

DECLARATION OF THESIS AND COPYRIGHT

Author's Full Name : GERALDINE CHAN SUE CHING

Date of Birth : 19 OCTOBER 1989

Title : DEVELOPMENT OF A GRAPHENE BASED IMMUNO-
BIOSENSOR FOR HEPATITIS B VIRUS SURFACE ANTIGEN
DETECTION

Academic Session :

I declare that this thesis is classified as:

<input type="checkbox"/>	CONFIDENTIAL	(Contains confidential information under the Official Secret Act 1997)*
<input type="checkbox"/>	RESTRICTED	(Contains restricted information as specified by the organization where research was done)*
<input type="checkbox"/>	OPEN ACCESS	I agree that my thesis to be published as online open access (Full Text)

I acknowledge that Universiti Malaysia Pahang reserve the right as follows:

1. The Thesis is the Property of Universiti Malaysia Pahanag
2. The Library of Universiti Malaysia Pahang has the right to make copies for the purpose of research only.
3. The Library has the right to make copies of the thesis for academic exchange.

Certified By: _____

(Student's Signature)

891019-13-5610

New IC/ Passport Number

Date:

(Supervisor's Signature)

Name of Supervisor

Date:

NOTE : * If the thesis is CONFIDENTIAL or RESTRICTED, please attach with the letter page 2 from the organization with the period and reasons for condentiality or restriction.

DEVELOPMENT OF A GRAPHENE BASED IMMUNO-BIOSENSOR FOR HEPATITIS
B VIRUS SURFACE ANTIGEN DETECTION

GERALDINE CHAN SUE CHING

Thesis submitted in fulfilment of the requirements for the award of the degree Master of
Engineering (Chemical)

Faculty of Chemical and Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

APRIL 2016



SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Master of Engineering in Chemical Engineering.

(Supervisor's Signature)

Full Name : CHONG FUI CHIN

Position :

Date :

(Co-supervisor's Signature)

Full Name : CHONG KWOK FENG

Position :

Date :



STUDENT'S DECLARATION

I hereby declare that the work in this thesis is based on my original work except for quotations and citation which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Malaysia Pahang or any other institutions.

(Author's Signature)

Full Name : GERALDINE CHAN SUE CHING

ID Number : MKC13019

Date : APRIL 2016

TABLE OF CONTENTS

	Page
DECLARATION	
TITLE PAGE	i
ACKNOWLEDGEMENTS	ii
ABSTRACT	iii
ABSTRAK	iv
TABLE OF CONTENTS	v
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	xii
CHAPTER 1 INTRODUCTION	1
1.1 Background of Study	1
1.2 Motivation	3
1.3 Problem Statement	4
1.4 Objectives of Study	6
1.5 Scope of Study	6
CHAPTER 2 LITERATURE REVIEW	8
2.1 Introduction	8
2.2 Hepatitis B Virus (HBV)	8
2.2.1. Infection of HBV	10
2.2.2. Clinical Diagnostics of HBV Infection	10
2.3 Reduced Graphene Oxide (RGO)	14
2.3.1. Synthesis of RGO	14
2.3.2. Properties of RGO	21
2.4 Biosensors	27
2.4.1. Immuno-Biosensors	30
2.5 Graphene Based Immuno Biosensor	32

2.6	Application of RGO as Electrochemical Biosensor	35
2.7	Summary	38
CHAPTER 3 METHODOLOGY		39
3.1	Introduction	39
3.2	Materials and Reagents	39
3.3	Sample Preparation	41
	3.3.1. Synthesis of Graphite Oxide	41
	3.3.2. Chemical Reduction of Graphite Oxide RGO	42
	3.3.3. Functionalization of RGO	42
	3.3.4. Direct Deposition of RGO on GCE	43
	3.3.5. Immobilization of HBsAg onto Functionalized RGO	44
3.4	Characterizations of Hydrazine Reduced Graphene Oxide	46
	3.4.1. Reduction Rate Measurements by UV-Vis	46
	3.4.2. Qualitative Measurements of RGO	47
3.5	Detection of HBsAg	48
	3.5.1. Incubation of Secondary Antibody, HBsAb-HRP)	48
3.6	Electrochemical Measurement	48
	3.6.1. Electrode Preparation for Cyclic Voltammetry Testing	48
	3.6.2. Electrochemical Characteristics on Electrode Surface via Cyclic Voltammetry	49
3.7	Adsorption of Hepatitis B Surface Antigen (HBsAg) Onto Functionalized RGO	49
	3.7.1. Preparation of Standard Calibration Curve	49
	3.7.2. Preparation of Samples for Adsorption Studies	50
	3.7.3. Experimental Data Analysis	52
CHAPTER 4 RESULTS AND DISCUSSION		53
4.1	Introduction	53
4.2	Qualitative Characterization of RGO	53
	4.2.1. Dispersion observation of RGO	53
	4.2.2. Ultraviolet-visible Absorption Spectrophotometry (UV-Vis)	55
	4.2.3. Fourier Transform Infrared Spectroscopy (FTIR)	56
	4.2.4. Scanning Electron Microscopy (SEM)	59
	4.2.5. Field Emission Scanning Electron Microscopy (FESEM)	61
4.3	Functionalized RGO	64

