DEVELOPMENT OF SANDWICH AND LABEL-FREE ELECTROCHEMICAL IMMUNOSENSOR FOR DETECTION OF HER2 BREAST CANCER BIOMARKER

ZUR MIRA AZIZAH @ NOR HAIZA BINTI LAH

FS 2019 72

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By

ZUR MIRA AZIZAH @ NOR HAIZA BINTI LAH

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

August 2018

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

DEVELOPMENT OF SANDWICH AND LABEL-FREE ELECTROCHEMICAL IMMUNOSENSOR FOR DETECTION OF HER2 BREAST CANCER BIOMARKER

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August 2018

Chairman : Shahrul Ainliah Alang Ahmad, PhD

Faculty : Science

An electrochemical immunosensor for determination of HER2 biomarker was developed using two different types of immunoreaction, which are label-free and sandwich assay. The fabrication of label-free immunosensor involved several steps; (1) pre-treatment of screen printed carbon electrode (SPCE) (2) the binding of primary anti-HER2 antibody to pre-treated SPCE via EDC/NHS (3) bovine serum albumin (BSA) blocking (4) the binding of HER2 antigen (5) electroanalytical performance of the label-free immunosensor. While, in sandwich immunosensor, lead sulphide quantum dots coated with secondary antibodies (Ab2-PbS QDs) were used as a label and the fabrication involved additional several steps which were; (1) synthesis and bioconjugation of PbS QDs with secondary onto-HER2 antibody (2) the sandwich of Ab2-PbS QDs onto label-free immunosensor (3) acid dissolution of Pb^{2+} from label and detection via square wave voltammetry (SWV). The electrochemical properties of the developed immunosensors were analysed by cyclic voltammetry (CV) and electron impedance spectroscopy (EIS) using $Fe(CN)_6^{-3/-4}$ as a redox probe. Under optimal condition, label-free and sandwich immunosensors were assessed by using differential pulse voltammetry (DPV) and SWV, respectively. The analytical performances revealed that both immunosensor give satisfactory sensitivity, selectivity and reproducibility towards HER2 cancer biomarker. For a linear range of 0.04-2.0 ng/ml of label-free immunosensor, the limit of detection obtained was 0.01 ng/ml while for sandwich immunosensor, the limit of detection obtained was 1.1 ng/ml for a linear range of 2.0-10 ng/ml Furthermore, excellent

recovery also obtained in human serum sample for both label-free and sandwich immunosensor. It was discovered that the recovery percentage of HER2 antigen in sandwich immunosensor was 102.06 % with RSD percentage of 7.51 %, while for label-free immunosensor, the percentage recovery is 97.10 % with RSD percentage of 10.89 %.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PEMBANGUNAN IMMUNOSENSOR ELEKTROKIMIA SANDWIC DAN TANPA LABEL BAGI PENGESANAN BIO PENANDA BARAH PAYUDARA HER2

Oleh

ZUR MIRA AZIZAH @ NOR HAIZA BINTI LAH

Ogos 2018

Pengerusi : Shahrul Ainliah Alang Ahmad, PhD

Fakulti : Sains

Immunosensor elektrokimia untuk penentuan HER2 biomarker telah dibangunkan menggunakan dua jenis imunoreaksi yang berbeza, yang bebas label dan sandwic assay. Pembuatan imunosensor bebas label melibatkan beberapa langkah; (1) pra-rawatan elektrod karbon bercetak skrin (SPCE) (2) proses immobilisasi antibodi anti-HER2 primer untuk SPCE yang telah diaktifkan melalui EDC / NHS (3) penyebaran serum albumin bovine (BSA) HER2 antigen (5) prestasi elektroanalisis imunosensor bebas label. Sementara itu, dalam imunosensor sandwic, titik kuantum yang dilapisi dengan antibodi sekunder (Ab2-PbS QDs) telah digunakan sebagai label dan fabrikasi melibatkan beberapa langkah tambahan; (1) sintesis dan biokonjugasi PbS QDs dengan antibodi ke-HER2 sekunder (2) sandwic Ab2-PbS QDs ke immunosensor bebas larutan label (3) pembubaran asid Pb^{2+} dari label dan pengesanan melalui voltammetry gelombang persegi (SWV). Sifat-sifat elektrokimia imunosensor telah dianalisis oleh voltammetry kitaran (CV) dan spektroskopi impedans elektron (EIS) menggunakan $Fe(CN)_6^{-3/-4}$ sebagai penyelidikan redoks. Di dalam keadaan optimum, imunosensor bebas label dan sandwic dinilai dengan menggunakan voltmeter nadi (DPV) dan SWV, masing-masing. Keputusan analitikal mendedahkan bahawa kedua-dua imunosensor memberikan sensitiviti, selektiviti dan keboleh ulangan yang memuaskan terhadap biopenanda HER2. Untuk julat linear 0.04-2.0 ng/ml immunosensor bebas label, had pengesanan yang diperoleh adalah 0.01 ng/ml manakala untuk immunosensor sandwic, had pengesanan yang diperoleh adalah 1.1 ng / ml untuk julat linear 2.0-10 ng / ml Selain itu, pemulihan yang sangat baik juga diperolehi dalam sampel serum manusia untuk kedua-dua bebas label dan imunosensor sandwic. Hasil penemuan

