# DEVELOPMENT OF WORKING ELECTRODE MODIFIED BY IRON OXIDE NANOPARTICLES FOR GLUCOSE BIOSENSOR APPLICATIONS

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by

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| | |
|---|---|
| AA | Ascorbic acid |
| Ag/AgCl | silver-silver chloride |
| Au | Gold |
| CA | Citric acid |
| $CeO_2$ | Cerium oxide |
| CE | Counter electrode |
| CNT | Carbon nanotubes |
| COOH | Carboxyl |
| CS | Chitosan |
| CuO | Copper oxide |
| CV | Cyclic voltammetry |
| DNA | Deoxyribonucleic acid |
| $E_{pa}$ | Anodic potential |
| $E_{pc}$ | Cathodic potential |
| FAD | Flavin adenine dinucleotide |
| $Fe_2O_3$ | Maghemite |
| $Fe_3O_4$ | Magnetite |
| FTO | Fluorine doped tin oxide |
| GCE | Glass carbon electrode |
| GDH | Glucose dehydrogenese |
| GOx | Glucose Oxidase |
| $H_2O_2$ | Hydrogen peroxide |

| | |
|---|---|
| HCl | Hydrochloric acid |
| HRP | Horseradish peroxidase |
| $I_{ch}$ | Background current |
| IDEs | Interdigitated electrode |
| IEP | Isoelectric point |
| IONPs | Iron oxide nanoparticles |
| $I_{pa}$ | Anodic current |
| $I_{pc}$ | Cathodic current |
| ISFETs | Ion-selective field-effect transistors |
| ITO | Indium tin oxide |
| LOD | Limit of detection |
| MGCE | Magnetic glass carbon electrode |
| MSPCE | Magnetic screen printed carbon elecctrode |
| MWCNT | Multiwall carbon nanotube |
| $N_2$ | Nitrogen |
| NAD | Nicotinamide adenine dinucleotide |
| NaOH | Sodium hydroxide |
| $NH_2$ | Amino |
| $NH_4OH$ | Ammonium hydroxide |
| OH | Hydroxyl |
| PANI | Polyaniline |
| PBS | Phospahte Buffer Saline |
| PEG | Polyethylene glycol |
| Ppy | Polypyrrole |

| | |
|---|---|
| PQQ | Pyrroloquinoline quinone |
| Pt | Platinum |
| PVA | Polyvinyl alcohol |
| PVP | Polyvinylpyrrolidone |
| RE | Reference electrode |
| rGO | Reduced graphene oxide |
| RSD | Relative standard deviation |
| SCE | Saturated calomel electrode |
| $SiO_2$ | Silica |
| Sn | Tin |
| $SnO_2$ | Tin Oxide |
| SPCE | Screen printed electrode |
| TEM | Transmission electron microscope |
| $TiO_2$ | Titania |
| UA | Uric acid |
| UV-Vis | UV-Visible |
| WE | Working electrode |
| XRD | X-ray diffractometer |
| ZnO | Zinc oxide |
| $ZrO_2$ | Zirconia |

## LIST OF SYMBOLS

| | |
|---|---|
| g/ml | Grams per milliliter |
| % | Percentage |
| $\Delta E_p$ | Potential different |
| µ | Micro |
| µA | Micro ampere |
| $\mu AmM^{-1}cm^{-2}$ | Microamperes per Centimetre Square per Millimolarity |
| µL | micro liter |
| $A_e$ | Electroactive surface area |
| cm | Centimeter |
| $cm^2$ | Centimeter square |
| D | Diffusion coefficient |
| F | Faraday constant |
| g | Gram |
| g/l | Grams per liter |
| g/mol | Gram per mole |
| $I^*$ | Surface concentration |
| K | Kevin |
| $K_m$ | Michaelis–Menten constant |
| L | Liter |
| mg/ml | Milligram per milliliter |
| min | Minute |

| | |
|---|---|
| ml | Mililiter |
| mM | Milimolar |
| n | No of electron |
| nm | Nanometer |
| º C | Degree Celsius |
| rpm | Revolutions per minute |
| s | second |
| T | Temperature |
| V | Voltage |
| $\nu$ | scan rate |
| V/s | Voltage per second |
| Vm | Maximum rate of reaction |
| σ | Standard deviation |

