INTEGRATION OF GLUTARALDEHYDE ONTO IMMUNO-MEMBRANE FOR POLYANILINE-BASED ELECTROCHEMICAL BIOSENSOR

ROSWANI BINTI SHAIMI

UNIVERSITI SAINS MALAYSIA

2013

INTEGRATION OF GLUTARALDEHYDE ONTO IMMUNO-MEMBRANE FOR POLYANILINE-BASED ELECTROCHEMICAL BIOSENSOR

by

ROSWANI BINTI SHAIMI

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

DECEMBER 2013

ACKNOWLEDGEMENT

In the name of Allah S.W.T. the most gracious and most merciful, Lord of the universe, with His permission, Alhamdulillah the project has been completed. Praise to Prophet Muhammad S.A.W., His companions and to those on the path as what He preached upon, might Allah Almighty keep us His blessing and tenders.

A special gratitude to my beloved parents, Mr. Shaimi bin Umat and Mrs. Siti Fatimah binti Hussin, my family members and also my partner, Che Amir Rajhan bin Che Jaffar for their endless support and prays.

I would like to express deepest gratitude to my beloved supervisor, Dr. Low Siew Chun for her excellent knowledge guidance, encouragement, valuable suggestion, guidance support and advises rendered throughout my research. Also, I would like to express my gratitude to my co-supervisor, Prof. Abdul Latif Ahmad for sharing his knowledge, opinion and assistance.

Not forgetting, thanks a lot to all technicians and staffs of School of Chemical Engineering for their cooperation. Deepest thank to all my friends for the help and moral support.

Last but not least, I would like to express my gratitude to whom that involved directly or indirectly for their unselfish advice and assistance toward performing in finishing this research within time.

Thank you so much and may Allah S.W.T. the Almighty be with us all the time.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS

^{o}C	Temperature
wt. %	weight percentage
µg	Microgram
cm^3	centimeter cubic
%	Percentage
mS	milli Semen
M Ω	Mega Ohm
ml	Milliliter
nm	Nanometer
M	Molar
e.g	Example
i.e.	which is
h	Hour
min	Minutes
rpm	revolution per minutes
µm	Micrometer
cm	Centimeter
V_E	membrane's existent volume
V_A	membrane's apparent volume
Ɛ	Porosity
mm	Millimeter
g	Gram

<	less than
v/v	volume/ volume
mV	Unit for zeta potential
kΩ	Kilo Ohm

LIST OF ABBREVIATIONS

PANI	Polyaniline
BSA	Bovine Serum Albumin
HF	High Flow
NC	Nitrocellulose
GA	Glutaraldehyde
RSM	Response Surface methodology
DLS	Dynamic Laser Scattering
PCR	Polymerase Chain Reaction
CA	Cellulose Acetate
PVDF	Polyvinylidene Fluoride
FESEM	Field Emission Scanning Electron Microscope
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared
TGA	Thermo-Gravimetric Analysis
AFM	Atomic Force Microscope
CCD	Central Composite Design
E. coli	*Escherichia coli*
APS	Ammonium Persulfate
SPR	Surface Plasmon Resonance
PPy	Polypyrrole
HCl	Hydrochloric acid
DMF	Dimethylformamide
PBS	Phosphate Buffer Saline

LiCl	Lithium Chloride
EAPM	Electrically active polyaniline magnetic
BCA	Bicinchoninic acid
Eq	Equation
ANOVA	Analysis of variance

INTEGRASI GLUTARALDEHID KE ATAS IMMUNO-MEMBRAN UNTUK BIOPENDERIA ELEKTROKIMIA BERASASKAN POLIANILINA

ABSTRAK

Pembangunan membran untuk aplikasi biopenderiaan untuk kawalan wabak penyakit mempunyai kesan global yang besar terutamanya untuk kesihatan awam. Kajian ini meneroka biopenderia elektrokimia dengan menggunakan polianilina (PANI) sebagai transduser melalui penganalisaan interaksi biotin-bovine serum albumin (BSA) sebagai sistem model. Memandangkan membran muncul sebagai pelantar untuk penjerapan protin, pemilihan membran yang sesuai amat diperlukan untuk menghasilkan biopenderia yang sensitif dan spesifik. Dalam pengukuran prestasi membran, kebolehan membran mengikat protin telah ditentukan secara kuantitatif. Antara membran-membran yang diuji, NC-180 muncul sebagai membran aliran sisi yang paling sesuai sebagaimana ia telah ditunjukkan pada kebolehan mengikat protin yang tinggi dan masa sisi penyumbuan yang pendek. Untuk meningkatkan kebolehan membran mengikat protin, NC-180 kemudiannya tertakluk kepada pengubahsuaian membran dengan menggunakan larutan glutaraldehid (GA). Pengikatan protin bagi membran yang diubahsuai dengan GA didapati dapat mengekalkan suhu sehingga 60^{o}C. Dalam kajian ini, kesan pelbagai faktor integrasi seperti kepekatan GA, masa integrasi dan bilangan lapisan integrasi pada keupayaan membran mengikat protin telah disiasat. Keadaan integrasi yang optimum telah ditentukan menggunakan *Response Surface Methodology* (RSM).