4.3.1.	Detection of HBsAg by Functionalized RGO	64
4.4	Electrochemical Characterization	66
4.4.1.	Cyclic Voltammetry (CV)	67
4.5	Adsorption Studies	71
4.5.1.	Adsorption of HBsAg onto Functionalized RGO	72
4.5.2.	Estimation of Amount of HBsAg Using ELISA Method	72
4.5.3.	Determination of Equilibrium Adsorption Isotherm	73
4.5.4.	Effect of pH	78
4.5.5.	Effect of Incubation Time	79
CHAPTER 5 CONCLUSION AND RECOMMENDATIONS		81
5.1	Introduction	81
5.2	Conclusion	81
5.3	Recommendations	82
5.3.1.	Evaluate Electrochemical Properties via Electrochemical Impedance Spectroscopy (EIS)	82
5.3.2.	Incorporate Biorecognition Elements into Graphene-based Electrodes	82
REFERENCES		83
APPENDICES		96
A	Adsorption Data (Effect of Concentration)	96
B	Adsorption Data (Effect of pH)	97
C	Adsorption Data (Effect of Incubation Time)	98
PUBLICATION AND AWARDS		99

LIST OF TABLES

Table	Title	Page
2.1	Summary of different methods of detection of HBV.	13
2.2	Linearized Langmuir and Freundlich isotherms. (Chen, 2015)	26
2.3	Summary of GO based immunosensor	34
2.4	Summary of RGO based electrochemical biosensor.	37
4.1	Adsorption isotherm data used for the modelling of Langmuir isotherm and Freundlich isotherm.	75
4.2	Summary of Langmuir and Freundlich isotherm parameters obtained from linear fitting.	77

LIST OF FIGURES

Figure	Title	Page
2.1	Graphical illustration of HBV (Gitlin, 1997)	9
2.2	Structure and organization of HBV genome (Mahoney, 1999)	9
2.3	Illustration on the fundamentals of a typical sandwich ELISA. (World Health Organisation, 2004)	11
2.4	Schematic diagram of graphene production via CVD process and transfer via polymer support (Kim et al., 2009)	16
2.5	Schematic diagram of the roll-to-roll production of the 30-inch graphene on copper foil and transferred to target substrate (Bae et al., 2010)	17
2.6	Structural layout of (a) graphene and (b) graphite (Kumar and Lee, 2013)	18
2.7	Graphical illustration of (a) oxidation of graphite to GO and reduction to reduced graphene oxide and (b) proposed reaction pathway for epoxy reduction by hydrazine (Hakimi and Alimard, 2012)	20
2.8	SEM micrographs of (a) graphite flakes and (b) RGO (Loryuenyong et al., 2013)	22
2.9	SEM micrographs of RGO produced via Hummer's Method. (Cao and Zhang, 2014)	22
2.10	SEM micrographs of (a) GO and (b) RGO (Fu et al., 2013)	23
2.11	Cyclic voltammograms of stepwise modified GCE with G and NG derivatives in 0.1 M NaPBS pH 7.0; $\nu = 100$ mV/s. (Prathish et al., 2013)	24
2.12	Adsorption behaviour of HBcAg which obeys Langmuir adsorption isotherm (Ng et al., 2007)	27
2.13	Schematic representation of a biosensor build up	28
2.14	Types of transducer used in biosensors	29
2.15	Antibody (Ab) molecular structure with the presence of antigen binding sites (triangles) and disulphide linkage (lines) (Conroy et al., 2009)	31
2.16	GO-based immunosensor proposed for rotavirus detection (Jung et al., 2010)	32
2.17	Treatment using APTES onto MLG prior to antibody attachment (Teixeira et al., 2014)	33