**FABRIKASI ELEKTROD TERMODIFIKASI DENGAN PARTIKEL NANO FERUM OKSIDA UNTUK APLIKASI BIOPENGESAN GLUKOS**

## ABSTRAK

Biopengesan glukos yang mempunyai ciri julat pengesanan yang besar, kepekaan yang tinggi dan tahap minima pengesanan yang rendah adalah ciri penting untuk alat ujian pengesan glukos. Ciri ini telah menjadi motivasi untuk menjalankan kajian pengesan glukus yang lebih baik. Dalam kajian ini, partikel nano ferum oksida (IONPs) yang stabil di dalam air telah dihasilkan menggunakan teknik pemendakan. Permukaan IONPs telah diubahsuai dengan mempelbagaikan kepekatan asid sitrik (CA) (0.10, 0.25, 0.50, and 0.70 g/ml). Teknik Pembelauan Sinar X (XRD) and Mikroskop Transmisi Elektron (TEM) telah digunakan untuk mengkaji kesan kepekatan asid sitrik ke atas susunan kristal dan morfologi IONPs yang terhasil. Hasil daripada ujian XRD, IONPs yang terhasil mempunyai susunan kristal yang baik dan padan dengan struktur kubik dan fasa *maghemite* ($\gamma$-$Fe_2O_3$) manakala hasil dari ujian TEM menunjukkan IONPs yang terhasil mempunyai saiz partikel 17-20 nm. Kepekatan CA yang optimum untuk menghasilkan IONPs yang stabil di dalam air dan mempunyai ciri elektrokimia yang baik ialah 0.25 g/ml. IONPs yang stabil telah digunakan untuk modifikasi elektrod berenzim dan elektrod tidak berenzim bagi ujian pengesan glukos menggunakan teknik penuangan bertitik. Untuk ubahsuai biopengesanan glukos berenzim, elektrod indium tin oxide (ITO) dan elektrod yang dicetak (screen printed carbon electrodes (SPCE)) telah diubahsuai dengan menggunakan IONPs–0.25 CA, enzim glukos oksida (GOx) dan lapisan *Nafion*. Untuk ubahsuai biopengesan glukos tidak berenzim, SPCE telah diubahsuai dengan menggunakan IONPs–0.25 CA dan lapisan *Nafion*. Kajian pembolehubah untuk fabrikasi elektrod berenzim dan elektrod

tidak berenzim bagi ujian pengesan glukos telah dijalankan antaranya adalah kesan kepekatan IONPs–0.25 CA, kesan kepekatan enzim GOx, kesan kuasa voltan, kesan pH elektrolit dan kesan suhu operasi. Ubahsuai elektrod berenzim dan elektrod tidak berenzim untuk ujian pengesan glukos yang dihasilkan dalam kajian ini mempunyai ciri yang baik dan mempunyai kepekaan yang tinggi. Kadar kepekaan elektrod yang terhasil adalah seperti berikut: Nafion/GOx/IONPs–0.25 CA/ITO (941 $\mu AmM^{-1}cm^{-2}$ dengan tahap minima pengesanan 0.10 µM), Nafion/GOx/IONPs–0.25 CA/SPCE (164 $\mu AmM^{-1}cm^{-2}$ dengan tahap minima pengesanan 14 µM), dan 5Nafion/IONPs–0.25 CA/SPCE (2802 $\mu AmM^{-1}cm^{-2}$ dengan tahap minima pengesanan 0.60 µM). Biopengesan glukos yang mempunyai ciri julat pengesanan yang besar, kepekaan yang tinggi dan tahap minima pengesanan yang rendah untuk ubahsuai elektrod berenzim dan elektrod tidak berenzim telah berjaya dihasilkan berdasarkan modifikasi elektrod dengan IONPs–0.25 CA.

