DEVELOPMENT OF ELECTROCHEMICAL BIOSENSOR BASED ON NYLON-6 MEMBRANE

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DEVELOPMENT OF ELECTROCHEMICAL BIOSENSOR BASED ON NYLON-6 MEMBRANE

by

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LIST OF SYMBOLS

E_{we}	Applied potential (V)
ca.	Approximately
R_{ct}	Charge transfer resistance (Ohm)
NYL	Commercial nylon-6 membrane
C	Concentration of AA (mM)
I	Current response (mA)
Z_{im}	Imaginary part of Nyquist plots on the *y*-axis
R^2	Linear regression correlation coefficients
I_{max}	Maximum current
V_A	Membrane‟s apparent volume (mm^3)
V_E	Membrane‟s existent volume (mm^3)
K_m	Michaelis–Menten constant (mM)
N-16	Nylon-6 polymer with 16 wt. %
N-18	Nylon-6 polymer with 18 wt. %
N-23	Nylon-6 polymer with 23 wt. %
N-25	Nylon-6 polymer with 25 wt. %
N-28	Nylon-6 polymer with 28 wt. %
N-16A	Nylon-6 membrane (polymer 16 wt. %) with water
N-16B	Nylon-6 membrane (polymer 16 wt. %) with methanol
ε	Porosity of the nylon-6 membranes (%)
Z_{re}	Real part of Nyquist plots on the *x*-axis
I_{ss}	Steady-state current
wt. %	Weight percentage

LIST OF ABBREVATIONS

AA	Ascorbic acid
Ag/AgCl	Silver/silver chloride
AM	Aniline Monomer
APS	Ammonium peroxodisulfate
ANOVA	Analysis of variance
BCA	Bicinchoninic acid assay
b-IgG	Biotinylated goat anti-mouse IgG
b-NHS	N-hydroxysuccinimidobiotin
BSA	Bovine serum albumin
CA	Citric acid
CP	Conducting polymers
CV	Cyclic voltammetry
DHA	Dehydroascorbic acid
DMF	*N, N*-dimethylformamide
DoE	Design of Experiment
DPV	Differential pulse voltammograms
EB	Emeraldine base
EIS	Electrochemical impedance spectroscopy
FESEM	Field emission scanning electron microscope
FTIR	Fourier transform infrared spectroscopy
GA	Glutaraldehyde
GCE	Glassy carbon electrode
HCl	Hydrochloric acid
Fe_2O_3	Iron oxide

LB	Leucoemeraldine base
LOD	Limit of detection
PANI	Polyaniline
PANI/Fe_2O_3	Polyaniline-iron oxide
PB	Pernigraniline base
Pt	Platinum
PVA	Polyvinyl alcohol
PVC	Polyvinyl chloride
PVDF	Polyvinylidene fluoride
TGA	Thermogravimetric analysis
TEM	Transmission electron microscopy

PEMBANGUNAN BIOPENDERIA ELEKTROKIMIA BERASASKAN MEMBRAN NILON-6

ABSTRAK

Jutaan manusia mati disebabkan ketiadaan dan ketidakbolehcapaian kemudahan diagnostik terutamanya dalam mengawal penyakit-penyakit penting, yang mana membawa kepada pembangunan biopenderia yang progresif. Malangnya, pengoksidaan secara langsung analit sasaran pada elektrod terdedah bagi biopenderia adalah proses tidak boleh balik dan memerlukan upaya lebih yang tinggi, menyebabkan kotoran elektrod dan kepekaan yang rendah. Elektrod yang terubahsuai membran nilon-6 telah dicadangkan untuk mengatasi masalah ini. Sebagai satu immunocerakin, tindakbalas biokimia antara analit sasaran dan tawanan berlaku pada permukaan membran nilon-6, kemudian diterjemahkan kepada isyarat rintangan yang boleh diukur. Kajian ini menjelaskan ciri morfologi bagi nilon-6 sebagai membran penjerapan protin dan pengaruh mereka dalam antaramuka pengecaman biologi. Membran nilon-6 disintesis melalui penyongsangan fasa kering dengan kepekataan berbeza bagi polimer nilon-6 dalam julat 16 wt. % hingga 28 wt. % dan pelbagai jenis bahan tambah (air dan metanol) dalam larutan '*dope*'. Membran nilon-6 yang dibangunkan, N-16B, dengan 16 wt. % nilon-6 polimer dan metanol sebagai bukan pelarut telah memperlihatkan kelajuan sisi penyumbuan yang terpantas (1.07 mm/saat) dan kapasiti penjerapan protin yang sangat baik, (1,650.00 $\pm$ 85.84 µg/cm^3). Kajian semasa mendedahkan kepentingan morfologi membran yang mempengaruhi kepekaan dan keberkesanan peranti pengesan imun. Isu kestabilan dalam penjerapan biomolekul telah diatasi dengan mengintegrasi glutaradehid (GA) ke atas membran nilon-6 sebelum penjerapan

protin. Kajian awal telah dijalankan untuk mengkaji kesan masa inkubasi, pH dan kepekatan GA pada pengikatan protin ke atas polimer nilon-6. Keadaan optimum bagi integrasi GA ditemui pada 40 minit masa inkubasi, pH 7.5 dan 1 wt. % kepekatan GA. Analisis statistik dengan program rekabentuk silang dilakukan dan keadaan integrasi GA optimum dari analisis statistik didapati pada 25 wt. % bagi polimer nilon-6, 75 wt.% campuran pelarut + tak larut, pH 9.0 dan 70 minit masa inkubasi. Keputusan eksperimen menunjukkan bahawa GA sebagai penyambung lintang pada keadaan optimum mampu mencapai sangkutan GA yang lebih baik untuk penjerapan protin yang terakhir. Kajian lanjut telah meneroka penyediaan polianilina-ferum (III) oksida (PANI/Fe_2O_3) yang konduktif yang berfungsi sebagai pemindaharuh isyarat elektrik, untuk menukar interaksi elektrokimia kepada satu isyarat ketahanan yang dapat diukur. PANI disintesis melalui pempolimeran beroksida bagi monomer anilina (AM) dengan kehadiran ammonium persulfat (APS). 0.2 M kepekatan AM and 1:3 nisbah sukatan AM:APS didapati menghasilkan PANI dengan tindakbalas konduktiviti ionik yang tertinggi pada 7.565 $\pm$ 0.262 mS/cm. Kajian telah mengkaji semula aspek menarik bagi keadaan yang berlainan oleh PANI sebagai satu bahan konduktif yang penting untuk peranti elektronik/elektrik. Seterusnya, penderia amperometri berasaskan-membran telah dipasang dan aktiviti elektrokimia antara asid askorbat (AA, analit sasaran) dan askorbat oksidase (analit tawanan) telah dinilai. Had pengesanan bagi penderia didapati pada 5.77 mM dan pemalar *Michaelis-Menten* (K_m) dikira sebanyak 26.76 mM. Tindakbalas bagi spektroskopi galangan elektrokimia (EIS), voltammetry kitaran (CV) dan voltammograms denyutan kebezaan (DPV) dijalankan untuk menganalis dua lapis elektrokimia ke atas elektrod kerja. Membran nilon-6 yang dibangunkan telah menyediakan satu pelantar pengesanan yang menjanjikan untuk