2.18	(A) Fabrication process of graphene based biosensor (B) Graphical illustrations of graphene based biosensor for the detection of rotavirus. (Liu et al., 2011)	36
2.19	Cyclic voltammogram of stepwise change occurring on the electrode for glucose electrochemical sensor (Unnikrishnan et al., 2013)	37
2.20	Schematic diagram of detection of BoNT/A using a RGO/Au electrode Chan et al. (2015)	38
3.1	Structural formula of nafion 117 solution (Chen and Hong, 2010)	40
3.2	Structural formula of thionine acetate salt (Katz et al., 2003)	41
3.3	Illustration of proposed reaction mechanism involved during immobilization of HBsAb and HBsAg onto functionalized RGO	45
4.1	Dispersion of (A) graphene oxide, (B) reduced graphene oxide in water and (C) functionalized RGO with 1 % Nafion. (D) dried RGO powder	54
4.2	Graph of absorbance vs. wavelength obtained from UV-Vis spectroscopy shows the peak shift from 231nm to 265nm	55
4.3	FTIR spectra of (A) graphite and (B) GO	57
4.4	FTIR spectra of (A) RGO, (B) functionalized RGO and (C) functionalized RGO attached with antigen antibody	58
4.5	SEM micrographs of (A) graphite, (B) GO and (C) RGO at magnification of 5000 X respectively	60
4.6	FESEM micrograph of (A) GO and (B) RGO	62
4.7	FESEM micrograph of functionalized (A) RGO and (B) HBsAg immobilized on functionalized RGO	63
4.8	Graphical illustration of possible reaction mechanism of HBsAg detection by functionalized RGO based biosensor	65
4.9	The changes that occur before (A, C) and after (B,D) addition of TMB substrates to controlled sample (A, B) whereby the presence of functionalized RGO is absent and tested sample (C,D) whereby functionalized RGO is present	66
4.10	(A) A representative cyclic voltammograms of bare functionalized RGO-modified GCE with varying scan rates (10 mV/s, 20 mV/s, 50 mV/s) in the K ₃ [Fe(CN) ₆]/K ₄ [Fe(CN) ₆] redox system. (B) Relationship between the peak current and square root of scan rate	68

4.11	The cyclic voltammetric curves (a) bare GCE (b) functionalized RGO-GCE (c) functionalized RGO-HBsAb-GCE (d) functionalized RGO-HBsAb-BSA-GCE and (e) functionalized RGO-HBsAb-HBsAg-GCE in 2.5 mM potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$) in 1:1 mixture with 0.1 M PBS containing 0.1 M KCl and with a scan rate of 20 mV s ⁻¹	70
4.12	The cyclic voltammetric curves of RGO-modified GCE incubated with HBsAg at (a) 0 ng/mL (b) 1 ng/mL (c) 0.9 ng/mL (d) 0.5 ng/mL (e) 0.4 ng/mL (f) 0.3 ng/mL for 30 minutes at room temperature	71
4.13	Graph of Standard ELISA calibration curve	73
4.14	Equilibrium adsorption isotherm of HBsAg adsorbed onto functionalized RGO for the prediction of adsorption behaviour	75
4.15	Linear fitting graph plots of Langmuir	76
4.16	Linear fitting graph plots of Freundlich isotherms	76
4.17	Effect of varying pH on the amount of HBsAg adsorbed at equilibrium (q_e , ng HBsAg/g) onto functionalized RGO	79
4.18	Effect of incubation time against the amount of HBsAg adsorbed at equilibrium (Q_e , ng HBsAg/g) onto functionalized RGO	80

LIST OF ABBREVIATIONS

Ab	Antibody
ACH	Adrenal cortical hormones
AFP	α -1-fetoprotein
AH	Aluminium hydroxide
APTES	3-Aminopropyl-triethoxysilane
Au@MGN	Multifunctional graphene nanocomposites
AuNP	Gold nanoparticles
BSA	Bovine serum albumin
BoNT/A	Botulinum neurotoxin serotype A
CEA	Carcinoembryonic antigen
CMG	Chemically modified graphene
CNT	Carbon nanotube
CuS	Copper monosulfide
CV	Cyclic voltammetry
CVD	Chemical vapour deposition
DI	Deionized
DNA	Deoxyribonucleic acid
DPV	Differential pulse voltammetry
EBA	Expanded bed adsorption chromatography
EIA	Enzyme immunoassay
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
FESEM	Field emission scanning electron microscopy
FET	Field effect transistor

FRET	Fluorescence resonance energy transfer
FTIR	Fourier transform infrared spectroscopy
GBP	Gold-binding polypeptide
GCE	Glassy carbon electrode
GO	Graphite Oxide
GO _x	Glucose oxidase
GNWs/GO	Gold nanowire functionalized graphene sheets
HBcAg	Hepatitis B core antigen
HBsAb	Hepatitis B surface antigen antibody
HBsAb-HRP	Hepatitis B surface antigen antibody conjugated with HRP
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
hCG	Human chorionic gonadotropin
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	Ionic liquid
IR	Infrared
ITO	Indium tin oxide
MGN	Magnetic graphene nanocomposites
MLG	Multilayer graphene
MWCNT	Multiwalled carbon nanotube
NADH	H ₂ O ₂ /β-nicotinamide adenine dinucleotide
NaPBS	Sodium phosphate buffered saline
NASBA	Nucleic acid sequence-based amplification
NG	Nitrogen doped graphene