Analisis statistik menggunakan RSM menunjukkan bahawa semua faktor integrasi (kepekatan GA, masa integrasi dan bilangan lapisan integrasi) telah menjejaskan kebolehan membran mengikat protin secara ketara dan mempunyai beberapa kesan-kesan interaksi antara pembolehubah tak bersandar. Pengikatan protin yang optimum (896.605 $\mu g/cm^3$) telah didapati pada 3 wt. % kepekatan GA pada lapisan tunggal dan 30 minit masa integrasi. Sisihan kecil dalam 2% antara reaksi ramalan dan sebenar telah mengesahkan kecukupan model statistik. Model empirikal tersebut boleh digunakan untuk meramalkan prestasi membran yang diubahsuai oleh GA bagi pembangunan biosensor elektrokimia. Dalam kajian ini, PANI bertindak sebagai transduser isyarat elektrik, yang menukarkan interaksi yang berlaku antara biotin-BSA kepada isyarat elektrik. PANI telah dikonjugasikan terlebih dahulu dengan ferum (III) oksida (Fe_2O_3) untuk bertindak sebagai ejen mengesan dan seterusnya mengikat kepada biotin (penangkap reagen) dalam biosensor ini. Dalam kajian ini, PANI telah disintesiskan melalui pempolimeran beroksida dengan kekonduksian 6.73 mS. Konjugasi daripada PANI-Fe_2O_3 dan PANI-Fe_2O_3-biotin telah disahkan dengan menggunakan *dynamic laser scattering* (DLS) dan spektrofotometer UV-Vis. Berdasarkan ujian prestasi melalui ukuran denyutan mod, konfigurasi pertama iaitu BSA yang telah dibarisi pada permukaan membran diikuti oleh rekaan elektrod perak, telah menjanakan rintangan yang paling tinggi pada 3.05 $\pm$ 0.32 MΩ. Keputusan menunjukkan bahawa elektrokimia biopenderia telah berjaya direka, dengan pengesanan secara kuantitatif antara PANI-Fe_2O_3-biotin dan BSA. Biopenderia yang pantas, pemeriksaan di tapak dan mesra pengguna ini menjadikan ia satu alat yang berpotensi dengan aplikasi pengesanan kehadiran virus atau bakteria yang tertentu dalam air minuman.

INTEGRATION OF GLUTARALDEHYDE INTO IMMUNO-MEMBRANE FOR POLYANILINE-BASED ELECTROCHEMICAL BIOSENSOR

ABSTRACT

Development of membrane for bio-sensing applications for epidemics control has a huge global impact especially for public health. This research explored the electrochemical biosensor with polyaniline (PANI) as a transducer through analyzing the biotin-bovine serum albumin (BSA) interaction as a model system. As membrane appeared as the platform for protein immobilization, selection of suitable membrane is required to create a sensitive and specific biosensor. In measurement of membrane performance, membrane protein binding abilities were quantitatively determined. Among the tested membranes, NC-180 appeared as the most suitable lateral flow membrane as it performed high protein binding ability and short lateral wicking time. To increase the membrane protein binding ability, NC-180 was then subjected to membrane modification using glutaraldehyde (GA) solution. The protein binding of the modified membrane with GA was able to retain up to temperature of 60^{o}C. In the present study, the effect of various integration factors such as the concentration of GA, integration time and number of the integration layer on membrane's protein binding ability was investigated. The optimum integration conditions were determined using response surface methodology (RSM). The statistical analysis using RSM showed that all the integration factors (concentration of GA, integration time and number of the integration layer) had significantly affected the membrane's protein binding ability and had some

interaction effects among the independent variables. The optimum protein binding (896.605 μg/cm^3) was obtained at 3 wt. % of GA concentration at single layer and 30 min of integration time. A small error within 2 % between predicted and actual responses confirmed the adequacy of the statistical model. This empirical model is applicable to predict the performance of the GA-modified membranes for the development of electrochemical biosensor. In this research, PANI served as an electric signal transducer, converting the interaction occurred between biotin-BSA to electrical signal. The PANI was first conjugated with iron (III) oxide (Fe_2O_3) to serve as the detecting agent and then further bound to biotin (target reagent) in the biosensor. In this work, the PANI was synthesized through oxidative polymerization with the conductivity of 6.73 mS. The conjugations of PANI-Fe_2O_3 and PANI-Fe_2O_3-biotin were confirmed using dynamic laser scattering (DLS) and UV-Vis spectrophotometer. Based on the performance test via pulse mode measurement, the first configuration i.e. the BSA was lined on the membrane surface followed by fabrication of the silver electrode, generated the highest resistance at 3.05 $\pm$ 0.32 MΩ. Results indicated that the electrochemical biosensor was successfully fabricated, with quantitative detection between PANI-Fe_2O_3-biotin and BSA. This rapid, on-site examination and user friendly biosensor make it a promising device with application in detecting the presence of selected viruses or bacteria in drinking water.

CHAPTER 1

INTRODUCTION

1.1 Biomedical Technology

Modern medical technology has undeniably achieved lots of advancement and sophistication, especially in the developed countries for providing treatment and controlling the spread of diseases. However, millions of people die annually due to waterborne outbreaks especially in third world countries (Bouwer, 2000). The root cause to the loss of millions human life is mainly due to the unavailability or inaccessible of diagnostics facilities in the underdeveloped or developing countries.

Existing diagnostics techniques do not address the needs of bottom billions. It was reported that 40 % of the approximately 50 million total annual deaths world-wide because of the infectious diseases (Leonard *et al.*, 2003). Of these infectious diseases deaths, 10-20 million was contributed from waterborne pathogens while non-fatal infection was more than 200 million people each year (Leonard *et al.*, 2003). Most of the major food-borne and water-borne diseases are caused of *Escherichia coli* (e.g., E. coli O157:H7). This pathogen is known to cause diarrhea, hemolytic uremic syndrome (HUS) and hemorrhagic colitis in humans (Zhu *et al.*, 2005). The outbreaks of illness are mainly due to ingestion of meats, water, and uncooked fruits and vegetables.

As popular awareness of the importance of health is increases, a cost effective approach would need to be considered to develop a rapid, on-site examination and user friendly device in monitoring and detecting the presence of selected viruses or bacteria in drinking water. In the past, polymerase chain reaction

(PCR) has been a common practice for the detection and identification of pathogen. This conventional method is sensitive and can be used to amplify small quantities of pure samples in detecting the presence of pathogen and viruses in drinking water. However, it needs relatively longer time to get the result, skilled personnel and advanced infrastructure to carry out the analysis (Waswa *et al.*, 2007), thus the focus has shifted to the development of biosensor. Biosensor appeared as one of the fastest growing pathogen detection tools with equally reliable results in faster time and on-site examination. In recent year, the biosensor possesses the fastest growth compared to other conventional methods, as illustrated in Figure 1.1.