pembinaan penderia dan sesuai untuk aplikasi praktikal dalam analisis farmaseutikal atau klinikal dan tanaman pertanian.

DEVELOPMENT OF ELECTROCHEMICAL BIOSENSOR BASED ON NYLON-6 MEMBRANE

ABSTRACT

Millions of people die due to the unavailability and inaccessible of diagnostics facilities especially in controlling crucial diseases, which led to the progressive development of biosensor. Unfortunately, direct oxidation of target analyte at the bare electrode of a biosensor is an irreversible process and requires a high overpotential, resulted in electrode fouling and low sensitivity. Nylon-6 membrane modified electrodes have been proposed to overcome this problem. As an immunoassay, the biochemical reaction between target and capture analyte takes place on the surface of the nylon-6 membrane, then translated to measurable resistance signal. The present study elucidates the morphology characteristic of nylon-6 as protein immobilization membrane and their influences in biological recognition interface. The nylon-6 membranes were synthesized via dry phase inversion with different concentration of nylon-6 polymer in a range of 16 wt. % to 28 wt.% and different types of additives (water and methanol) in dope solution. The developed nylon-6 membrane, N-16B, with 16 wt. % nylon-6 polymer and methanol as non-solvent had demonstrated the fastest lateral wicking speed (1.07 mm/sec) and excellent protein immobilization capacity (1,650.00 $\pm$ 85.84 μg/cm^3). The current study revealed the importance of membrane morphology that affects the sensitivity and effectiveness of an immuno-sensing device. The stability issue in biomolecule immobilization has been overcomed by integrating glutaraldehyde (GA) onto nylon-6 membrane prior to protein immobilization. The preliminary study was carried out to study the effect of incubation time, pH and concentration of GA on protein

binding of the nylon-6 polymer. The optimum conditions of GA integration were found at 40 minutes of incubation time, pH 7.5 and 1 wt. % of GA concentration. Statistical analysis using crossed design programme was performed and the optimum GA integration conditions from the statistical analysis were found at 25 wt. % of the nylon-6 polymer, 75 wt. % of mixture solvent + nonsolvent, pH 9.0 and 70 minutes of incubation time. The experimental results showed that the GA as a cross-linker reagent at optimum conditions was able to achieve better GA attachment for latter protein immobilization. Further study has been explored on the preparation of conductive polyaniline-iron oxide (PANI/Fe_2O_3) that served as the electrical signal transducer, to convert the electrochemical interactions to a measurable resistance signal. PANI was synthesized via oxidative polymerization of aniline monomer (AM) in the presence of ammonium persulfate (APS). 0.2 M concentration of AM and 1:3 volume ratio of AM:APS were found to produce PANI logged with the highest ionic conductivity response at 7.565 $\pm$ 0.262 mS/cm. The study had reviewed the interesting aspect of different state of PANI as one of the important conducting material for electronic/electrical devices. Subsequently, a membrane-based amperometric sensor was assembled and electrochemical activities ascorbic acid (AA, target analyte) and ascorbate oxidase (capture analyte) were evaluated. The detection limit of the sensor was found at 5.77 mM and the Michaelis–Menten constant (K_m) was calculated as 26.76 mM. Electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV) and differential pulse voltammograms (DPV) responses were carried out to analyze the electrochemical double layer on the working electrode. The developed nylon-6 membrane has provided a promising detection platform for sensor construction and suitable for practical application in pharmaceutical or clinical analysis and agricultural crops.

CHAPTER 1

INTRODUCTION

1.1 Membrane as biological recognition interface for immunosensor

Modern technology has achieved better enhancement and sophistication in providing treatment, monitoring and controlling the spread of crucial diseases, especially those related to waterborne and foodborne outbreaks (Ivnitski *et al.*, 2000, Low *et al.*, 2012). Due to the unavailability or inaccessibility of diagnostic facilities, a rapid construction, on-site examination, and user-friendly detection system is progressively developed to overcome such problem. Under such a scenario, immunosensor has emerged as a potential detection system for an effective monitoring of pathogen (Donmez *et al.*, 2015, Hosseini *et al.*, 2014, Kolosovas-Machuca *et al.*, 2015, Nandakumar *et al.*, 2008, Pohanka *et al.*, 2007, Waiyapoka *et al.*, 2015), contaminant and toxic material in the biotechnology applications (Grover *et al.*, 2015, Hsiue *et al.*, 2004, Khaksarinejad *et al.*, 2015, Srivastava *et al.*, 2014, Zahedi *et al.*, 2016). The biomolecules immobilization is the key factor that determined the sophisticated development of an immunosensors (Dong *et al.*, 2013, García *et al.*, 2012).