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## ABSTRACT

Glucose biosensor that is capable to provide wide linearity of detection, high sensitivity, and low limit of detection is important in clinical diagnosis. This has motivated the research into development of a better glucose biosensor. In this study, iron oxide nanoparticles (IONPs) were synthesized using the precipitation method and surface functionalized with varying citric acid (CA) concentration (0.10, 0.25, 0.50, and 0.70 g/ml) to produce stable colloidal IONPs in water. The effect of varying CA concentration on the crystallinity and morphology, of the IONPs–CA in water were studied using X-ray diffraction (XRD) and transmission electron microscope (TEM). From the XRD patterns, high crystallinity of spinel cubic lattice of maghemite (γ-$Fe_2O_3$) was obtained, while observation using transmission electron microscopy (TEM) showed particle size was in the range of 17–22 nm. The optimum CA concentration to functionalize IONPs–CA forming stable colloidal IONPs in water and exhibited excellent electrochemical performance was 0.25 g/ml. The stable colloidal IONPs–0.25 CA in water was then applied for fabrication of enzymatic and non-enzymatic glucose biosensor by modification of working electrode using drop casting method. In enzymatic glucose biosensor, the indium tin oxide (ITO) electrode and screen printed carbon electrode (SPCE) were modified with IONPs–0.25 CA, glucose oxidase (GOx) enzymes and Nafion layer. As for non-enzymatic glucose biosensor, the SPCE electrode was modified with IONPs–0.25 CA and Nafion layer. The optimization parameters of enzymatic and non-enzymatic glucose biosensors performance were conducted, such as effect of IONPs concentration, effect of GOx

enzyme loading concentration, effect of working potential, effect of buffer solution pH and effect of operating temperature. The sensing performance of the developed enzymatic and non-enzymatic glucose biosensor exhibit excellent glucose detection performance with sensitivity of Nafion/GOx/IONPs–0.25 CA/ITO (941 $\mu AmM^{-1}cm^{-2}$ and limit of detection of 0.10 µM), Nafion/GOx/IONPs–0.25 CA/SPCE (164 $\mu AmM^{-1}cm^{-2}$ and limit of detection of 14 µM), and 5Nafion/IONPs–0.25 CA/SPCE (2802 $\mu AmM^{-1}cm^{-2}$ and limit of detection 0.60 µM). The wide linearity of detection, high sensitivity and low limit of detection of enzymatic and non-enzymatic glucose biosensors were successfully developed based on modification of working electrode with IONPs–0.25 C

# CHAPTER ONE

# INTRODUCTION

## 1.1 Research background

Diabetes mellitus has become the major health problem in most developed societies worldwide and has encouraged the development of glucose biosensors for monitoring the blood glucose level of diabetic patients. It is very important to observe the blood glucose level to prevent diabetes complications and other health issues. The normal range of glucose concentration in human serum before meal is around 4–6 mM (70–110 mg/dl) and after 2 hours of meal < 7.8 mM (< 140 mg/dl) (Güemes *et al.*, 2016, Nice, 2012). As for diabetic patients, the normal range of glucose concentration in human serum before meal is around 5.6–6.9 mM (100–125 mg/dl) and after 2 hours of meal 7.8–11 mM (140-199 mg/dl) (American Diabetes, 2010). Common ways of blood glucose monitoring involve finger pricking of blood using lancet and placed on the glucose test strip, then analysed using glucometer. Numerous commercial glucose test strips for monitoring blood glucose level are available in the market, somehow the difference lies on their performance (Dzyadevych *et al.*, 2008; Vashist *et al.*, 2011). Common commercial glucose test strips have linearity of detection in the range of 0.5–33 mM, required 0.3–2 µl of blood and 4–5 s of assay time. Glucose biosensor that is capable to provide fast and quantitative determination is important not only in the field of clinical chemistry, but also in industrial quality analysis and food analysis (Arduini *et al.*, 2016; Ozdemir *et al.*, 2012). Thus, the fabrication of glucose biosensor to

improve the performance of biosensor in terms of accuracy, response time, reliability and cost effectiveness are on demand.