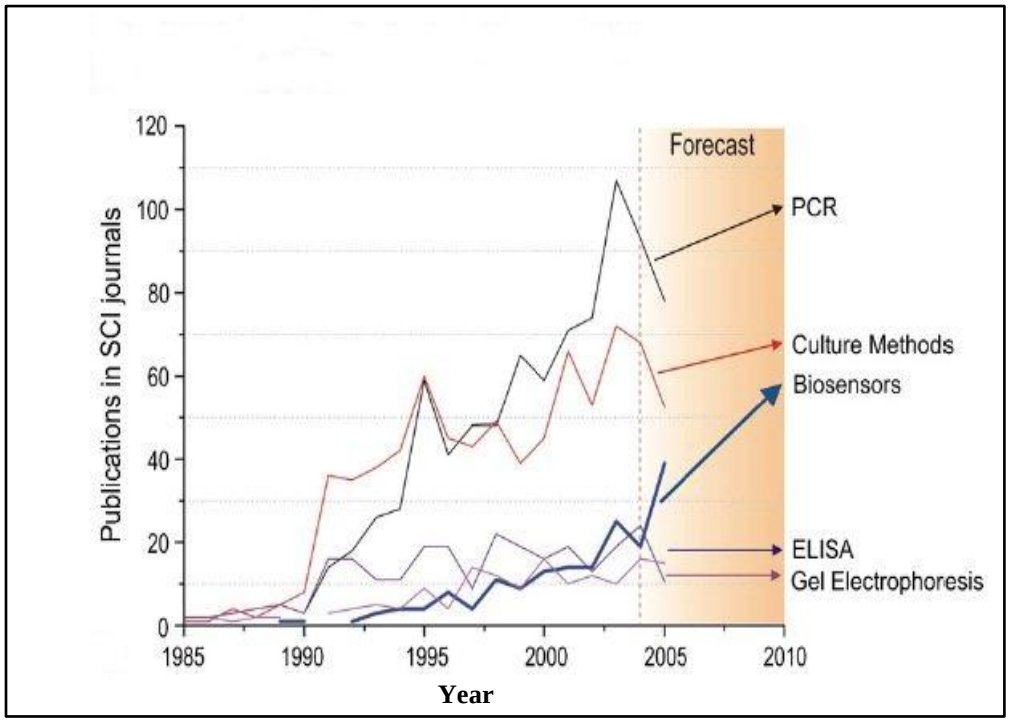

Figure 1.1: Growing technology in detecting pathogen (Lazcka *et al.*, 2007).

1.2 Biosensor

Several biosensors approaches based on electrochemical, optical and piezoelectric detection principles are developed as illustrated in Figure 1.2. The primary aim of those configurations are of low sample consumption, fast response time and high throughput (Zhang *et al.*, 2011). Among these approaches, the electrochemical biosensors present to be an ideal choice compared to the other transduction methods: (1) the electrochemical electrodes are relatively low cost for production which promising for practical use (2) the applied voltage are sufficiently small, thus minimize the sensor's power consumption, (3) easily integrated with electronic devices and less susceptible to environmental effects and contaminants and (4) reduce response time due to the smaller diffusion distances.

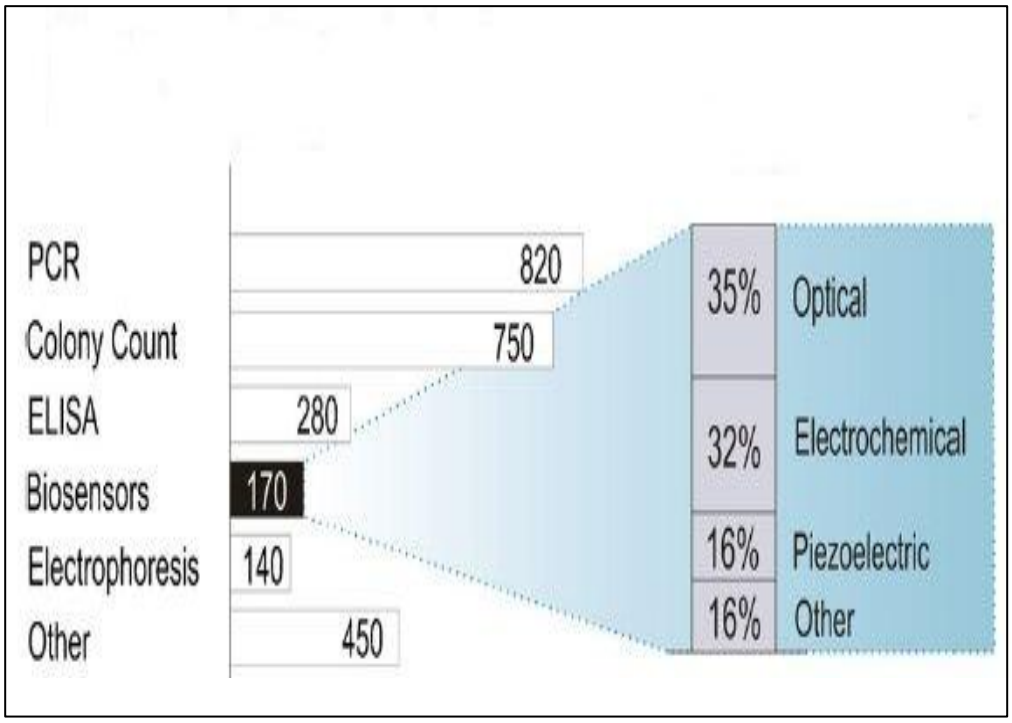

Figure 1.2: Biosensor transduction methods in pathogen detection (Lazcka *et al.*, 2007).

The biosensor consists of two major parts which are the immuno-sensor and the electronic data collection system. The immuno-sensor comprises of the membrane separation pad, sampling pad and also absorption pad. Meanwhile, the electronic data collection system consists of two main components which are the bio-receptor element and the transducer. The bio-receptor element can be a tissue, microorganisms, organelle, cell, enzyme, antibody, nucleic acid or bio-mimic (Velusamy *et al.*, 2010) while the transducer may be in the form of optical, electrochemical or piezoelectric. For an electrochemical biosensor, the bio-receptor element recognizes the target analyte and the biological responses are converted into electrical signal by the transducer. A schematic diagram for main component of an electrochemical biosensor is shown in Figure 1.3.