menunujukkan peratusan pemulihan antigen HER2 dalam imunosensor sandwic adalah 102.06% dengan peratusan RSD sebanyak 7.51%, manakala bagi imunosensor bebas label, pemulihan peratusan adalah 97.10% dengan peratusan RSD sebanyak 10.89%.

ACKNOWLEDGEMENT

First and foremost I would like to thank Allah the almighty for giving the strength to finish up this thesis. Without His love and blessing, this thesis would not be possible.

I would like to express my gratitude to my main supervisor, Dr. Shahrul Ainliah Alang Ahmad for her guidance and advice through this research project. Without her knowledge and expertise, this research project would not be possible.

I am highly indebted to my co-supervisor, Dr. Mazliana Ahmad Kamarudin on her help during my process in learning on synthesis of quantum dots. I also would like to extend my gratitude to my other co-supervisors, Dr. Jaafar Abdullah and Dr. Janet Lim Hong Ngee for their advice for my research project.

Special appreciations also go to my lab mates in lab 103 for their useful guide in conducting experiments.

Finally, I would like to express my gratitude to my parents, Puan Jamaliah and Encik Lah for their prayer and encouragement. Without their support, I would not be able to finish up this thesis.

Thank you.

ZUR MIRA AZIZAH @ NOR HAIZA BINTI LAH

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Shahrul Ainliah Alang Ahmad, PhD
Senior Lecturer
Faculty of Science
Universiti Putra Malaysia
(Chairman)

Mazliana Ahmad Kamarudin, PhD
Senior Lecturer
Faculty of Science
Universiti Putra Malaysia
(Member)

Jaafar Abdullah, PhD
Senior Lecturer
Faculty of Science
Universiti Putra Malaysia
(Member)

Janet Lim Hong Ngee, PhD
Senior Lecturer
Faculty of Science
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Name of
Member of
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Committee: _____

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LIST OF ABBREVIATIONS

Ab1	Primary Antibody
Ab2	Secondary Antibody
AFP	Alpha Fero Protein
Anti-CEA	Anti-Carcinoembryonic
Anti-cTnT	Anti-Cardiac Troponin T
Anti HER2	Anti-Human Epidermal Growth Factor Receptor 2
APTES	(3-Aminopropyl)triethoxysilane
AuNPs	Gold nanoparticles
BSA	Bovine serum albumin
CDI	Carbonylimidazole
C-Dot	Carbon dot
CDSe/CdZns	Cadmium Selenide/Cadmium Zinc
CDTe	Cadmium telluride
CEA	Carcinoembryonic Antigen
CLB	Clenbuterol
CNT	Carbon Nanotube
CV	Cyclic Voltammetry
DNA	Deoxyribonucleic acid
DPV	Differential Pulse Voltammetry
DTG	Dithioglycerol
ECL	Electrochemiluminescence
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EIS	Electrochemical Impedence Spectroscopy
ELISA	Enzyme-Linked Immunosorbent Assay
EV	Extracellular Vesicles
FISH test	Fluorescence In Situ Hybridization Test

