode was successfully fabricated. GR-PEDOT:PSS ink was directly dropped on the screen printed carbon electrode (SPE), and then ChOx and AChE in the optimized loading ratio were immobilized on GR-PEDOT:PSS/SPE by the 10% glutar-aldehyde (GA) vapor cross-linked for 8 minutes. 1% Nafion was coated on the outermost layer of AChE-ChOx/GR-PEDOT:PSS/SPE for performing as a protective film. For amperometric responding, our acetylcholine biosensor showed a low limit detection, high linearity and sensitivity, which were 50 µM, 50 to 250 µM and 486.1 nA/mM, respectively, at -200 mV versus Ag/AgCl reference electrode. Moreover, this biosensor has a simple preparation, low-cost fabrication, and stable detection, thus it is a novel tool for acetylcholine determination.**

Keywords—biosensor; acetylcholine; graphene-PEDOT/PSS; enzyme immobilization

I. INTRODUCTION

Alzheimer's disease (AD) is one of neurodegenerative diseases which mostly occur in elderly people [1]. From the previous research, Hebert et al. estimated the prevalence of AD increases largely in people age 65 above, and it exponentially increases every five years of age [2]. The previous research showed that AD patients have decreased ACh concentration in cerebrospinal fluid (CSF) [3]. Currently, treatment options for AD patients are using cholinesterase inhibitors drugs, such as galantamine, donepezil, and rivas-tigmine to increase ACh concentration level. Jia Jian Ping et al., studied about the differentiation of vascular dementia (VD) and AD by using high-performance liquid chromato-graphy with electrochemical detector (HPLC-ECD) for measurement the ACh and choline concentration in CSF. The results showed that decrease of ACh concentration in CSF significantly correlates with AD and VD patients [4].

Many applications for ACh detection were developed. The correlation between ACh concentration and neurodegenerative diseases, such as AD, Parkinson's disease, and Huntington's disease, were reported. Much research focused on the application to measure *in vitro* and *in vivo* ACh concentration [5]. There are several methods for ACh measurement, such as, electrochemical measurement [6], microdialysis technique, and high-performance liquid chromatography (HPLC) [7, 8]. Each measurement method has different advantages and drawbacks, such as, micro-dialysis and HPLC which have high accuracy and very low LOD. On the contrary, they require complicated methods that must be operated by specialist and specific device in the laboratory [8].

Amperometric biosensors carry on many advantages. For example, the low-cost fabrication, easy to use and rapid response. The principle of amperometry is the current detection, which is generated from redox reaction [9]. The conductivity of working electrodes is an importance factor, in order to increase the sensitivity and facilitate an electron transfer [10]. Therefore, most research tend to modify working electrode surface with nanomaterials, such as, graphene, carbon nanotubes, and gold nanoparticles, for increasing surface to volume ratio and conductivity [11].

Graphene (GR), a monolayer of carbon atom consisting of sp^2-bonded carbon, contains unique properties, which can improve the performance of electronic devices, such as, high electric conductivity, high thermal conductivity, high surface to volume ratio, and robust structure [12, 13]. The

combination of GR with another materials: polymer, metal, and oxide, were used to enhance the thermal conductivity, electrical conductivity and flexibility. GR exhibits a very high surface area of 2630 $m^2 g^{-1}$, while graphite and single walled carbon nanotubes (SWCNT) is only 10 $m^2 g^{-1}$, and 1315 $m^2 g^{-1}$, respectively [14]. S. Alwarappan et al. reported the conductivity of GR (64 mS cm^{-1}) is higher than SWCNT about 60-fold [15]. GR modified glassy carbon electrode for detection paracetamol was fabricated by X. Kang et al. The experiment showed the increase of oxidation and reduction peak of cyclic voltammetry when was compared with bare glassy carbon electrode [16]. From the previous study, Chakrit S. et al. used graphene-PEDOT:PSS (GR-PEDOT:PSS) modified electrode which showed a high efficiency in electrochemical sensing [17].