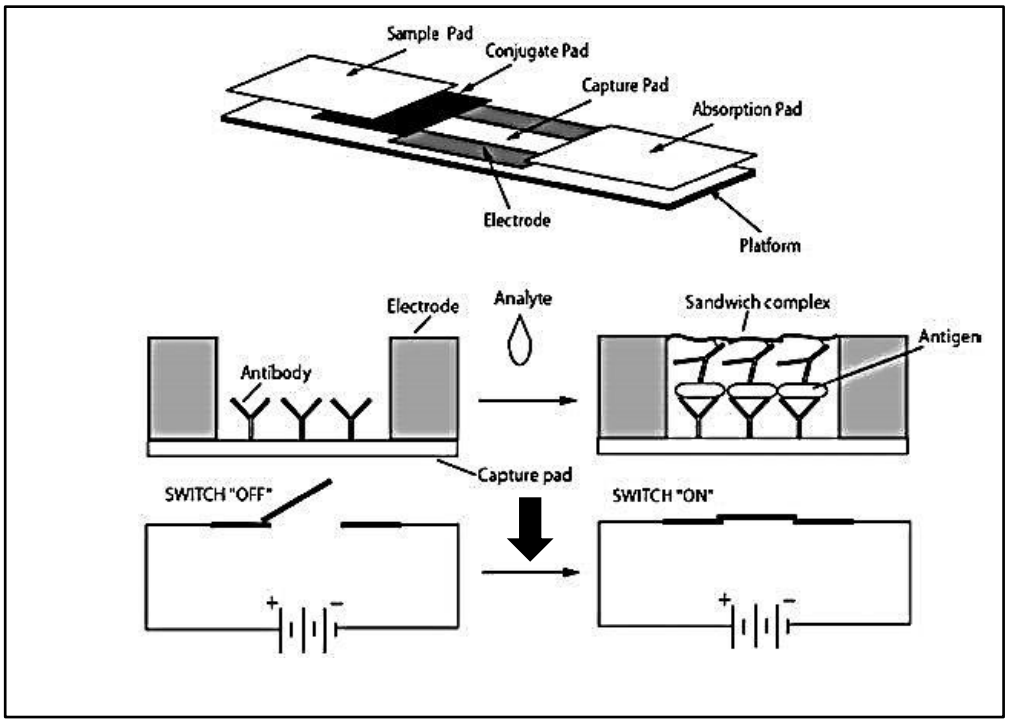

Figure 1.3: Schematic diagram showing the main component of an electrochemical biosensor (Pal *et al.*, 2007).

1.3 Membrane for bio-sensing applications

The unique separation principle of a membrane, namely selective transport processes and efficient separation makes it an ideal candidate as the key functional element in a biosensor. Membrane provides the capillary action and induces a flow of aqueous medium along the lateral side of the strip that transports the target analyte to the immobilized binding partner. The binding reaction between antigen and antibody takes place on the surface and unbound molecules are subsequently separated by the medium flow. Selection of membrane as the support material to bind with bio-molecules oftentimes a protein is not a trivial task. A wide range of porous membranes such as cellulose acetate membrane (CA), polyvinylidene fluoride membrane (PVDF), nylon membrane and nitrocellulose membrane (NC) have found to be compatible in bio-sensing development (Singh *et al.*, 2008, Fang *et al.*, 2011). NC membrane has long occupied a position of central importance in medical and immunological analysis due to its excellent wetting properties, high binding capacity and low background staining (Morais *et al.*, 1999).

Protein is the most common reagent to be applied onto the membrane surface in an immunoassay. The interaction between protein and membrane is generally affected by the membrane morphology, and eventually reflected the effectiveness of the biosensor. Protein immobilization onto the membrane material remains a key issue in development of the lateral flow biosensor (Kim *et al.*, 2000, Minett *et al.*, 2002, Cho *et al.*, 2007, Apostolova *et al.*, 2011).

1.4 Membrane modification by glutaraldehyde

Immobilization of protein by covalent bonding onto membrane surface is one of the effective methods in development of biosensor. To assure the required sensitivity level of a biosensor, the membrane surface is always modified using the cross-linker reagent. Aldehyde has found to be an excellent cross-linking agent in fields such as medicine, electron microscopy study of proteins, cells, and etc. (Fathima *et al.*, 2004). Among the aldehyde groups, glutaraldehyde (GA) has found great success in protein immobilization due to its thermally and chemically stable (Migneault *et al.*, 2004). The cross-linked protein by GA presents a stable, compact protein structure (Emre *et al.*, 2011), low impact on the activities recoveries and improves the stability (Alonso *et al.*, 2005). As a result, more stable and high sensitivity biosensor is obtained.

Glutaraldehyde, a bi-functional aldehyde serves covalent bonding to the polymer's backbone and protein (Busto *et al.*, 1997). Two identical reactive groups react with the residual free amino acid groups of protein and the polymer's backbone, make the protein immobilization in development of biosensor to achieve easily (Romero *et al.*, 2008). The three-dimensional linking between protein and polymer's backbone through intramolecular and intermolecular cross-linking resulting the modified protein to completely insoluble in water and can be adsorbed onto the support (Albareda-Sirvent *et al.*, 2000).

1.5 Problem statement and importance of the research

Although sophisticated medical technology has undeniably achieved a lot of advancement, however, millions of people die due to waterborne outbreak, especially in the third world countries. The root cause to the loss of millions human life is mainly due to the unavailability or inaccessible of diagnostics facilities in the underdeveloped or developing countries. Existing diagnostics techniques do not address the needs of bottom billions. To address this problem, the biosensor has been found to be promising device for the rapid detection of pathogen and viruses in drinking water.