The glucose biosensor based on electrochemical system has been widely applied owing to its excellent sensitivity, selectivity, and simplicity. The electrochemical glucose biosensor can further be classified into the measuring types which are potentiometric (measure the change in electrode potential), conductometric (measure the change in charge transfer resistance) and amperometric (measure the change in currents produced). Among all, the enzymatic electrochemical glucose biosensor based on amperometric measurement is the most commonly explored and commercially available (Yoo and Lee, 2010; Zaidi and Shin, 2016). Commercial glucose test strips normally involve the three electrodes system which are reference, counter and working electrodes. The reference electrode is a stable and well-known electrode potential that is usually made of silver-silver chloride Ag/AgCl electrode or saturated calomel electrode (SCE). The working electrode is known as a sensing electrode which serves as a transducer responding to the electrochemical reaction. The counter electrode functions to complete the current circuit in electrochemical cell where it provides a current connection between the electrocatalytic solutions and the working electrode. The counter electrode usually made of an inert material e.g. platinum (Pt), gold (Au), graphite, and glassy carbon (Grieshaber *et al.*, 2008; Shruthi *et al.*, 2014). Among these electrodes, sensitivity and specificity of detection are dependent on the working electrode.

There are two categories of glucose biosensor; the enzymatic and non-enzymatic. The enzymatic glucose biosensor based on immobilized GOx enzyme is

the most popular due to the high specificity and sensitivity of GOx enzyme can offer to the glucose biosensor (Witkowska Nery *et al.*, 2016). The immobilization of GOx enzyme on the working electrode surface is an important factor to be considered in biosensor fabrication. In GOx enzyme, the active group of Flavin adenine nucleotide (FAD) that will be responsible for the redox reaction for the GOx enzyme surrounded in thick layer protein. This has made the exchanging the electrons between GOx enzyme and electrode surface difficult. Besides, the GOx enzyme shape can shift slightly after being immobilized on the electrode surface (Bankar *et al.*, 2009; Peng *et al.*, 2013). Therefore, immobilization of GOx enzymes on the suitable matrix is important.

Recent development of glucose biosensor involves modifying the working electrode with nanomaterials, such as metals, metal oxides, and carbon materials (Eskandari *et al.*, 2015; Shi *et al.*, 2014; Xu *et al.*, 2014b). Nanomaterials play a role as a matrix to modify the electrode surface and prepare the environmental friendly area for enzyme immobilization because of nanomaterials properties that are large surface area for reaction activity, good catalytic efficiency, and strong adsorption ability (Saei *et al.*, 2013; Zhu *et al.*, 2012). Several metal oxides nanomaterial have been used in glucose detection such as zinc oxide (ZnO) (Atan *et al.*, 2014; Ma and Nakazato, 2014), iron oxide ($Fe_3O_4$) (Chen *et al.*, 2011b; Kaushik *et al.*, 2008), titanium oxide ($TiO_2$) (Haghighi *et al.*, 2017; Wang *et al.*, 2014; Yang *et al.*, 2015), copper oxide (CuO) (Huang *et al.*, 2013; Liu *et al.*, 2014; Xu *et al.*, 2014a), cerium oxide ($CeO_2$) (Patil *et al.*, 2012; Saha *et al.*, 2009), and zirconia ($ZrO_2$) (Cai *et al.*, 2012; Vilian *et al.*, 2014). Amongst all, electrode modification based on iron oxide nanoparticle (IONPs) attracts attention owing to properties of chemically inert, biocompatible,

strong superparamagnetic, easy to synthesize, and has high electric conductivity (Shi *et al.*, 2014; Teymourian *et al.*, 2013; Yuan *et al.*, 2016). The biocompatible properties promote strong absorption ability of the GOx enzymes on the electrodes and allow direct electron transfer between GOx enzymes and electrode. The superparamagnetic property is important during functionalization process because can separate the functionalized nanoparticles with unreacted precursor using a magnet.

However, IONPs that have large surface energy and strong magnetic attraction tend to agglomerate, thereby limiting their applications especially in biological environment. Electrostatic and steric stabilizations of IONPs in liquid carrier can be obtained by surface functionalization of the IONPs with inorganic, organic and polymeric materials (Abdul Amir Al-Mokaram *et al.*, 2016; Baby and Ramaprabhu, 2010; Baratella *et al.*, 2013; Eskandari *et al.*, 2015). Surface functionalization prepares the IONPs to obtain colloidal stability in suspension, biocompatibility and provides functional group as the attachment site for biomolecule. In glucose biosensor applications, the coating of IONPs using single inorganic coating material or combination of inorganic coating materials that is known as hybrid materials have been applied (Baby and Ramaprabhu, 2010; Chen *et al.*, 2011b; Li *et al.*, 2010). The inorganic materials coating of platinum (Pt), silica ($SiO_2$), silver (Ag), and gold (Au) not only offer stability to IONPs but also improve the catalytic properties, biocompatibility, and electrical conductivity of the functionalize IONPs.