FRET	Fluorescence Resonance Energy Transfer
FTIR	Fourier Transform Infrared Spectroscopy
GCE	Glassy carbon electrode
GS	Graphene Sheet
LOD	Limit of Detection
NHS	N-Hydroxysuccinimide
HER2	Human Epidermal Growth Factor Receptor 2
HNT	Halloysite Nanotube
HRP	Horse Radish Peroxidase
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IR	Infrared
ITO	Indium Tin Oxide
KCl	Potassium chloride
mAb	Monoclonal Antibody
MB	Magnetic Beads
MCGCE	Magnetic Controlled Glassy Carbon Electrode
MES	2-(N-morpholino)ethanesulfonic Acid
MNP	Magnetic Nanoparticles
MSN	Mesoporous Silica Nanoparticles
OA	Okadaic Acid
PbS QDs	Lead Sulphide Quantum Dots
PDA	Polydopamine
PSA	Prostate Specific Antigen
QDs	Quantum Dots
rGO	Reduced Graphene Oxide
SPCE	Screen Printed Carbon Electrode
SPE	Screen Printed Electrode
TEM	Transmission Electron Microscopy

TGL	Thioglycerol
TPSA	Total Prostate Specific Antigen
UV-VIS	Ultraviolet Visible
XPS	X-Ray Photoelectron Spectroscopy
XRD	X-Ray Diffraction

CHAPTER 1

INTRODUCTION

1.1 Background

Breast cancer is a tumour that can either be malignant or benign in which latter does not invade other tissues. However a malignant tumour can invade other parts of body through lymph system, hence causing a cancer.

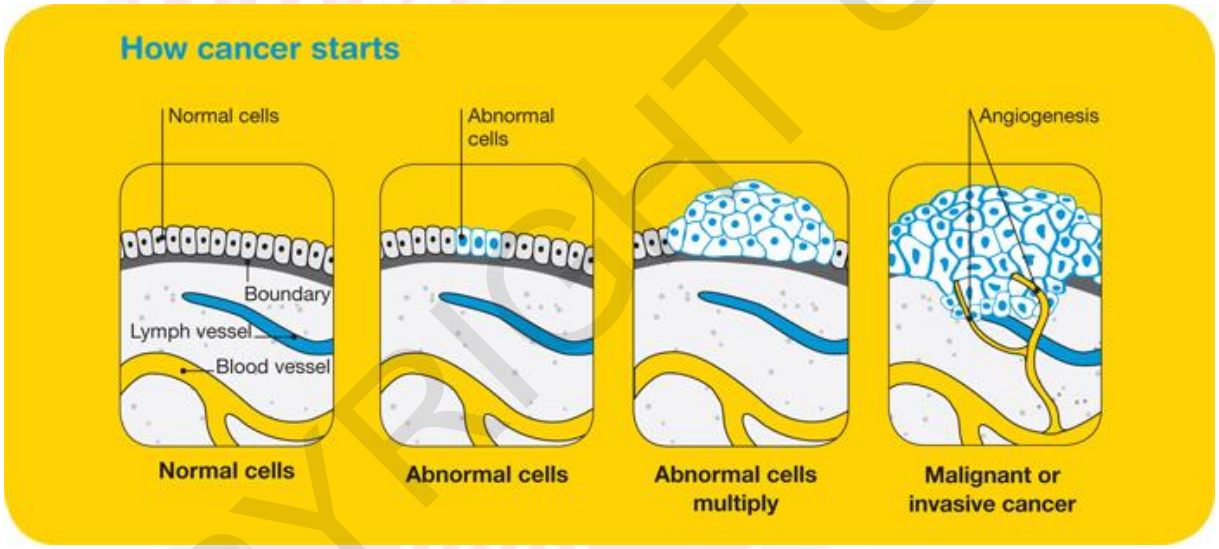

Figure 1.1 : The progression of normal cells into malignant cancer (Australia, 2017)

Figure 1.1 shows the cancer progress when there are changes in cells. These cells produce signals to control how much and how often the cells divide. However, the faulty in this cell can cause the cells to multiply abnormally and cause it to form tumour. This tumour becomes a cancer once it invades the lymphatic system and blood vessels.