Membrane is the most important key element in the development of biosensor as it serves capillary action for the target analyte to flow and place for the interaction between target analyte and capture reagent that is immobilized onto membrane surface. Biosensor will not fully function without applying the membrane as a support material. As membrane shows its importance, selection of the membrane should be focused and studied for the development of bio-sensing applications. The suitable membrane could contribute towards the high effectiveness of the developed biosensor.

The main factor on the sensitivity of biosensor is based on the protein immobilization technique onto the membrane surface. Adsorption technique is commonly applied for protein immobilization. However, the leaking of the bio-receptor and possible diffusion barriers are the common problems that occur by applying this conventional immobilization technique, which eventually rendered the low sensitivity of the biosensor. A sensitive biosensor could be obtained if some improvement on protein immobilization technique is performed. Modification by

GA as a cross-linker reagent is introduced to assure that the sensitive biosensor could be developed.

This research explores the integration of GA onto immuno-membrane for polyaniline-based electrochemical biosensor which is based on the pulse mode measurement technique, by analyzing the biotin-BSA interaction as a model system and polyaniline as a transducer. The selected membrane is modified with GA solution for further enhancement for protein immobilization on the membrane surface. By governing the membrane modification by GA and optimization of GA integration operational procedure mechanism, biosensor with specific characteristics and desired performances can be manufactured.

1.6 Research objectives

The aim of the study is to develop an electrochemical biosensor with enhancement performance through the integration of glutaraldehyde onto polymeric membrane. The study comprises of following objectives:

1) To select the potential transport media by quantitatively determine the binding capacity and lateral wicking time of polymeric membranes
2) To modify the membrane surface using glutaraldehyde in order to enhance the protein immobilization ability of the polymeric membrane
3) To optimize the integration operational procedures of glutaraldehyde onto the membrane surface using Central Composite Design (CCD) of Response Surface Methodology (RSM)
4) To prepare a complex polyaniline-Fe_2O_3 conjugated biotin and carry out the performance test through pulse-mode measurement.

1.7 Scope of study

In the development of lateral flow membrane for polyaniline-based electrochemical biosensor, this research was consisted of four major investigations. i.e. the selection of potential transport media (membrane) which governs the performance of the lateral flow biosensor, modification of selected membrane with glutaraldehyde (cross-linker reagent), optimization of GA integration operational procedure using RSM and the last section was the preparation of a complex polyaniline-Fe_2O_3 conjugated biotin and subsequently performance test via pulse-mode measurement.

In this research, the interest was focused on determining the protein binding ability and lateral wicking time for NC, Nylon, CA and PVDF membranes. Both factors reflect the effectiveness of the biosensor in direct and eventually, developed an in-depth understanding of membrane selection for the development of biosensor. The characterization of the membrane such as membrane morphology, pore structure and bonding characteristic were performed with the aid of different analytical instruments such as field emission scanning electron microscope (FESEM) and attenuated total reflectance fourier transform infrared (ATR-FTIR).

The selected membrane was subjected to membrane modification using GA solution. In this section, we studied the integration operational procedure of GA onto the membrane surface. The effects of GA concentration (0.5 wt. % to 5.0 wt. %), integration time (10 min-90 min), the number of integration layer (single to triple) as well as the thermal effects of integration (40^oC-70^oC) were taken into considerations. For the measurement of membrane performance, membrane protein

binding abilities were quantitatively determined. Furthermore, the functionality of the GA-modified membrane was verified through ATR-FTIR and TGA analyses.

The interactive effects among the independent variables and optimizing the GA integrated were presented through central composite design (CCD) of RSM. Same effects as preliminary study (GA concentration, number of integration layers and integration time) were taken into considerations. In measuring the membrane performances, the membrane protein binding abilities were quantitatively determined. In this section, the topographic of the GA-modified NC membrane was determined through FESEM and AFM analyses.

In the last section, the biosensor was assembled by using BSA as the bio-receptor element and polyaniline (PANI) nanowires as the molecular transducer. The working principle of the developed biosensor was based on the antigen-antibody interaction where the target reagent (biotinylated IgG) was conjugated with PANI maghemite nanoparticles. PANI, an electric signal transducer for monitoring of the bio-specific binding event between biotinylated IgG-BSA, was synthesized through the oxidative polymerization of aniline monomer in the presence of ammonium persulfate as oxidizing agent. The synthesized PANI was then integrated with maghemite nanoparticles and subsequently, conjugated with biotinylated IgG. Next, the biosensors were assembled into different biosensor assembly configurations, and the performance of this polyaniline-based biosensor was demonstrated using the pulse mode measurement technique.

1.8 Organization of the thesis

The thesis comprises of five chapters providing all the details and findings of the research. Chapter One (Introduction) focuses on the biomedical technology that implement the conventional method in pathogen detection, then eventually shift to the development of biosensor. The chapter also included the application of the membrane as a platform in protein immobilization and then further, the enhancement of protein immobilization by modification of membrane using GA solution. The problem statement and importance of the research are also highlighted in this chapter. Besides, the research objectives of present study are elaborated in detail together with the scope of the study to be covered. The thesis organization is given in the last section of this chapter.

Chapter Two (Literature Review) reviews all the important literatures from the researchers especially in the development of biosensor in detection of pathogen and viruses in drinking water. At first, the chapter reviews the problem of waterborne pathogens and the conventional method to detect the pathogens or viruses in this waterborne problem. Biosensor is introduced to overcome the problems of using the conventional method. As biomolecule immobilization is the main stage in the development of biosensor, hence next section discusses on the biomolecule immobilization.

Besides, this chapter also demonstrates the biosensor detecting systems based on different transducer properties and then the implementation of the electrochemical transduction method in development of biosensor. The rapid growth of conducting polymers which lead to an innovation in development of electrochemical based biosensor is also presented while the interesting of magnetic

polymer nanoparticle that is employed as magnetic concentrator and signal transducer in biosensor is discussed in the next section. The polymeric membrane that serves as platform for protein immobilization is reported and then, the final section in the chapter, a future direction is given to illustrate the future study in development of biosensor.