The effectiveness of biomolecule immobilization strongly depends on the membrane material (the detection platform) (Betty, 2016, Sadeghi *et al.*, 2015) that provides excellent stability, fast lateral wicking speed and reduces biomolecule desorption (Yong *et al.*, 2010). As an immunoassay, the biochemical reaction between antigen and antibody takes place on the surface of the porous membrane, which is greatly influenced by the membrane morphology and its intrinsic chemical composition. The membrane material should be insoluble in water and has a high

binding capacity to the specifically targeted biomolecules (Pundir *et al.*, 2008). By manipulating the structure of the membrane support, a sensor's bio-catalytic efficiency can be easily achieved to produce an effective and accurate immunological analysis (Ahmad *et al.*, 2016). Recently, polymeric porous membranes offer the most available support material for recognition interface in biosensing applications. The drive to improve the response signal, increase sensitivity, lower detection limit and better reproducibility are the reason of the growing need of the polymeric porous membrane (Luo *et al.*, 2017, Santhy *et al.*, 2018, Tang *et al.*, 2019). Polyvinyl alcohol (PVA) (Braiek *et al.*, 2018, Luo *et al.*, 2017), polyvinyl chloride (PVC) (Afkhami *et al.*, 2014, Ezzeldin *et al.*, 2012, K Gupta *et al.*, 2011, Santhy *et al.*, 2018), polysulfone (Zhao *et al.*, 2016), nylon-6 (Tang *et al.*, 2019, Yaqoob *et al.*, 2016) and polyvinylidene fluoride (PVDF) (Chen *et al.*, 2015, Zhao *et al.*, 2015) are the most potential polymeric porous membrane in development of biosensing application.

Among these polymeric membranes, nylon-6 is the most desirable material and is proven to be a suitable immobilization platform (Jackeray *et al.*, 2010). This polymer comprises of high mechanical strength (Salapare *et al.*, 2015, Shakaib *et al.*, 2013, Zhou *et al.*, 2013), good stability and high resistance towards microbial attack (Pant *et al.*, 2013b). Nylon-6 membrane is commonly produced by phase inversion technique through immersion precipitation (Chang *et al.*, 2013, Lin *et al.*, 2002, Shakaib *et al.*, 2013, Shih *et al.*, 2012, Sobhanipour and Karimi, 2013) or dry phase inversion (Fatarella *et al.*, 2014, Leo *et al.*, 2011, Pant *et al.*, 2013a, Pant *et al.*, 2013b, Yan *et al.*, 2007). Liu *et al.* (2013), reported the use of nylon-6 membrane as a porous matrix to immobilize antibody in the detection of two main serum biomarkers for down syndrome, which were β-human chorionic gonadotrophin (β-

HCG) and α-fetal protein (AFP). The nylon-6 membrane was chosen as the sensor's detection platform because of its large total surface area, high mechanical strength, and good biocompatibility. Antibodies were first immobilized onto the nylon-6 membrane surface, so as to capture the target antigens to form the immunocomplexes (antibodies–antigens) prior to react with the labelled antibodies to form the triplex "sandwich" structures. Then the target antigen will be detected through photoluminescence. A low detection limit of the biosensor at 1×10^{-6} IU/L for β-HCG and 1 ng/mL for AFP were detected within the short assay time (Liu *et al.*, 2013).

Biosensor is the most effective method in monitoring and detection of target interest. In biosensing application, biomolecules immobilization is the key factor to determine the effectiveness of the developed biosensor. Biomolecule immobilization strongly depends on the membrane material (the detection platform). Polymeric membrane is the most desirable material for biomolecules immobilization. Among of the polymeric membrane, nylon-6 has proved to be an effective detection platform. However, due to the lower hydrophilicity of the membrane, had limit the usage of the membrane in biosensing application.

1.2 Challenges of protein immobilization

Immobilization process is defined as the attachment of biomolecules to a substrate surface resulting in a reduction or loss of mobility (Rusmini *et al.*, 2007). The attachment of the protein onto a surface should not affect the protein conformation and its function for fully retain biological activity. There are many immobilization techniques, includes physical adsorption, covalent and bioaffinity

immobilization (Kim and Herr, 2013, Zhang *et al.*, 2013). For physical immobilization, proteins can adsorb on surfaces via intermolecular forces which could be the ionic bonds, hydrophobic interactions or polar bindings. Thus, the layer is likely to be heterogeneous and randomly oriented since each molecule can form many contacts in different orientations to minimize the repulsive interactions with the substrate. However, the main disadvantage is its random orientation and weak attachment of protein (Kim and Herr, 2013, Rusmini *et al.*, 2007). This is because protein may be removed by some buffers or detergents during performing the immobilization process.

Proteins commonly are covalently bound to the immobilization support through accessible functional groups of the exposed amino acids (Rusmini *et al.*, 2007). The protein-substrate covalent bonds are mostly formed between side-chain-exposed functional groups of the proteins, which resulting in a high surface coverage. However, the covalent reaction is usually slow, hence, the protein and the binding substrate required longer incubation times (Kim and Herr, 2013). The disadvantages of covalent linkage are reduced activity of proteins, toxic reagents and complicated chemistry (Kim and Herr, 2013).

Integration of supports material by glutaraldehyde is one of the most popular techniques to modify physicochemical properties of materials, subsequently overcome the drawbacks of protein immobilization. (Betancor *et al.*, 2006). Glutaraldehyde is introduced to chemically cross-link between proteins and the membrane support in order to improve the protein adsorption capacity on the membrane surface. Indeed, chemical cross-linking is one of the most effective and

simplest methods to bind the biomolecules onto the membrane surface (Tian *et al.*, 2016).

In biomolecule immobilization on the support matrix, the biomolecule was commonly covalently bound to the immobilization support. However, due to the several disadvantages of the covalent reaction especially in biomolecule stability, gluataraldehyde was introduced to overcome the drawback of the biomolecule immobilization.

1.3 Polyaniline as conductive electric transducer in immunosensor

Polyaniline (PANI) is among the most studied conducting polymers found in variety of applications such as rechargeable batteries, photonics, optoelectronic devices, biochemical sensing devices (Casado *et al.*, 2014, Chowdhury *et al.*, 2008, Patil *et al.*, 2015, Rakić *et al.*, 2015), membrane separation as well as modified electrodes for electro-reduction (Bober *et al.*, 2013, Yang *et al.*, 2014). PANI is produced by chemical oxidative polymerization of aniline (Choi *et al.*, 2005, Mascaro and Gonçalves, 2007, Thakur *et al.*, 2015), is well known to have good environmental stability (Casado *et al.*, 2014, Choi *et al.*, 2005, Patil *et al.*, 2015, Wu *et al.*, 2007), controllable electrical properties (Choi *et al.*, 2005, Devi and Pundir, 2014, Ndangili *et al.*, 2010, Yılmaz and Küçükyavuz, 2009), easy to synthesis (Golmohammadi *et al.*, 2007, Wu *et al.*, 2007), as well as its high pseudo-capacitance (Yang *et al.*, 2014) that make them highly utilized in the development of an electrochemical biosensor (Devi and Pundir, 2014, Mascaro and Gonçalves, 2007, Moghadam and Zareh, 2010, Shamagsumova *et al.*, 2015). As for biosensing application, PANI performed as the transducer where this conductive polymer will

integrate with the biological element, relays any antigen-antibody binding as a measured electrical quantity (Jagur-Grodzinski, 2012, Tahir *et al.*, 2005).