In conclusion, this work focused on the development of amperometric ACh biosensor by immobilization of bienzyme, acetylcholine esterase and choline oxidase based on GR-PEDOT:PSS modified electrode.

II. METHODS

A. Reagents and Materials

The enzyme acetylcholine esterase (AChE), and choline oxidase (ChOx), the substrate acetylcholine chloride (AChCl) and choline chloride (ChCl), crosslinking agent glutaral-dehyde, and nanomaterial multi-walled carbon nanotubes (MWCNT) were obtained from Sigma. Graphene X4011 conductive inkjet ink (GR) was purchased from Innophene Co. (Thailand). Screen-printed electrodes were purchased from Quasense Co. (Thailand). All the other reagents were analytical grade.

B. Apparatus

The electrochemical detection was carried out with potentiostat (910PSTAT mini, Metrohm, Thailand). The amperometry was used for determination of H_2O_2 and substrate activity at 700 mV and -200 mV, respectively, versus Ag/AgCl in pH 7.4 phosphate buffer solution (PBS)

C. Preparation of MWCNT dissolved in chitosan

1 mg/ml MWCNT-CS was prepared by dissolving 5 mg chitosan in 1 ml 1% acetic acid. This mixture was stirred by magnetic stirrer for 15 minutes. Then added 1 mg MWCNT powder in 1 ml solution, and sonicated for 2 hours. The homogeneous colloid suspension of MWCNT-CS was achieved.

D. Preparation of nanomaterial modified electrode

The nanomaterial modified electrode was consisted of two different nanomaterials, GR-PEDOT:PSS and MWCNT-CS. 2 µl of GR-PEDOT:PSS was directly deposited onto working electrode surface of SPE and allowed to dry 1 hour at 37 °*C*. GR-PEDOT:PSS modified screen-printed electrode (GR-PEDOT:PSS/SPE) was achieved. MWNCT-CS

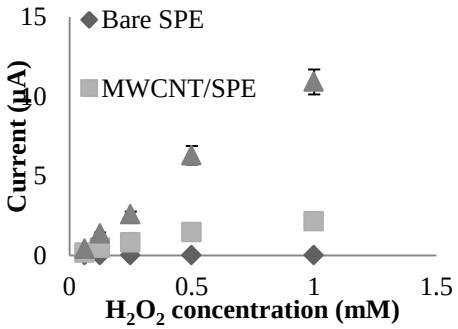

Fig. 1. Amperometric detection of different modified electrodes in H_20_2 at 700 mV vs Ag/AgCl reference electrode

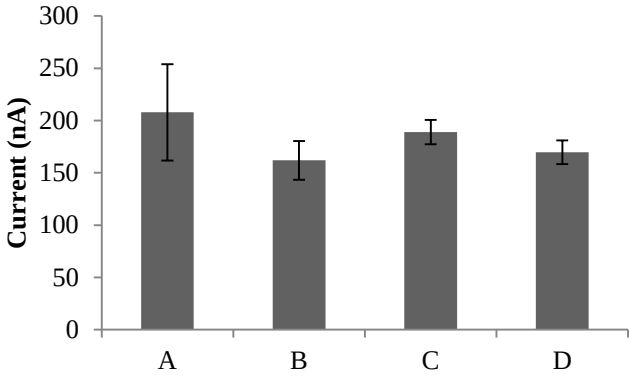

Fig. 2. Amperometric detection of different enzyme immobilization methods, A: ChOx/GR-PEDOT:PSS/SPE, B: Nafion/ChOx/GR-PEDOT:PSS/SPE, C: ChOx/GR-PEDOT:PSS/SPE with GA vapor, and D: Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vapor, in 1 mM choline chloride at -200 mV versus Ag/AgCl reference electrode

modified screen-printed electrode (MWCNT-CS/SPE) was prepared by the same process, but used the 2 µl MWCNT-CS instead of GR-PEDOT:PSS