Chapter Three (Research Methodology) presents the experimental procedures that were performed in this study. All details on materials and chemicals and experimental procedures regarding the selection of lateral flow membrane, modification of polymeric membrane by GA solution, optimization of GA integration, preparation of complex PANI-Fe_2O_3 conjugated biotinylated IgG as well as performance test were described. Detail equipment used together with the analysis methods is incorporated in this section toward to achieve the objectives of the study.

Chapter Four (Results and Discussion) presents the experimental results and discussion obtained from the study. This chapter is divided into five sub-chapter that is generally based on each objectives studied. The results and discussion covers the selection of membrane as the potential transport media, membrane modification using glutaraldehyde, optimization of GA integration operational procedures, preparation of complex PANI-Fe_2O_3 conjugated biotinylated IgG and further the performance test via pulse mode measurement.

Chapter Five (Conclusion and Recommendation) concludes the finding in current study. In the second part of this chapter, several recommendations related in this field are reported for improvement in future work.

CHAPTER 2

LITERATURE REVIEW

2.1 Emerging waterborne pathogens

Microorganisms such as bacteria and viruses are widely found in environment like food, marine and estuarine waters, soil and intestinal tracts of human and animals. These microorganisms play a few essential functions, especially important in the biotechnological applications such as the in vitro evolution of the enzyme usage as whole cell bioconversion, pesticide detoxification, removal of heavy metal and traditional food fermentation process (Kim *et al.*, 2005). Besides, the microorganisms were also employed as a host in manufacturing the recombinant proteins (Serrano-Heras *et al.*, 2005, Choi *et al.*, 2006, Westers *et al.*, 2004), as well as used for the biological control of plant diseases caused by microbial infections (Hwang and Wang, 2012). However several of these organisms are harmful which are profound to become negative effects to both human and animals. These harmful microorganisms can contaminate food and water, eventually causes a plethora of infectious diseases in both animals and humans. It is estimated that infectious diseases caused about 40 % of the approximately 50 million total annual deaths world-wide. Waterborne pathogens caused 10-20 million of these deaths with additional non-fatal infection of more than 200 million people each year (Leonard *et al.*, 2003).

Enterohemorrhagic *Escherichia coli* (e.g., *E. coli* O157:H7) is a major food-borne and water-borne pathogen. This pathogen is known to cause diarrhea and hemolytic uremic syndrome (HUS) and hemorrhagic colitis in humans (Zhu *et al.*,

2005). *E. coli* O157:H7 is a facultative gram negative bacillus that has been implicated in outbreaks of illness due to ingestion of meats, water and uncooked fruits and vegetables. The strain of Shiga toxin-producing *E. coli* is the most common outbreaks in many developed countries include Canada, Europe, Australia, and Japan (Tims and Lim, 2003). It is estimated that *E. coli* O157:H7 causes nearly 75,000 human infections in the U.S. each year by The Center for Disease Control and Prevention (Zhu *et al.*, 2005) .

Another pathogen that is responsible for numerous waterborne outbreaks is Protozoan parasite *Cryptosporidium parvum* that causes diarrheal disease. The organism poses a significant public health threat because of the resistance of oocysts to chlorine treatment and large number of unfiltered surface water supplies. Generally, the *C. parvum* infection is initiated by the ingestion of oocysts, which then undergo excystation to release sporozoites. Crucial primary steps in the pathogenesis of cryptosporidiosis is by attachment of sporozoites to host epithelial cells and subsequent invasion (Kang *et al.*, 2006). There are no effective drug treatments for this infection and the routine chlorination treatment could not eradicate the dormant *Cryptosporidium* organisms in drinking water (Wang *et al.*, 1997).

The third example of common pathogen is the white-spot syndrome virus (WSSV). Samanman *et al.* (2011) reported that the cultivation and exportation of shrimp is the most common outbreaks affected by this infectious virus. This virus causes high mortality rates, reaching 100% within 3–10 days. Water is one of the major pathways for the WSSV pathogen to enter the aquaculture facilities.

2.2 Conventional methods in pathogen and viruses detection

In the past, polymerase chain reaction (PCR) has been a common practice for detection and identification of any pathogen and viruses. This conventional method is sensitive and can be used to amplify small quantities of pure samples in detecting the presence of pathogen and viruses in drinking water (Ivnitski *et al.*, 1999). However, the PCR method requires pure samples and need longer time for processing as well as expertise in molecular biology (Leonard *et al.*, 2003). For example, the PCR method provided high sensitivity for toxigenic *V. cholerae* detection but they required pre-enrichment, molecule labeling, high skill operator and multiple detection steps (Sappat *et al.*, 2010). Although this technique is sensitive but it is a time consuming method and is not able to detect the viable but non-cultureable form of organisms, hence made it a big obstacle for the PCR development (Sappat *et al.*, 2010).

Chan *et al*. (1994) reported on the use of PCR kinetics by using a positive internal control target to quantitatively detect *cytomegalovirus* (CMV) target sequences. In this report, CMV PCR primer pair was used to detect the native sequence target of CMV. The detection was carried out under co-amplification conditions by a plasmid containing CMV control target. The quantitation of PCR using co-amplification PCR was feasible, however, this co-amplification had introduced a number of kinetic and analytical issues that need to be overcome before this PCR method could be applied in clinical diagnostic testing. Further study on PCR method was carried out by Chen *et al*. (1997) on the specificity and sensitivity of the assay for pure cultures of *Salmonella* and *Salmonella*-contaminated food samples. This PCR method presented a significant step in food microbiology due to

its automatable and direct means of analyzing PCR products. However, simplification of the DNA isolation procedures was needed to be carried out before it could be implemented in routine lab.

In another study by Riyaz-Ul-Hassan *et al*. (2004), primers based on *Salmonella* enterotoxin gene were used to develop a specific PCR assay for the detection of *Salmonella*. This method was developed for rapid detection in blood, milk and water and took for 75–90 min as to complete the PCR reaction. However, proper procedures for template preparation need to be developed before this method could be implemented in food and clinical samples. As discussed earlier, *E. coli* appeared as the most common infectious disease. Ellingson *et al*. (2005) published an article describing the PCR based assay in detection of enterohemorrhagic *E. coli* in bovine food products and feces. This assay could detect and confirm the presence of *E. coli* O157:H7 with a great sensitivity but only able to provide result after around 12 h upon receipt of sample.