Chemically synthesized PANI has been subjected to electrochemical characterization. Its electrical conductivity is reversibly controlled both by changes in the oxidation state of the main chain and by protonation of the imine nitrogen atoms (Chowdhury *et al.*, 2008, Ndangili *et al.*, 2010, Song and Choi, 2014). The potentiodynamic behavior of PANI obtained through chemical oxidation could be varied in the presence of different oxidants such as ammonium persulfate ($(NH_4)_2S_2O_8$), iron (III) chloride ($FeCl_3$), hydrogen peroxide (H_2O_2) or dichromate (Cr_2O7^{2-}) (Shamagsumova *et al.*, 2015). Particularly, PANI exists stable in three different oxidation states: fully reduced leucoemeraldine base, partially oxidized emeraldine base and fully oxidized pernigraniline (Ebrahim *et al.*, 2014, Golmohammadi *et al.*, 2007, Gomes and Oliveira, 2012, Gospodinova and Terlemezyan, 1998, Yılmaz and Küçükyavuz, 2009). Each oxidation state can exist in the protonated form after treatment with an acid.

In recent year, there has been increasing interest in the synthesis of conductive polymer-inorganic composites with an organized structure. Such polymer/inorganics composites have found a number of practical applications in molecular electronics, electronics displays, telecommunication, electrochemical storing systems and the most recent in biosensor (Kamikawa *et al.*, 2010, Pal *et al.*, 2008). Looking more specifically to the hybrid composites with magnetic and electric properties (Pal and Alocilja, 2009), the colloidal PANI-iron oxide composite is synthesized through *in situ* oxidation polymerization in a micellar medium where the gamma iron oxide cores are embedded in a PANI matrix layer (Pal and Alocilja,

2009). Numerous researches have been done by employing this conductive hybrid composite as a transducer in the development of electrochemical sensor. For examples, the PANI-iron oxide composite has been applied to a direct-charge transfer sensing device for the detection of *Bacillus anthracis* endospores in contaminated food samples (Pal and Alocilja, 2009). Recent literature shows that different types of magnetic cores (Kamikawa *et al.*, 2010, Stejskal *et al.*, 2006) and doping agents have been used for the synthesis of the magnetic polyaniline nanoparticles. Typical examples of the magnetic core include iron (II, III) oxide, hydroxyl iron, Ni Zn Ferrite and Li Ni Ferrite (Pal and Alocilja, 2009).

Conductive PANI has served as as the transducer to integrate the biochemical reaction between biological element and target interest to a measured electrical quantity. Polyaniline exists in various forms with different properties in chemical and physical. The transformations between these different states make PANI suitable transducer material in various applications such as textile fibers, photovoltaic devices, etc.

1.4 Membrane-based electrochemical biosensor for ascorbic acid detection

An excess intake or a sudden intake of high-acidity foods will lead to excessive secretion of gastric juices in the stomach. Thus, increased the amount of pepsin secretion in the mucous membrane chief cells which led to exposure to the gastric juice, subsequently induce the damage of the stomach wall. Ascorbic acid has very high acidity and may induce these gastrointestinal side effects when ingested on an empty stomach. (Lee *et al.*, 2018). As humans cannot synthesize AA *in vivo*, ascorbic acid must be uptake from an external source. The biggest consumer of ascorbic acid is the food and beverage industry, which counted approximately

60%; while 30 % in the pharmaceutical industry and the remaining in the feed industry in 2016 (Pune, 2019). Thus, quick monitoring of AA levels and consumption is needed.

A large number of methods of analysis have been applied to fruits, vegetables, biological fluids, and pharmaceuticals, which includes spectrophotometric (Khan *et al.*, 2006, Wu *et al.*, 2003), chemiluminescence (Ma *et al.*, 2002), titrimetric (Lenghor *et al.*, 2002, Suntornsuk *et al.*, 2002) as well as optical sensor and (Teixeira *et al.*, 2003) electrochemical sensors (El-Moghazy *et al.*, 2018, Gopalakrishnan *et al.*, 2018, Kwasny *et al.*, 2018). The conventional techniques such as titrimetric and spectrophotometric methods have some disadvantages, including complex sample pretreatment procedures, high manufacturing costs, time-consuming manipulations, and low sensitivities (Barberis *et al.*, 2010, Nakamura and Karube, 2003).

Hence, a need has arisen for a fast, sensitive, and inexpensive method to detect ascorbic acid, and substitution of analytical methods to a biosensor. Given the advantages of low cost of the required equipment, fast response, simple instrumentation, high sensitivity, facile miniaturization, and low power requirement, amperometric/electrochemical technique appears as an attractive detection tool compared to the titrimetric or instrumental methods mentioned earlier (Akhgar *et al.*, 2012, Beitollahi and Sheikhshoaie, 2012, Thomas *et al.*, 2013).

However, fouling effects on the bare working electrode of the electrochemical methods remain the drawback which could lower the sensitivity of the response signal. A considerable effort had been devoted to a strategy of electrode

surface modification. Electrode surface modification by attachment of membrane is an interesting method that could apply in the development of the biosensor.

The membrane-based electrochemical biosensor is beneficial for improving the stability of the biosensors. Therefore, it is a good idea to practically applying the in-house developed nylon-6 membrane in the electrochemical sensing device, which can be used as the detection platform for the immobilization of ascorbate oxidase in detection of ascorbic acid.