Several natural polymers e.g. chitosan, dextran, starch, albumin, and liposomes (He *et al.*, 2016; Kaushik *et al.*, 2008) and several synthetic polymers e.g. polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), polypyrrole

(Ppy), and polyaniline (PANI) (Sanaeifar *et al.*, 2017; Yang *et al.*, 2014b) have been used recently to functionalize the IONPs for the applications in glucose biosensor. Polymer coating reduces the aggregation between the IONPs by providing steric stabilization and thus increases the surface-to-volume ratio for biomolecule immobilization. Moreover, a simpler functionalization technique using surfactant and small molecules such as citric acid, oleic acid, 3-aminopropyltriethyloxysilane (APTES), and mercaptopropyl triethoxysilane agents attracts attention from researchers (Bloemen *et al.*, 2012; Sharma *et al.*, 2015b). This functionalization able to introduce the functional group of amino ($-NH_2$), carboxyl (-COOH), hydroxyl (-OH), and silane, which offers good water dispersible properties to the IONPs and allows the IONPs for further modification with enzymes for the application in enzymatic glucose biosensor.

However, the dependent of enzymes activity with temperature, pH, humidity, and toxic chemicals have advanced the research in the glucose biosensor based on non-enzymatic system (Dhara *et al.*, 2015; Fu *et al.*, 2014; Tian *et al.*, 2014). The non-enzymatic glucose biosensor offers high sensitivity performance, good stability and ease of fabrication whereby the current response of the glucose biosensor depends directly on the oxidation of glucose on the modified electrode. The common limitation of the non-enzymatic glucose biosensor is in terms of specificity (Nouira *et al.*, 2013). Recently, scholars reported the high sensitivity non-enzymatic glucose biosensor based on modification of electrode with iron oxide nanostructured (Ahmad *et al.*, 2017; Chen *et al.*, 2017; Zhang *et al.*, 2015a).

## 1.2 Problem statement

In commercial glucose test strip, a mediator was applied to shuttle the electrons transfer between active redox center of the GOx enzyme to the surface of the working electrode. The problem of using mediator is the electrode tends to be unstable due to the leaching of the implemented mediator. Another problem of using mediator is the dependence of the electron transfer process on the capability of the mediator to be rapidly oxidized and reduced for electron transfer (Dominguez-Benetton *et al.*, 2013; Toghill *et al.*, 2010). Recently, glucose biosensor research was directed towards producing direct electron transfer in between GOx enzymes and electrode by modification of working electrode with nanomaterials (Ahmad *et al.*, 2017; Haghighi *et al.*, 2017; Pakapongpan and Poo-arporn, 2017).

As for types of electrode used in glucose biosensor, the demand lies on good sensitivity, good reproducibility and lower in cost types of electrode. Common works reported on modification of electrode for glucose biosensor using solid carbon electrode e.g. magnetic glass carbon electrode and carbon paste electrode (Baghayeri *et al.*, 2017; Comba *et al.*, 2010; Li *et al.*, 2010) and solid noble metal electrode e.g. Pt and Au (He *et al.*, 2016; Wan *et al.*, 2010). The limitation of using solid electrode was difficulty to transfer the development process into disposable electrode to suit the application for home blood glucose monitoring. Disposable electrode offers advantages for fast screening and on-site monitoring because of its low cost, high sample throughput, and easy integration into mass production processes (Cardosi and Liu, 2012; Yamaoka and Sode, 2007).