In summary, PCR method had shown to have some weakness including longer time to complete the PCR reaction (due to extensive washing and incubation steps), need skilled personnel as well as advanced infrastructure. Moreover, proper procedures for DNA isolation and template preparation should be considered in applying this conventional method. This is why it is difficult to consider these immunoassays as rapid diagnostic test. Therefore, a device that performs rapid detection and on-site examination should be developed to replace this conventional method.

2.3 Biosensor in pathogen and viruses detection

As popular awareness of the importance of health increases, the devices and methods which can perform quick and accurate tests are becoming more and more compelling. Biosensor appeared as one of the fastest grown pathogen detection tools. Several biosensors approaches based on electrochemical, optical and piezoelectric (mass-based/mass-sensitive) detection principles are developed (Zhang *et al.*, 2011). A biosensor is an analytical instrument that converts a biological response into a measurable signal. The major potentials are on its specificity, sensitivity, reliability, portability, real time analysis and simplicity of operation.

Generally, a biosensor is a device incorporating a biological molecular recognition component connected to a transducer that can output a measurable signal proportional to the concentration of the analyte being sensed (Zhang *et al.*, 2006). Recently, biosensors are increasingly utilized for the detection of point mutations to facilitate the early diagnosis of genetic diseases. Specifically, the antibody-based fiber-optic biosensors have received a lot of attention. Advances in luminescence detection, research in biotechnology, and fiber-optics have opened opportunities for developing biosensors for environmental monitoring (Ajit, 1998). In addition, biosensor has also become an attractive research area with a wide variety of important biomedical applications, ranging from diagnostics of cells, DNA and protein micro-arrays, to novel materials for biosensors, tissue engineering, surface modification, systems for drug delivery, and so on (Sang and Witte, 2010).

There are many types of biosensors that are used for pathogens detection in water. For example, Banerjee and Bhunia (2010) have introduced cell-based biosensor for rapid screening of pathogens and toxins. The biosensor detecting

analyte is interacted with mammalian cells and distinguish pathogenic from non-pathogenic and active from inactive toxins, rendering accurate estimation of the risk associated with the agents. Besides, Baeumner *et al*. (2003) have introduced the RNA biosensor for the rapid detection of viable *E. coli* in water. The high specificity, rapid, ease of use and high sensitivity make this biosensor detects as few as 40 viable *E. coli* in water.

Another report by Wang *et al*. (1997), demonstrated electrochemical biosensor for the detection of DNA sequences from the pathogenic protozoan *Cryptosporidium parvum.* The sensor relied on the immobilization of a 38-mer oligonucleotide unique to the *Cryptosporidium* DNA onto the carbon-paste transducer. LaGier *et al*. (2007) also reported the detection of microbes in environment sample using electrochemical biosensors. The research was conducted by using *Karenia brevis*, fecal-indicating bacteria (*Enterococcus spp*.), markers indicative of human sources of fecal pollution (human cluster *Bacteroides* and the *esp gene* of *Enterococcus faecium*), bacterial pathogens (*E. coli 0157:H7, Salmonella spp., Campylobacter jejuni, Staphylococcus aureus*), and a viral pathogen (adenovirus) as the model. The integration waveguide biosensor has been reported by Zhu *et al*. (2005) for the detection of water-borne *E. coli* O157. The principle of the integrating waveguide biosensor was based on a fluorescent sandwich immunoassay performed inside a glass capillary waveguide. The genomic DNA of captured *E. coli* O157 cells was extracted and quantitative real-time PCR subsequently performed to assess biosensor-capture efficiency.

2.4 Biomolecule Immobilization

2.4.1 Classical method of biomolecule immobilization

The main step in the construction of bio-sensing devices is the immobilization of the sensing bio-receptor, which recognizes the analyte onto a transducing surface. The immobilization method that applied should be simple to carry out and highly reproducible (more to large scale production of biosensor). Immobilization is not only helps in forming the required close proximity between the biomaterial and the transducer, but also helps in stabilizing the immobilized biomolecules. Non–specific binding and extreme environmental condition should be avoided during the immobilization processes. The immobilized biomolecules must be easily accessible after immobilization and chemically inert towards the host structure. The immobilization could be performed through adsorption, entrapment, covalent binding, cross-linking or a combination of all these techniques (Kumar and D'Souza, 2008). The proteins are immobilized onto the polymeric carrier surface such as membranes or microsphere beads via the above stated methods through physical adsorption or covalent binding, which creates avenues for viable processes involving the use of biomolecules (Yong *et al.*, 2010).

The simplest method to immobilize biomolecules is by physical adsorption technique. The electrostatic nature of physical adsorption makes it susceptible to pH. The binding forces include hydrogen bonds, multiple salt linkages and van der Waal's forces. However, this technique can cause easy desorption of the bio-receptor from the surface and eventually leach to the sample solution during measurement. This is because the adsorption originates from the weak electrostatic binding (Teles and Fonseca, 2008).

2.4.2 New trend of biomolecule immobilization

Adsorption of protein solution onto solid surfaces plays a major role in biological systems for development of bio-sensing applications. The immobilization of the bio-receptor i.e. tissue, microorganisms, organelle, cell, enzyme, antibody, nucleic acid as well as bio-mimic appeared as one of the main stages in the design and development of a biosensor. The main challenge in this process is to retain the protein active conformation after the immobilization procedure and avoiding several limitations such as the leaking of bio-receptor and the occurrence of diffusion barriers. These problems rendered low sensitivity, un-stable and longer response time of the developed biosensor.

Cross-linking procedures was introduced to overcome these problems which tend to create better biomolecule activity and greater stability. Different types of cross-linkers are commonly studied to modify physicochemical properties of bio-polymers materials. Heat-treatment, chemical method, irradiation and physical cross-linking were also been considered in these cross-linking methods (Pedram *et al.*, 2013).