1.5 Problem statement and importance of the research

Biosensors are increasingly becoming practical and useful analytical tools in medicine, food quality control, environmental monitoring, and research. It uses a biological recognition element (bio-receptor) and a transducer in direct special contact with the target analyte to provide selective quantitative or semi-quantitative analytical information (Jeong *et al.*, 2018). The most concern in a biosensing application is about the sensitivity and capacity of protein immobilization. The development of lateral flow membrane as an immunosensor substrate remains a challenge, due to the difficulty in controlling the desired polymer matrix functionality for specific and stable biomolecule immobilization. This drawback eventually limits their worldwide application. Therefore, it is of great interest to elucidate the fundamental perceptive of membrane morphology in order to produce a polymeric support matric with improved material properties and enhances its influence in the immunosensing application.

Biomolecule stability is the most concern factor of a biosensor after solving the challenge in the development of polymeric support. In order to develop a well-

functioning and high binding ability of biosensor, the immobilized biomolecule needs to be retained stable on the polymeric support and have low nonspecific (unwanted) bindings of biomolecules. Nonetheless, the biomolecule could be encountered in unfolding whereby the binding site may be disrupted and the biomolecule may lose its biological functions. Hence, this contributes to the low binding capacity and reduce sensitivity of a biosensor. Cross-linking by glutaraldehyde has found to be the most effective cross-linking reagent and is widely used for biomolecule immobilization stability. Depth understanding of the cross-linking conditions needs to be study in order to increase the stability of biomolecule immobilization, consequently results in excellent binding ability.

As for an immunosensing application, a transducer is needed to transform the biological binding into a readable and measurable output signal. Polyaniline (PANI) has appeared as a great conducting material that made it suitable as a transducer in biosensing development as an electrical signal. Unfortunately, the morphology of the PANI formed is still hard to be controlled and mainly depends on its oxidation state. The synthesis properties which depend on the protonation level and the particle-polymer interactions such as concentration effects of the aniline monomer, the ratio of the aniline monomer to the oxidizing agent, concentration of protonating agent as well as pH are still limited and lack of exposure. In fact, the understanding of these properties could help to enhance the charge transfer from the biochemical reaction into the measurable signal as well as increase the sensitivity of the biosensor.

Numerous studies have approached to deal with the amperometric-based biosensor in the determination of various samples using three-system electrodes. The

interferences problem confronted from the electroactive compounds/bioreceptor molecules attached to the electrode has contributed to a false current measurement and low reproducibility. In fact, the electrode fouling due to the non-specific interaction between the bioreceptor molecule and the electrode surface had made the limitation of the usage of the amperometric technique. Therefore, a new approach has heightened to a renewed interest in applying the polymeric materials coated/wrap onto the surface of electrodes as the support material for biomolecules immobilization. This polymeric-modified electrode is expected to perform high sensitivity and specificity of the developed electrochemical sensing.

In this current work, some of the raw fruits such as plum, guava, blackcurrant and kiwi that have a high content of ascorbic acid were chosen for evaluation and comparison. If the developed electrochemical biosensor could detect the ascorbic acid of these fruits, it could be concluded that the electrochemical biosensor had successfully developed. Therefore, the detection of these types of fruit had been a benchmark in this successfully developed biosensor.

1.6 Research objectives

Based on above problem statements, this study is aimed to synthesize nylon-6 membrane to achieve enhancement performances of an electrochemical biosensor. The study comprises of following objectives:

1. To synthesize and characterize nylon-6 membrane as a biological recognition platform for an electrochemical sensing device.

2. To optimize cross-linking conditions of glutaraldehyde on the membrane surface through statistical analysis to achieve better glutaraldehyde attachment for latter protein immobilization.
3. To synthesize conductive polyaniline-iron oxide (PANI/Fe_2O_3) nanocomposite and to explore the different state of PANI as an important conducting material for electronic/electrical devices in convert biochemical interaction to measurable resistance signal.
4. To assemble nylon-6 membrane onto the carbon electrode as a membrane-based electrochemical biosensor for amperometric transduction for the uptake of ascorbic acid.

1.7 Scope of study

The first part of the research is focused on the membrane synthesis. This work elucidates the morphology characteristic of nylon-6 as a protein immobilization platform and their influences in biological recognition for a single-use electrochemical immunosensor. In this work, additives (water and methanol) and different concentration of nylon-6 polymer (16 wt. % to 28 wt. %) are introduced to control the membrane morphology. Nylon-6 membranes with different intrinsic properties are assembled as electrochemical immunosensors, which are used to find the correlation between the membrane structure and to its closely connected biomolecule immobilizing ability, lateral wicking rate as well as electrochemical detection sensitivity.

Subsequently, cross-linking of nylon-6 membrane using glutaraldehyde is subjected to improve the protein adsorption capacity on the membrane surface.

Glutaraldehyde cross-linking conditions was studied by investigating the effect of time incubation (10-90 minutes), pH (4.5-9.0) and concentration of glutaraldehyde (0.5 wt. %-4.5 wt. %). The D-Optimal design in Design Expert (DoE) is introduced to study the interactions between the membrane formulation and its effects on different glutaraldehyde cross-linking conditions.

To fulfill the requirement of the excellent biosensor, the study of polyaniline as an electrical signal transducer is conducted. In this work, PANI and magnetic PANI/Fe_2O_3 nanocomposite are chemically prepared at different imine nitrogen protonation level, that depends on the concentration of aniline monomer (AM) at 0.2 M to 1.0 M and the volume ratio of AM to the oxidizing agent (ammonium peroxodisulfate, APS) at 1:1 to 1:5. The surface morphologies and conductivity properties of the nanocomposites are characterized using transmission electron microscopy (TEM) as well as the ionic conductivity measurement. In this work, the conductive PANI/Fe_2O_3 nanocomposites are subjected to modification with the biotinylated goat anti-mouse IgG for complex antigen-antibody biomolecules detections in an assembled pulse-mode electrochemical sensing platform.

The last part of the study is practicability of applying the in-house developed nylon-6 membrane as a recognition interface on a carbon electrode for amperometric-based sensor in the determination of ascorbic acid. If the concept of biosensor with applying protein for immobilization in determining level of protein binding ability success, therefore for real application, ascorbic acid is chosen. Ascorbic acid is one of the elements required for the maintenance of human health. This biosensor is the most effective device to monitor and detect the consumption level of ascorbic acid. The conjugation of ascorbic acid to PANI/Fe_2O_3 is first

conducted prior to demonstrate the voltammetry performance. UV-Vis absorbance is measured to prove the successful conjugation of ascorbic acid to PANI/Fe_2O_3. In this work, a membrane-based sensor is assembled for measuring electrochemical activities between AA as the target analyte and ascorbate oxidase (immobilized onto the membrane surface) as the capture analyte. Electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV) and differential pulse voltammograms (DPV) responses are performed to analyze the electrochemical double layer on the electrode. The selectivity study has also been conducted for the determination of ascorbic acid (AA) in the presence of citric acid (CA), a common interference compound for AA in most food samples.