E. Preparation of enzyme immobilization

Enzyme immobilization was performed by four different methods, ChOx/GR-PEDOT:PSS/SPE, ChOx/GR-PEDOT:PSS/SPE with GA vapor, Nafion/ChOx/GR-PEDOT:PSS/SPE and Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vapor. The homogeneous solution of ChOx prepared in 0.01 mM HEPES buffer containing 3 µl/U, was deposited on the GR-PEDOT:PSS/SPE and allowed to dry at room temperature. ChOx/GR-PEDOT:PSS/SPE was acquired. Dropped 3 µl Nafion on ChOx/GR-PEDOT:PSS/SPE and let it dried at room temperature to acquire Nafion/ChOx/GR-PEDOT:PSS/SPE. To achieve ChOx/GR-PEDOT:PSS/SPE with GA vapor, ChOx/GR-PEDOT:PSS/SPE was exposed with 5 ml 0f 10% GA solution in close system for 8 minutes. Excess free enzyme was washed by pH 7.4 PBS. Dropped 3 µl of 1% Nafion onto ChOx/GR-PEDOT:PSS/SPE with GA vapor and let it dried at room temperature to achieve Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vapor.

III. Result and Discussion

A. Detection of hydrogen peroxide

Hydrogen peroxide (H_2O_2) detection was performed by amperometry. The electrochemical system containing 4.95 ml of pH 7.4 PBS with constant applied potential, 700 mV versus Ag/AgCl reference electrode. Working electrode surface was immersed into the solution and the measurement was started. After acquired the stabilized baseline, H_2O_2 substrate was added to achieve the final concentration. Three different types of electrode modification were performed by these procedures in the different H_2O_2 final concentration: 0.063, 0.125, 0.250, 0.500 and 1.000 mM H_2O_2. The current detection is shown in Fig.1 which exhibits the linearity relation between current detection and H_2O_2 concentration. The sensitivity of bare/SPE, MWCNT-CS/SPE, and GR-PEDOT:PSS/SPE was 0.006 µA/mM, 2.038 µA/mM, and 11.25 µA/mM, respectively. GR-PEDOT:PSS/SPE which showed the highest sensitivity was selected for the further study.

,

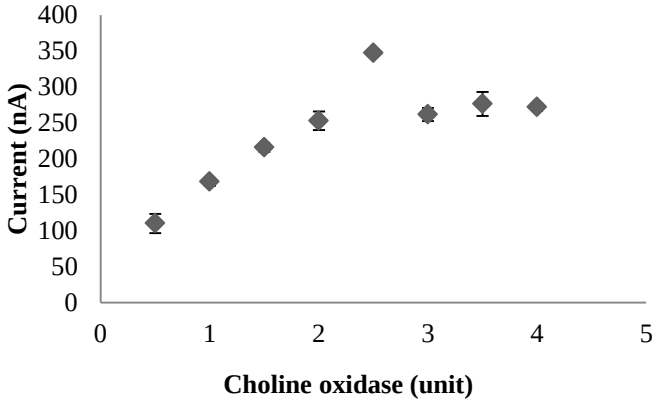

Fig. 3. The optimization of ChOx unit for 1 mM choline chloride detection at -200 mV versus Ag/AgCl reference electrode

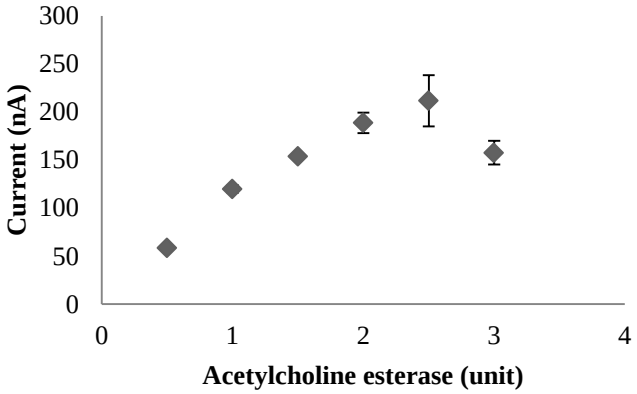

Fig. 4. The optimization of AChE unit for 1 mM acetylcholine chloride detection at -200 mV versus Ag/AgCl reference electrode