There are three current tests that have been used extensively for detection of breast cancer. They are screening, diagnostic and monitoring tests. These tests depend on categories of breast cancer related issues. Screening tests such as mammogram is used to identify breast cancer at early stage before the cancer cell is further developed so that the treatment is easier. While, diagnostic tests such as biopsy are tested in order to identify the symptoms of breast cancer, which further can identify types of treatment needed. Furthermore, the test can also determine if the cancer cells have spread or

not. Lastly, monitoring tests are used to determine how well the treatment is and can be used to check any recurrence.(Breastcancer.org, 2017)

Among the available test, screening test is significant to obtain information of breast cancer cell before the symptoms arise because early detection of cancer can increase the chances of successful treatment. There are different kinds of screening test available including the physical exam and history, laboratory tests, imaging procedures and genetic tests. From the test mentioned, laboratory tests are sensitive and more reliable detection which involves the use of blood marker formed from the response of body towards the tumours. These biomarkers can be found in blood, urine, or body tissue of a cancer patient.

In this project, a simple and disposable of label-free and label immunosensor integrating the lead sulfide quantum dots (PbS QDs) as a reader for detection of HER2 cancer biomarker was constructed. PbS QDs was synthesized with thioglycerol (TGL) and dithioglycerol (DTG) as a capping agent to produce PbS QDs with hydroxyl surfaces. The as-synthesized PbS QDs was conjugated with antiHER2 antibody by using carbonylimidazole (CDI) as a linker. Screen printed carbon electrode (SPCE) was used as the platform for the immunosensor development. In order to improve the electrode interface, the transducer surface was functionalized. The surface of SPCE was electrochemically pre-treated to form carboxyl functional group which then activated with EDC/NHS linker for immobilization of primary anti-HER2 (Ab1). The quantification of HER2 biomarker was performed through acid dissolution of the bioconjugated PbS QDs. The released cations were transferred to an electrochemical cell and measured using square wave voltammetry (SWV).

1.2 Immunosensor

Immunosensor is a biosensor with affinity-ligand based in which the immunological reaction is coupled with transducer (Azam *et al*., 2014). The key to the development of an immunosensor is the non-covalent specific binding of antibodies to antigens. There are numerous analytical technique that took advantage of this specific interaction in many industries such as clinical diagnostic and analysis, food control safety, environmental monitoring, forensic analysis, drug screening and etc. In this regard, immunosensor emerges as a significant, continuously growing scientific industry (Kokkinos *et al*., 2016).

There are many types of technique used for detection in immunosensor, for instance, electrochemical, optical, microgravimetric and thermometric. All type of detection can either be labelled or non-labelled. The non-labelled usually detect the physical changes during the immunology reaction while the

labelled sensor used the signal generating label which is more sensitive and versatile detection mode (Luppa *et al*., 2001)

Recently, electrochemical immunosensor appear as a more promising alternative for immunosensing. This is due to the fact that it is possible for miniaturize modern microelectronics which allows building of microelectrodes that useful for multiplexing. Furthermore, it is well suited for detection of very small volumes of samples ranging from microliters to nanoliters. In addition, electrochemical approach is favourable due to low cost and large scale production of electronic devices for high-throughput analysis. Apart from that, it is suitable for complex and coloured sample because of the scarcity of electrochemical interference in real samples (Ricci *et al*., 2012).

The detection mechanism of an electrochemical immunosensor employs antibodies as capture and detection means to produce electrical charges for the quantitative analysis of target molecules. One of the main issues in developing immunosensor system is to achieve high sensitivity of detection. Numerous current nanotechnologies allowed the development of innovative electrochemical biosensors with high sensitivity. This is achieved by employing various nanomaterials which able to facilitate the electron transfer and carry signal tracers in combination with surface modification and bioconjugation techniques (Cho *et al*., 2018).

There are many different formats that can be used to build an electrochemical immunosensor. In this case, two approaches can be used which are label-free and labelled formats. For the label-free format (Figure 1.2 (b)), it able to sense directly the protein bind to the antibody which allow detecting proteins as they bind in real-time. (Mehrabani *et al*., 2014). While for labelled format (Figure 1.2 (a)) or most commonly known as sandwich format, it used the immobilized antigens and labelled antibodies. Sandwich format generally adopted to prevents all the problems related to antibody immobilization such as loss of affinity and orientation of the immobilized protein.