1.8 Thesis organization

This thesis comprises of five chapters. Introduction on the membrane as a biological recognition interface in immunosensor, protein immobilization, polyaniline as transducer as well as the membrane-based electrochemical biosensor is provided to give a brief overview on this study background. The problem statement is also highlighted to reflect the current scenario regarding the restriction in development of biosensor. The research objectives are elaborated in detail together with the scope of the study to address the issues in the development of biosensor. The thesis organization is also provided in the last section of this chapter.

Chapter Two represent the comprehensive review (Literature Review) on the perspective and the principle in an electrochemical biosensor. This chapter has highlighted the key element in development of the electrochemical biosensor based on the existing researches involve the availability of the sensing technique,

membrane in biosensing application, cross-linking in biomolecule immobilization, as well as the advances in the application of conductive polymer as a transducer. With the existing literature, future direction in the innovation of effective in-house membrane-based electrochemical biosensor is discussed.

Chapter Three starts with the illustration of a research flow chart represent the overall experimental run in the current study. The details on materials and chemical, experimental procedures, characterization of the developed membrane, analyses as well as the related performances are included in this chapter.

Chapter Four presents the experimental results from the findings of the research with the in-depth discussion. The results and discussion covers the research objectives which, at first, the membrane is synthesized through dry phase inversion with the addition of the additive to influence the morphological structure of the membrane. The in-house nylon-6 membrane is then integrated with the glutaraldehyde in expectation to modify physicochemical properties of materials, eventually encounter the protein immobilization issues. In fact, the preparation of conductive polyaniline-iron oxide (PANI/Fe_2O_3) nanocomposite is focused to reveal the role of PANI as an electric signal transducer to convert biochemical interaction into the measurable resistance signal. This chapter ended with the assembly of developed nylon-6 membrane as a recognition interface on a carbon electrode for electrochemical-transmission in the determination of ascorbic acid.

Summarization of the findings in accordance with the research objectives are concluded in Chapter Five (Conclusion and Recommendation). Several recommendations and consideration related to this field are proposed for improvement in future work.

CHAPTER 2

LITERATURE REVIEW

2.1 Biosensor

A biosensor is an analytical device that comprises of a biological sensing element coupled to a physicochemical transducer for target compounds detection. The global market value of biosensor is designated to grow rampantly (Figure 2.1), driven from medicine, process control, food and environmental monitoring, with global market valued USD 17.5 billion in 2016 and is estimated to reach to USD 41.9 billion by 2026 at a CAGR of 9.2% (Maximize, 2019). The need for biosensor is expected because of the rising numbers of chronic diseases and demands for point-of-care-testing (Future, 2019). Emerging awareness and concern on food safety, environmental monitoring and the elevated threat of bioterrorism that triggered the design of biosensors especially in the detection of pesticides, heavy metals, organic pollutants, pathogens and toxins are also contributed to the growing market of the biosensor (Jeong *et al.*, 2018).

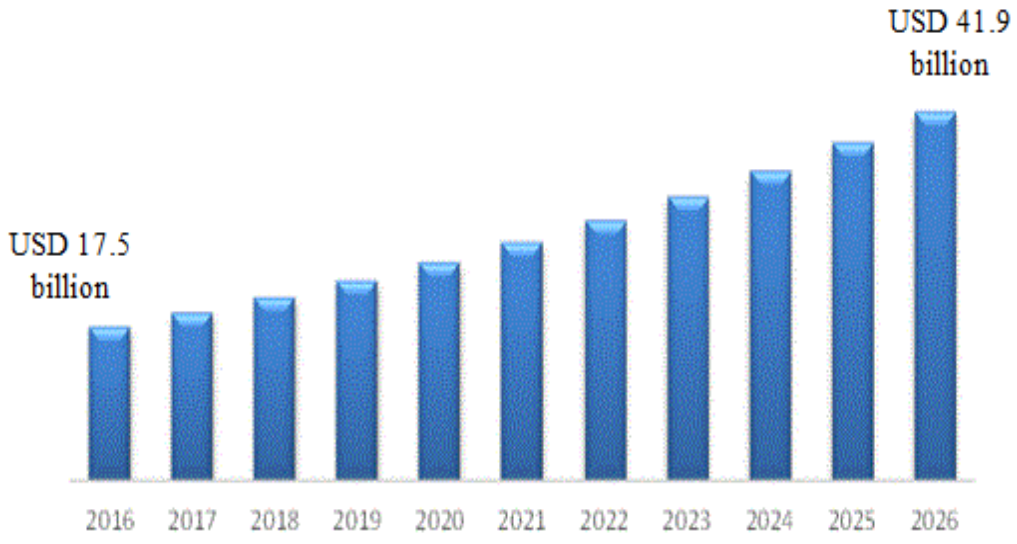

Figure 2.1 Market demand of biosensor through 2016-2026 (Maximize, 2019)

Biosensor can be categorized into electrochemical biosensors, optical biosensors, thermal biosensors, piezoelectric biosensors and others (Xu *et al.*, 2017a). The analytical device consists of a re-usable or single-used immobilized biological ligand that immobilized on a porous substrate and a physical transducer (Jongerius-Gortemaker *et al.*, 2002). The immobilized biological ligand is used to "capture" the targeted analyte, while the physical transducer will convert the interaction between the targeted and biological ligand into physicochemical changes. Then, this physicochemical change will be amplified by processor to a measurable signal. The overall sensing concept is illustrated in Figure 2.2 (Jenkins *et al.*, 2011, Jeong *et al.*, 2018).