B. Enzyme immobilization

Four types of enzyme modified electrode were used in this experiment. All contained 1U ChOx. The efficacy of enzyme immobilization was analyzed by amperometry, -200 mV versus Ag/AgCl reference electrode. Current detection was measured from H_2O_2 which was hydrolyzed from 1 mM ChCl by ChOx. The results were shown in Fig.2. ChOx/GR- PEDOT:PSS/SPE showed the highest current detection 207.8 ±45.9 nA, but highest fluctuation. ChOx was immobilized by the physical absorption. It is very fragile and easy to elute from the working electrode surface which was immersed in electrochemical cell. The lowest fluctuation of current detection was received from Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vapor, 169.63±11.23 nA. The use of Nafion as a thin protective film layer could prevent the enzyme from elution. Meanwhile, GA enhances the enzyme immobilization efficacy by acting as a bridge between amino groups of enzyme and carboxylic groups of GR-PEDOT:PSS, consequently the enzyme was immobilized inside the matrix formation. Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vapor showed the lowest fluctuation, therefore it was considered for the next experiment. PEDOT:PSS/SPE showed the highest current detection 207.8 ±45.9 nA, but highest fluctuation. ChOx was immobilized by the physical absorption. It is very fragile and easy to elute from the working electrode surface which was immersed in electrochemical cell. The lowest fluctuation of current detection was received from Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vapor, 169.63±11.23 nA. The use of Nafion as a thin protective film layer could prevent the enzyme from elution. Meanwhile, GA enhances the enzyme immobilization efficacy by acting as a bridge between amino groups of enzyme and carboxylic groups of GR-PEDOT:PSS, consequently the enzyme

was immobilized inside the matrix formation. Nafion/ChOx/GR-PEDOT:PSS/SPE with GA vapor showed the lowest fluctuation, therefore it was considered for the next experiment.

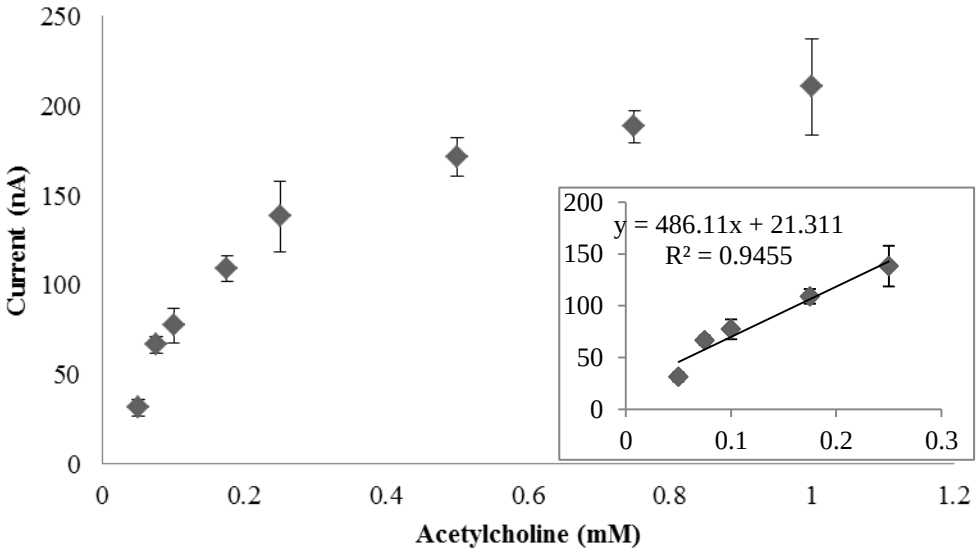

Fig. 5. A: Amperometric calibration curve of Nafion/AChE-ChOx/GR-PEDOT:PSS/SPE with GA vapor cross-linked, B: Linearity of acetylcholine chloride detection.