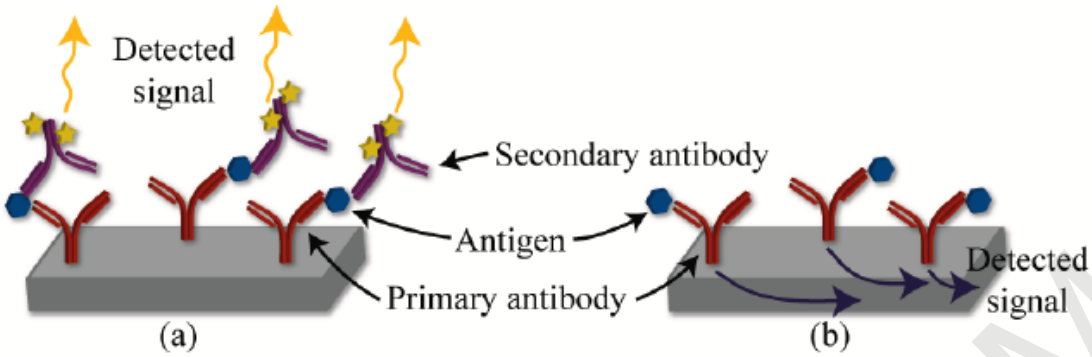

Figure 1.2 : Comparison between (a) labelled and (b) label-free detection method (Mehrabani *et al*., 2014)

1.3 Lead Sulfide Quantum Dots (PbS QDs)

Lead sulphide is an inorganic compound with a chemical formula of PbS. The naturally occurring ore of this compound is known as galena. It is one of the earliest semiconductors found due to the fact that it is easily crystallized in sodium chloride. PbS most commonly used in infrared detector, photo optic application and to enhance heat conduction and regulating friction coefficient in friction industry. It has a molecular weight of 239.90 g/mol with a lattice constant of 5.936 Å. While for the electrical properties, it has a small band gap which is 0.37 eV (Azo Materials, 2018).

Lead sulphide quantum dots (PbS QDs) have attracted much attention in recent years due to the excellent properties compare to the bulk PbS. It is most used in single junction and tandem photovoltaics (PV) applications due to a band gap that can be size-tuned from 0.41 to 1.6 eV(Rekemeyer *et al*., 2017). Furthermore, PbS QDs with optical transitions in the near infrared region are now used in third-generation photovoltaic cells, photodetectors, and light-emitting diodes (Litvin *et al*., 2017). The availability for synthetic route for creating size tuned PbS QDs and high stability as compared to PbSe QDs making it more favourable for these applications (Márquez *et al*., 2017).

Although PbS QDs mostly used in electronics, energy conversion and storage (Pal *et al*., 2017), several research have generated enormous application of this material into biosensing technology. Therefore, attempts have been made in recent years for the inclusion of PbS QDs in biosensor. For instance, an electrochemical biosensor based on PbS QDs for detection of carcinoembryonic antigen tumor marker (Wang *et al*., 2008), Escherichia Coli O157:H7 Detection (Wang *et al*., 2015), DNA hybridization (Zhu *et al*., 2004b)and simultaneous detection of Rabbit immunoglobulin G antigen (IgG) and carcinoembryonic antigen (CEA) (Qian *et al*., 2011) was developed.

In thid regard, the present study explores the application of PbS QDs for detection of breast cancer biomarker using HER2 antigen.

The toxicity of lead is the main concern for the PbS QDs to be employed for clinical study purposes. The toxicity of lead is very much dependent upon the route of exposure whether, dose, chemical structure, solubility, particle size, matrix incorporation and other physiological and physicochemical factors (Dieter *et al*., 1993). In this regard, several researchers have been investigated the effect of lead concentration towards biological organism. For instance, Dieter and co-workers (1993) have initiated a study on lead bioavailability in F344 rats that have been fed with lead acetate, lead oxide, lead sulphide, or lead ore concentrate from Skagway Alaska. From the study, there is no mortality and symptoms of lead toxicity observed (Dieter *et al*., 1993). Apart from that it was found that PbS compound have no effect on red blood cells and is highly insoluble and stable at the pH of bloods hence almost non-toxic to human (Bischoff *et al*., 1928). According to world health organization (WHO), there is also limited evidence of lead compounds carcinogenicity. However, there is sufficient evidence of carcinogenic of lead compounds towards experimental animal. The evaluation of lead compounds overall bring to the probability of carcinogenic to human (Group 2A) (World Health Organization (WHO), 2018). Overall, the toxicity of PbS QDs is negligible attributed to low bio-availability which consequently leads to low risk.