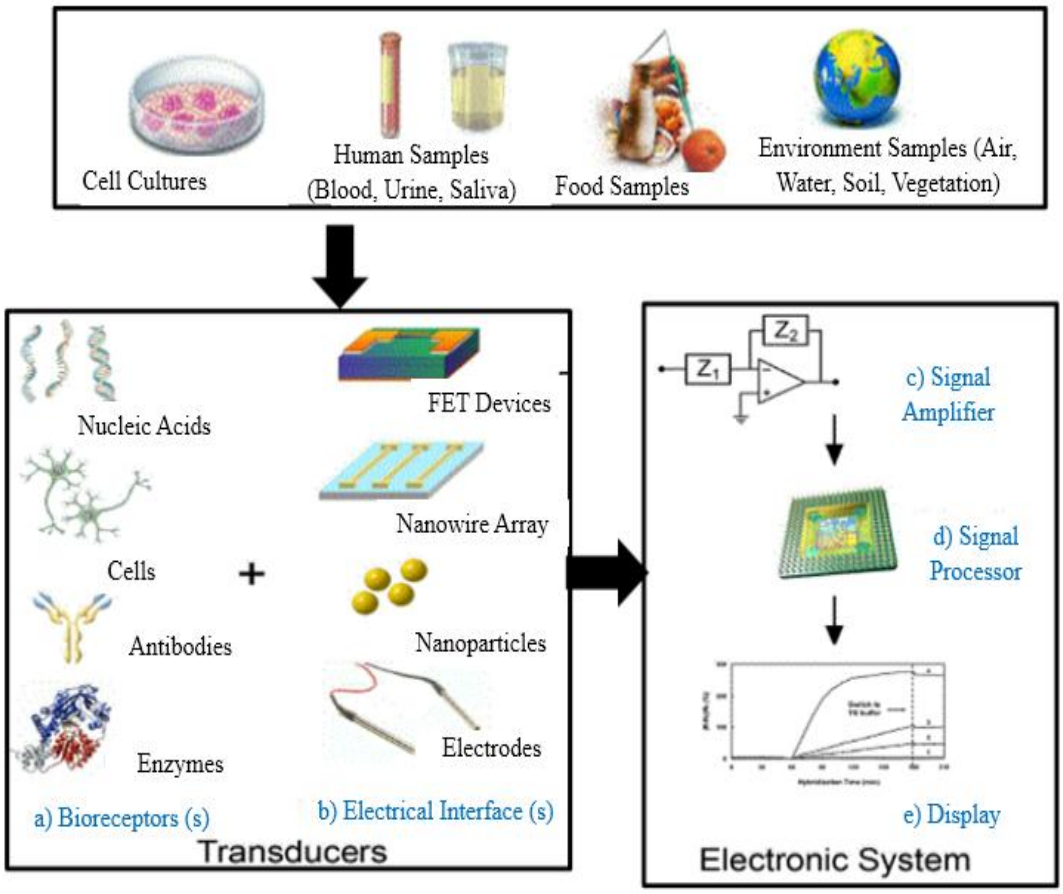

Figure 2.2 Element and components of a biosensor (Grieshaber *et al.*, 2008)

As shown in Figure 2.2, the biosensor systems convert biochemical signals into measurable physiochemical signals, which could be used to quantify the amount of analytes in the solution. Meanwhile, transduction, which is a signal conversion, could be generated through various methods based on numerous detection system (Jeong *et al.*, 2018). An effective biosensor needs an efficacious biosensing platform to perform high sensitive detection with low sensing limits. The bio-sensitive zone is referred to a specific area used to immobilize the biological recognition element (enzyme, receptor protein, probe molecule, cell-receptor etc.) on the sensing platform (Tereshchenko *et al.*, 2016, Xu *et al.*, 2017a). This biorecognition zone, also known as the functionalized surface, will serve to capture the target analyte such as low molecular compound (LMC), protein, nucleic acid or cell. Indeed, the effective surface area, roughness, porosity, functional groups and hydroscopic nature of the biosensing platform will affect the biointerface formation to provide precise and rapid detection of the target biomolecules (Tereshchenko *et al.*, 2016). Membrane is identified as one of the biorecognition platform. Details of membrane in affecting overall sensing applications will be discussed in section 2.3.

Based on the literature review, a biosensor is the most effective method in monitoring and detection of target interest. In biosensing application, a transducer will convert the biochemical reaction occurred between target and capture analyte to a measurable signal. The biochemical reaction occurred on the support surface or commonly referred to the membrane. Therefore, support matrix and transducer are the key elements to fulfill the requirement of the sensitive biosensor.

2.2 Electrochemical signal transduction

Among different configuration of sensors (electrochemical, optical, thermal, etc.), the electrochemical biosensor has been well recognized as it showed to have good ability in sensitivity, specificity, and accuracy (Cui *et al.*, 2014, Wang *et al.*, 2014). Besides, electrochemical biosensing is an inexpensive sensing application and easy to operate, which made this type of biosensor received high attention (Cui *et al.*, 2014). In recent years, numerous studies have been done to implement the electrochemical biosensor in the detection of waterborne and foodborne pathogens. For example, Dong *et al.* (2013) had done the research on the detection of *Salmonella typhimurium* in milk by using a stable and sensitive label-free electrochemical impedance immunosensor. The anti-*Salmonella* antibodies (capture analyte) were immobilized onto the gold nanoparticles and poly(amidoamine)-multiwalled carbon nanotubes-chitosan nanocomposite film. The nanocomposite thin film was then attached to the modified glassy carbon electrode (AuNPs/PAMAM-MWCNT-Chi/GCE). In verification of the stepwise assembly of the immunosensor, the electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were used. The fabricated electrochemical biosensor had been successful in determining *S. typhimurium* in milk with 94.5% to 106.6% of recoveries. Moreover, the biosensor had also performed well in reproducibility and acceptable stability.

In 2013, Singh *et al.* (2013) had employed the graphene oxide (GO)-Chitosan (CHI) nanocomposite for the development of DNA based electrochemical biosensor to diagnose the typhoid disease. The studies had revealed that differential pulse voltammetry (DPV) showed good specificity and able to distinguish

complementary, non-complementary and one base mismatch sequences. This biosensor had demonstrated low detection limit of 10 fM in phosphate buffer and 100 fM in spiked serum. In fact, the large surface-to-volume ratio, good graphene oxide activity and good biocompatibility of chitosan which had improved the DNA immobilization and facilitate electron transfer between DNA and electrode surface were among the important factors that contributed to the excellent performance of a biosensor. In another work by García *et al.* (2012), a disposable electrochemical biosensor was developed for selective *Salmonella* detection in presence of *Lysteria* and *E. coli* in the sample solution. Researchers used an electrochemical chip based on an array of eight gold electrodes and a silver pseudo reference configuration to carry out the test. In this study, the detection of *Salmonella* in samples containing other pathogens had successfully achieved.