C. Optimization of enzyme ratio

Because of ACh detection was achieved by the consecutive reaction of bi-enzyme, AChE and ChOx, the enzyme ratio must be chosen judiciously. The concentration of ChOx was varied from 0.5U to 4.0U. Fig.3 showed the current detection in 1 mM ChCl by amperometry. In the range of 0.5U to 2.5U ChOx, the current detection increased proportionally with enzyme concentration, but decreased at more than 3U ChOx. 2.5U ChOx performed the highest current detection which was 347.35 nA. Due to cost-effectiveness, our group decided to use 1.5U ChOx. AChE was varied in 0.5U to 3.0U for mixed with 1.5U ChOx, and used in 1 mM AChCl detection. At 2.5U:1.5U (AChE:ChOx) showed the highest current detection, 211.59 nA (Fig.4). Increasing enzyme concentration facilitated the chance of enzyme and substrate binding, consequently increasing reaction rate. Nevertheless, the excess enzyme immobilization could act as a barrier which prevented the diffusion and electron transfer rate on an electrode surface, thus lessened reaction rate.

D. Calibration curve of acetylcholine detection

Nafion/AChE-ChOx/GR-PEDOT:PSS/SPE with GA vapor with the optimized enzyme ratio 2.5U:1.5U (AChE:ChOx) was used to measure ACh concentration. The range of concentration measurement is 0.50 µM to 1 mM AChCl. The calibration curve was shown in Fig.5. The current detection increased from 31.35 nA to 210.70 nA with the increase of AChCl concentration. The linear relation of current detection located in the range of 50 µM to 250 µM (R^2= 0.945). For the concentration higher than 250 µM AChCl, the curve tend is an approximate plateau. This sensor showed the LOD at 0.50 µM, and sensitivity at 486.1 nA/mM.

IV. Conclusion

GR-PEDOT:PSS and MWCNT was selected for electrode modification. The sensitivity of H_2O_2 detection, at 0.7V vs Ag/AgCl, based on bare SPE, MWCNT/SPE, and GR-PEDOT:PSS/SPE was 6.193 nA/mM, 2.038 µA/mM, and 11.25 µA/mM, respectively. GR-PEDOT:PSS/SPE exhibited the highest sensitivity, and was selected for the further study. ChOx was immobilized on GR-

PEDOT:PSS/SPE by 10% GA vapor cross-linked for 8 minutes and 1% Nafion solution coating (NAFION/ChOx/GR-PEDOT:PSS/SPE). It showed an extremely low fluctuation of current detection when compared with physical absorption. Thus it confirmed that this method can prevent the elution of enzyme from SPE surface. For the amount of enzyme optimization, 0.5U to 4.0U of ChOx was varied for amperometric detection in pH 7.4 PBS containing 1 mM ChCl at -200 mV versus Ag/AgCl. 2.5U of ChOx showed the highest current response. However, we opted to use 1.5U of ChOx for the low-cost fabrication. The variation of AChE, 0.5U to 3.0U, was mixed with 1.5U of ChOx. The results showed that 2.5U of AChE exhibited the highest current detection of PBS containing 1 mM AChCl. The optimization of enzyme ratio is 1.5:2.5 (ChOx:AChE) and the calibration curve shows the limit of detection, linearity and sensitivity, 50 µM, 50 to 250 µM and 486.1nA/mM, respectively. Therefore, Nafion/AChE-ChOx/GR-PEDOT:PSS/SPE with GA vapor is promising candidate for determination of acetylcholine.

REFERENCES

[1] Sonkusare SK, Kaul CL, Ramarao P. Dementia of Alzheimer's disease and other neurodegenerative disorders--memantine, a new hope. Pharmacological research : the official journal of the Italian Pharmacological Society. 2005 Jan;51(1):1-17. PubMed PMID: 15519530.

[2] Hebert LE, Scherr PA, Bienias JL, Bennett DA, Evans DA. Alzheimer Disease in the US Population. Arch Neurol. 2003;60:1119-22.

[3] Croxson PL, F. PG, Browning, Gaffan D, Baxter MG. Acetylcholine facilitates recovery of episodic memory after brain damage. J Neurosci. 2012;32(40):13787-95.