1.4 Problem Statement And Research Motivation

Enzyme-Linked Immunosorbent Assay or ELISA is one of the current clinical diagnostic tools for early detection of breast cancer. It is often used in biomedical research for quantification of specific antigen or antibodies in a given sample. It is highly sensitive and most of ELISA kits are portable and ready to use. However, ELISA has few limitations observed. Firstly, over a long period of time, the enzyme-mediated colour most likely to change, hence result in a false result. Secondly, the non-specific binding of antigen and antibody could occur lead to false-positive result (Gan and Patel, 2013).

Substantial studies on immunosensors have been carried out based on piezoelectric transducer. The working principle of the piezoelectric immunosensor is based on formation of antigen-antibody complexes that increases the mass as compared with antigen or antibody alone which detected through a device such as quartz crystal balance or a microcantilever (Moina and Ybarra, 2012b). Due to its simplicity, low cost and real time response, it gains significant attention in the field of bioanalytical assays and characterization as one of the competitive tools (Montoya *et al*., 2009). However, it possesses some drawbacks that set as a challenge for clinical analysis such as complex instrumentation and long incubation time with samples.

As an alternative, optical immunosensors have been widely used as a mean of immunosensing. Typically, biological element is immobilized on the surface of transducer where it will generate optical signal when interacted with target analyte, such as fluorescence.

Fluorescence technique for detection of breast cancer is one of the most widely used immunosensor where the conjugate antibody is labelled with fluorescent probe. The antigen antibody complexes formed cause optical changes towards surrounding. However, most fluorescence immunoassay requires a complex instrumentation. Furthermore, fluorescence tends to have photobleaching effect (Moina and Ybarra, 2012a).

To overcome these issues, electrochemical technique was used as a method for breast cancer biomarker detection. This technique have several advantages over other methods, such as, miniaturised, easy to use and portable (Hammond *et al*., 2016). Apart from that, electrochemical immunosensor is a rapid, simple and cost effective method (Tian *et al*., 2016). Electrochemical detection involves the conversion of the event of antigen-antibody interaction into electrical signal. This technique able to display high specificity and selectivity of antibody-antigen complexes by monitoring the current response. In this regard, this technique was employed for this study as a method on detection of HER2 breast cancer biomarker based on label-free and sandwich immunosensor.

Recently, quantum dots (QDs) attract much attention due to their superior properties. For instance, effective suppression of non-specific adsorption of biomolecules at the surface and less aggregation is the most significant criteria. Furthermore, the colloid stability can be improved through functionalization of the surface of QDs (Drbohlavova *et al*., 2009). Due to this pursuit, QDs was employed as a label for the fabrication of sandwich immunosensor. In this regard, PbS QDs was synthesized and bioconjugated with secondary anti HER2. The synthesis process involved the use of lead(II) acetate and sodium sulphide as the precursors with two additional ligand which are thioglycerol (TGL) and dithioglycerol (DTG). Previous reported procedure have shown that the PbS QDs were more stable for several months upon usage of TGL/DTG instead of using either one of them (Zhao *et al*., 2005). Apart from that, the hydroxyl tail groups available are easily tunable for the bioconjugation process through carbonylimidazole (CDI) linker.

The utilization of PbS QDs as a label sensing for detection of HER2 electrochemical sandwich immunosensor has not yet been explored. Hence, the suitability and stability of the PbS QDs conjugation is still unknown. In order for the anti-HER2 conjugated PbS QDs effectively immobilized onto the

surface of modified screen printed carbon electrode, the optimum condition of layer formation was studied.

1.5 Objectives

The aim of the research is to develop label-free and label electrochemical immunosensors for the detection of cancer biomarkers. In sandwich-type (label) immunosensor, conjugated PbS QDs was used as a label. In order to achieve this overall goal, the specific technical objectives are:

1. To synthesize and bioconjugate PbS QDs with antibody
2. To develop label-free and sandwich immunosensor
3. To analyse the performance of label-free and sandwich immunosensor towards different concentration of HER2

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