As previously mentioned, electrochemical-based sensor platform is the most common and frequently used in biosensing applications. There are several approaches that applied in detection of the electrochemical changes during a biorecognition event. These electrochemical detection techniques were categorized as amperometric, potentiometric, impedance, and conductometric (Mongra *et al.*, 2012). The electrochemical changes by a generation of measurable current are known as amperometric technique. A charge accumulation/measurable potential called as potentiometric technique, while the measurable alteration of the conductive properties of a medium between electrodes are referred as conductometric techniques. All these specific sensing techniques were discussed in following sub-sections (Grieshaber *et al.*, 2008) .

2.2.1 Amperometric

An amperometric-based biosensor is a type of electrochemical technique which measure current response generated from the redox reaction (oxidation and reduction process) of electroactive elements in a biochemical reaction. Amperometry is referred to the current generated at a constant potential while voltammetry is the current generated with the controlled variations of the potential (Grieshaber *et al.*, 2008). Typically, the peak value of the measured current at a linear potential range is directly proportional to the concentration of the testing solution (Grieshaber *et al.*, 2008). The signal output in the amperometric technique has resulted from the controlled potential of the working electrode at a constant value relative to a reference electrode (Terzi *et al.*, 2017). The applied potential acts as the driving force for the reaction of electron transfer while the current generated is a measurement of the electron transfer rate (Mongra *et al.*, 2012). Cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and differential pulse voltammetry (DPV) are the common resulting current signal obtained from the amperometric measurements.

Amperometric biosensors constitute an appealing alternative for the certain molecules that can be oxidized or reduced at the working electrode. Commonly, the working electrode used is the inert electrode which could be gold, platinum, carbon, and others. The working electrode driven to a positive potential is referred to the oxidation reaction while a negative potential is known as reduction reaction (Mongra *et al.*, 2012). Diffusion of the analyte solution to the working electrode led to the generation of the current flow. Thence, the current signal is reflected the concentration of the analyte solution diffusing towards the working electrode. The measurement of the current flow is aided by the counter/ auxiliary electrode. This

amperometric transduction signal is great potential used in various fields as this technique is highly sensitive, rapid, a possibility of real-time monitoring and economically (do Prado *et al.*, 2015).

In the most study, the biomolecule was immobilized on the working electrode, and the target analyte diffused towards the electrode surface which finally generated the current signal. The current signal was reflected in the reaction occurring between the biomolecule and the target analyte. Several studies following this direction had been applied based on the occurrence of electrochemical reactions on the electrode surface. For example, the work carried out by do Prado *et al.* (2015) had described an electrochemical biosensor based on the amperometric and differential pulse voltammetric for penicillin G detection. Cobalt phthalocyanine (CoPc) was acted as an electron mediator and penicillinase was immobilized on the working electrode surface. The interaction between the enzyme and CoPc induced the electrochemical reduction which the increment of the current reduction represents of substrate concentration in the cell. In another study by Švorc *et al.* (2012), an electrochemical sensor with an application of bare boron-doped diamond (BDD) as an electrode had been demonstrated in determination of the penicillin V. The electrode surface was used without any chemical modifications and electrochemical pretreatment. This simple, economic and practically used approach was able to quantify penicillin V in in pharmaceutical formulations (tablets) and human urine samples.

According to a study by Yilmaz *et al.* (2008), cyclic (CV) and differential pulse voltammetry (DPV) technique were applied to determine ascorbic acid in the tablet dosage form and some fruit juices. The electrochemical behavior of ascorbic

acid was conducted using a bare glassy carbon (GC) electrode at different pH range in various aqueous solutions. The experimental finding revealed a fast response of amperometric sensing which only require about 5 minutes with no sample preparation to the supporting electrolyte. With the same objective for determination of ascorbic acid, Pisoschi *et al.* (2011) conducted experiment through voltammetry sensing using platinum (Pt) and carbon paste electrodes. Its DPV results had demonstrated good results in food quality control.

However, there is also drawback for this amperometric-based biosensor. One of it was the interferences problem that driven from the electroactive compounds/bioreceptor molecule which contributed to a false current measurement (Mongra *et al.*, 2012). The problem in good reproducibility in electrochemical oxidation has also been confronted by unmodified electrodes (Pisoschi *et al.*, 2011). In fact, the electrode fouling due to the non-specific interaction between the bioreceptor molecule and the electrode surface had made the limitation of the usage of the amperometric technique (Wu *et al.*, 2012). However, these drawbacks have been solved by using electrodes coating/attaching polymers on electrodes. Recent development in the electrochemical approach has heightened to a renewed interest in the usage of electrodes coated/attachment with various polymers, as tabulated in Table 2.1, to overcome the above discussed limitations.

Table 2.1 Summary of different modified electrode/transducer

Electrode /transducer	Electrode	Detection technique	Sample	Reference
Reduced graphene oxide/iron nanoparticles nanocomposite glassy carbon modified electrode	Glassy carbon electrode (GCE)	Voltammetry	Hydrogen Peroxide	(Amanulla *et al.*, 2017)
Gold screen printed electrodes (SPE) surface with peptide nanotubes-encapsulating horseradish peroxidase	Gold Screen printed electrodes (SPE)	Voltammetry	Hydrogen Peroxide	(Feyzizarnagh *et al.*, 2016)
Carbon cloth electrode	carbon cloth electrode	Voltammetry	Riboflavin	(Yu *et al.*, 2017b)
Nafion®-coated titanium oxide (TiO_2)-carbon nanotube modified electrode	GCE	Voltammetry	Hydrazine	(Kim and Choi, 2015)
Multi-walled carbon nanotube (MWCNT)-modified electrode	GCE	Voltammetry	4-chloroaniline	(Montes *et al.*, 2016)
Multiwall carbon nano-tubes (MWCNTs)-screen-printed carbon electrode (SPCE).	SPCE	Voltammetry	Bromate (BrO_3^-)	(Lee *et al.*, 2017b)