[4] Jian-ping J, Jian-min J, Wei-dong Z, Min X, Chang-biao C, Xin Y, et al. Differential acetylcholine and choline concentrations in the cerebrospinal fluid of patients with Alzheimer's disease and vascular dementia. Chinese Medical Journal. 2004;117(8):1161-4.

[5] Perry M, Li Q, Kennedy RT. Review of recent advances in analytical techniques for the determination of neurotransmitters. Anal Chim Acta. 2009 Oct 19;653(1):1-22. PubMed PMID: 19800472. Pubmed Central PMCID: 2759352.

[6] Yamamoto K, Sato K, Chikuma T, Kato T. A highly sensitive and stable detection of acetylcholine by HPLC-osmium-horseradish peroxidase redox polymer electrode coated on a gold radial flow ring disk. Analytica Chimica Acta. 2004;521(2):209-13.

[7] Uutela P, Reinila R, Piepponen P, Ketola RA, Kostiainen R. Analysis of acetylcholine and choline in microdialysis samples by liquid chromatography/tandem mass spectrometry. Rapid communications in mass spectrometry : RCM. 2005;19(20):2950-6. PubMed PMID: 16180202.

[8] Nirogi R, Mudigonda K, Kandikere V, Ponnamaneni R. Quantification of acetylcholine, an essential neurotransmitter, in brain microdialysis samples by liquid chromatography mass spectrometry. Biomedical chromatography : BMC. 2010 Jan;24(1):39-48. PubMed PMID: 19877295.

[9] Grieshaber D, MacKenzie R, Voros J, Reimhult E. Electrochemical Biosensors - Sensor Principles and Architectures. Sensors. 2008;8:1400-58.

[10] Le Goff A, Holzinger M, Cosnier S. Enzymatic biosensors based on SWCNT-conducting polymer electrodes. The Analyst. 2011 Apr 7;136(7):1279-87. PubMed PMID: 21311804.

[11] Wei D, Bailey MJ, Andrew P, Ryhanen T. Electrochemical biosensors at the nanoscale. Lab on a chip. 2009 Aug 7;9(15):2123-31. PubMed PMID: 19606287.

[12] GEIM AK, NOVOSELOV KS. The rise of graphene. nature materials. 2007;6.

[13] Chen XM, Wu GH, Jiang YQ, Wang YR, Chen X. Graphene and graphene-based nanomaterials: the promising materials for bright future of electroanalytical chemistry. The Analyst. 2011 Nov 21;136(22):4631-40. PubMed PMID: 21975368.

[14] Zhu Y, Murali S, Cai W, Li X, Suk JW, Potts JR, et al. Graphene and graphene oxide: synthesis, properties, and applications. Advanced materials. 2010 Sep 15;22(35):3906-24. PubMed PMID: 20706983.

[15] Alwarappan S, Erdem A, Liu C, Li C-Z. Probing the Electrochemical Properties of Graphene Nanosheets for Biosensing Applications. J Phys Chem. 2009;113:8853-7.

[16] Kang X, Wang J, Wu H, Liu J, Aksay IA, Lin Y. A graphene-based electrochemical sensor for sensitive detection of paracetamol. Talanta. 2010 May 15;81(3):754-9. PubMed PMID: 20298849.

[17] Sriprachuabwong C, Karuwan C, Wisitsorrat A, Phokharatkul D, Lomas T, Sritongkham P, et al. Inkjet-printed graphene-PEDOT:PSS modified screen printed carbon electrode for biochemical sensing. Journal of Materials Chemistry. 2012;22(12):5478.

BIOGRAPHY

NAME	Mr. Piyawat Pitikultham
DATE OF BIRTH	5 December 1989
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 2008 – 2011 Bachelor of Applied Thai Traditional Medicine**,** Siriraj Hospital Mahidol University, 2011-2015 Master of Engineering (Biomedical Engineering)
HOME ADDRESS	165/88 Moo 8 Bangkae 10 Lane, Bangkae Road, Bangkae Sub-District, Bangkae District, Bangkok, Thailand, 10160 E-mail : piyawat.ptkt@gmail.com