NMR	Nuclear magnetic resonance
OPV	Organic photovoltaic cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDDA	Polydiallyldimethylammonium
PDMS	Poly(dimethylsiloxane)
PSE	1-pyrenebutyric acid N-hydroxysuccinimide ester
PTFE	Polytetrafluoroethylene
RGO	Reduced graphene oxide
RIA	Radio immunoassay
RNA	Ribonucleic acid
RT	Room temperature
SEM	Scanning electron microscopy
SPR	Surface plasmon resonance
TEM	Transmission electron microscopy
TMB	3,3',5,5'-Tetramethylbenzidine
TPA	Tissue polypeptide antigen
USA	United States of America
UV	Ultraviolet
UV-Vis	Ultraviolet-visible spectroscopy
WHO	World Health Organisation

DEVELOPMENT OF A GRAPHENE BASED IMMUNO-BIOSENSOR FOR HEPATITIS
B VIRUS SURFACE ANTIGEN DETECTION

GERALDINE CHAN SUE CHING

Thesis submitted in fulfilment of the requirements for the award of the degree Master of
Engineering (Chemical)

Faculty of Chemical and Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

APRIL 2016

ABSTRACT

Hepatitis B virus (HBV) is a blood-borne and transfusion-transmitted human pathogen that has a large impact on blood safety and public health worldwide whereas reduced graphene oxide (RGO) is a derivatives of graphene which has gain much attention in electrochemical immunosensors. HBV infects the liver and causes chronic and acute Hepatitis. The best treatment for this disease is for the early detection before the occurrence of severe liver damages. The presence of Hepatitis B surface antigen (HBsAg) is evidence of presence of HBV infections. Presently, the conventional methods shows weakness in terms of time and efficiency. In this research, it aims to immobilize the HBsAg antibody (HBsAb) onto functionalized RGO as well as to study the interaction between HBsAb and HBsAg by electrochemical probe of the functionalized RGO. RGO was synthesized via modified Hummer's methods and reduced using hydrazine hydrate. RGO is functionalized with nafion and thionine prior to immobilization of HBsAb. Qualitative analysis of functionalized RGO was conducted via ultraviolet-visible spectrophotometry (UV-Vis), fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and field emission scanning electron microscopy (FESEM). Electrochemical analysis of functionalized RGO probe electrode on the detection of HBsAg was done via cyclic voltammetry (CV). As a conclusion, successful immobilization of HBsAg on functionalized RGO was proven to obey Freundlich adsorption isotherm with maximum adsorption capacity of 31 ng HBsAg/g and further confirmed with the change in surface structure observed as well as the presents of functional groups detected. Interaction between HBsAg and HBsAb causes the colour change when tetramethylbenzidine (TMB) was added and step changes to occur during CV with a limit of detection at 0.5 ng/mL.

ABSTRAK

HBV berjangkit melalui pemindahan dan transfusi darah yang mempunyai kesan yang besar terhadap keselamatan darah dan kesihatan awam di seluruh dunia manakala RGO adalah variasi graphene yang telah menarik perhatian ramai para penyelidik dalam pelbagai bidang. HBV menjangkiti hati dan menyebabkan Hepatitis kronik dan akut. Rawatan yang terbaik untuk penyakit ini adalah pengesanan awal sebelum berlakunya kerosakan hati yang teruk. Jangkitan HBV dikesan melalui pengesanan HBsAg. Pada masa kini, kaedah pengesanan HBV konvensional menunjukkan kelemahan dari segi masa dan kecekapan. Kajian ini bertujuan untuk mempekenalkan HBsAb ke atas RGO yang telah difungsikan serta untuk mengkaji interaksi antara HBsAb dan HBsAg dengan menggunakan kaedah electrokimia. RGO telah dihasilkan melalui kaedah Hummer yang telah diubahsuaikan dan melalui penurunan dengan menggunakan agen penurunan hydrazine hidrat. RGO difungsikan dengan nafion dan thionine sebelum diperkenalkan dengan HBsAb. Analisis kualitatif ke atas RGO yang telah difungsikan adalah melalui ultraviolet-visible spektrofotometri (UV-Vis), spektrokopi Fourier transform infrared (FTIR), mikroskop imbasan elektron (SEM), dan 'field' emisi mikroskop imbasan elektron (FESEM). Analisis electrokimia dijalankan dengan menggunakan elektrod yang mengandungi RGO yang telah difungsikan untuk mengesan HBsAg melalui voltammetry kitaran (CV). Had pengesanan diperolehi ialah kira-kira 0.5 ng / mL. Tambahan pula, ekuilibrium penjerapan isoterma RGO yang telah difungsikan dan HBsAg didapati mematuhi Freundlich penjerapan isoterma dengan kapasiti maksimum, 31 ng HBsAg / g.