Chemical cross-linking is a new trend method to modify polymers to improve mechanical, thermal and chemical stability to the immobilized materials (Reis *et al.*, 2006). Several cross-linker containing aldehyde groups such as glutaraldehyde (GA), formaldehyde as well as glyoxal were considered to cross-link with the biomolecules (Gerrard *et al.*, 2005). Reis *et al*. (2006) has reported the possibility utilization of the chemical cross-linking agents such as GA, formaldehyde, glyoxal, adipic aldehyde, acrolein as well as terephthaldehyde in their preparation of PVA membrane. As reported (Reis *et al*., 2006), the prepared PVA

membrane that cross-linked with GA showed no necessity to any thermal treatments and the produced membrane demonstrated less swollen.

Previous study has shown the usage of GA as bifunctional agent to coimmobilized 6-phospho-d-gluconate dehydrogenase and bovine serum albumin onto screen-printed electrodes (SPCEs) by cross-linking method (Román *et al.*, 2013). This study presented an interesting alternative to determine gluconic acid using amperometric biosensor. According to Jin *et al*. (2013), the interaction between soy proteins and intercalated montmorillonite (MMT) platelets coated with soy proteins was enhanced through the chemical cross-linking of GA. In this research, the incorporation of the intercalated MMT using GA has significantly promoted the development of the elastic modulus of nanocomposites hydrogels. Moreover, Beppu *et al*. (2007) has reported on the use of GA as a cross-linking agent in preparing chitosan membrane. A drastic change was found in some macro and micro properties such as water absorption, ion permeability, chemical as well as mechanical properties for the chitosan membrane that modified using glutaraldehyde.

The enhancing usage of GA as a cross-linker agent was also reported by Wilson *et al*. (2013). They reported a study on development of terpolymer (i.e.three-component) sorbent materials by cross-linking of β-cyclodextrin (b-CD) and chitosan with glutaraldehyde. This incorporation of the β-CD into the copolymer framework promoted the potential GA in tuning the uptake efficiency of inorganic/organic anion species.

Figure 2.1 shows the three representation of a molecule of monomeric GA structure that shows the bi-functional aldehyde groups. The deposited –CHO

functional groups onto the membrane will react with amino groups of protein through covalent bonding between –CHO and NH_2 groups, as illustrated in Figure 2.2. Till now, numerous papers have been published related to the use of GA as the cross-linker agent, involving in protein immobilization activity. Some other research applications of GA for improved membrane performances are summarized in Table 2.1.

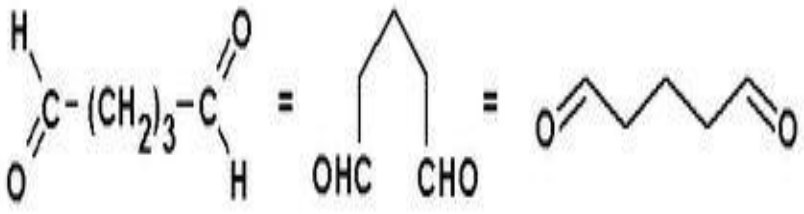

Figure 2.1: Three representation of a molecule of monomeric glutaraldehyde (Kiernan, 2000)

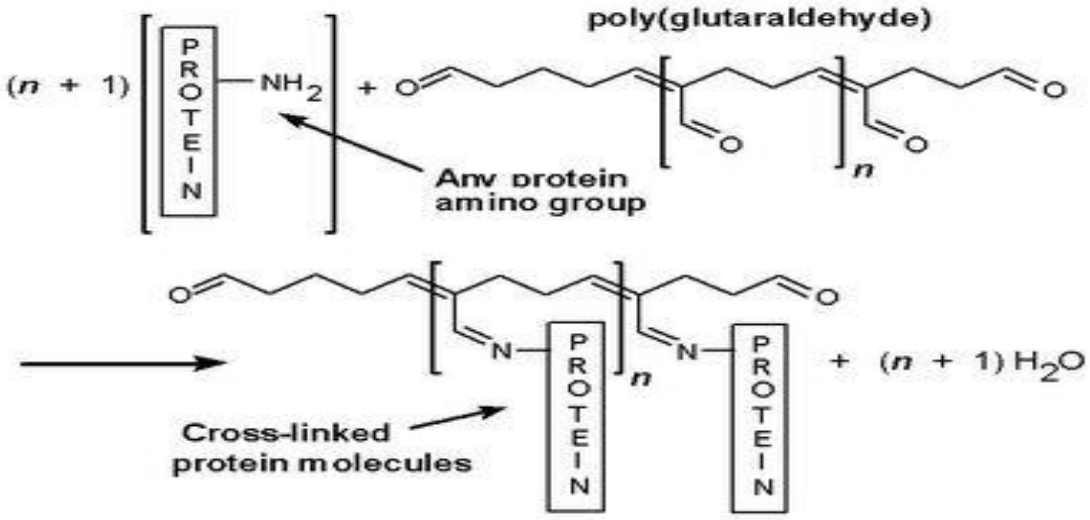

Figure 2.2: Reaction of GA with amino groups of protein (Kiernan, 2000)

Table 2.1: Importance of the glutaraldehyde as cross-linker for improved membrane performances

References	Description	Advantages of cross-linking
Pedram *et al.* (2013)	Cross-linking of diethanolamine (DEA) impregnated poly(vinyl alcohol) (PVA) on polytetrafluoroethylene (PTFE) by glutaraldehyde (GA). The membranes were evaluated for CO_2/CH_4 separation.	(1) The glass transition temperature for cross-linking of GA with PVA was dramatically increased. (2) The homogeneous pattern was created and no macro-voids were observed. (3) The highest selectivity was achieved in PVA/ GA (1 wt. %)/PTFE membrane.
Jin *et al.* (2013)	Improvement of the mechanical strength of composite hydrogels with constant soy protein and montmorillonite (MMT) concentrations by studying cross-linking variables of GA concentration, pH, and temperature.	(1) Can be beneficial for preparing bio nanocomposite materials with enhanced mechanical properties.