The screen printed carbon electrode (SPCE) features simplicity, rapidness, long shelf lifetime, cost efficiency, high mechanical robustness, and miniaturization potential (Arduini et al., 2016; Xin et al., 2013). As for ITO electrodes, the good electrical properties, wide electrochemical working window and high transparency to optical wavelengths have made ITO as the potential candidates for application in electrochemical or optical biosensors. In recent years, the use of IONPs with various types of functionalization or the combination of IONPs with other nanomaterials forms hybrid/composite materials applied for the modification of SPCE and ITO electrode in enzymatic glucose biosensor have attracted a lot of attention (Abbasi *et al.*, 2016; Pakapongpan and Poo-arporn, 2017; Zhang *et al.*, 2015b). The hybrid/composite nanomaterials function to improve the GOx enzyme immobilization and electron transfer behavior in glucose detection (Fu et al., 2014; Tian et al., 2013).

Samphao *et al*. (2015) developed mediated glucose biosensor with sensitivity of 2.52 $\mu AmM^{-1}cm^{-2}$ and limit of detection of 0.3 mM for linearity of 0.2–9.0 mM. The SCPE was modified with hybrid/composite nanomaterials of IONPs-Au, GOx enzyme and manganese oxide ($MnO_2$) as a mediator (IONPs-Au/GOx-modified SPCE($MnO_2$)). Pakapongpan and Poo-arporn (2017) developed high sensitivity (5.90 $\mu AmM^{-1}cm^{-2}$) and low limit of detection (0.1 μM) of glucose biosensor based on modification of magnetic screen printed carbon electrode (MSPCE) with IONPs-reduced graphene oxide (rGO) and GOx enzyme (GOx/rGO–IONPs/MSPCE). The combination of various types of nanomaterials known as hybrid/composite resulted in the enhancement of the electrode analytical performance for glucose detection. However, the fabrication of the hybrid/composite based glucose biosensor is

complicated, requires multiple step, various types of nanoparticles preparation and functionalization and also numerous parameters to be controlled.

There are limited works have been performed for the modification of disposable electrode (SPCE and ITO) using IONPs only without hybrid/composite. The main hindrance is in controlling the morphology and distribution of IONPs on the electrode to ensure consistency of glucose biosensor performance. This happen because of the agglomeration in between IONPs. Therefore, formation of stable dispersion of IONPs in liquid carrier is important to control the morphology of IONPs on the electrodes and ensure effective GOx enzyme immobilization on the IONPs matrix (Baratella *et al.*, 2013). Scholars reported on many types of surface functionalization of IONPs using organic, inorganic, and biopolymeric materials (Samphao et al., 2015; Sanaeifar et al., 2017; Wang et al., 2012).

Organic polymers such as chitosan, polyethylene glycol, polyaniline (PANI), polyvinyl alcohol (PVA) and polypyrrole (PPy) were applied to stabilize the IONPs and improved the immobilization of GOx enzyme in fabrication of glucose biosensors. Yang *et al.* (2009) developed high sensitivity (11.54 $\mu A cm^{-2} mM^{-1}$), low detection limit (6 µM) glucose biosensor based on Nafion/Chitosan–IONPs–GOx/Pt for linearity of 6 µM–2.2 mM. IONPs was functionalized with chitosan to provide stability and functional group on the IONPs surface. The limitation of using chitosan is in their low solubility in aqueous and alkaline solution. Sanaeifar *et al.* (2017) reported self-assembly of GOx–PVA–IONPs on the tin (Sn) electrode for glucose biosensor applications in which GOx enzyme was physically absorbed on the PVA–IONPs then drop-casted on the Sn electrode. PVA provided biocompatible environment for GOx

enzymes immobilization thus promoted excellent electron transfer between GOx enzymes and electrode. The GOx–PVA–IONPs/Sn electrode had high sensitivity of 9.36 $\mu Acm^{-2}mM^{-1}$, limit of detection of 8 µM for wide linearity of 5 µM–30 mM. The limitation of using PVA and PEG polymer is low electrical conductivity. As for conducting polymer such as PANI and PPy, the limitations are in their complicated synthesis process and limited solubility (Kausaite-Minkstimiene *et al.*, 2011; Lai *et al.*, 2016).

The challenge of using long polymer chain and large surfactant molecule to functionalize IONPs is the loss of some binding affinity through steric hindrances. This challenge can be overcome by utilizing small molecules, such as citric acid (CA). CA is a small molecule that has three carboxyl groups and one hydroxyl groups. The carboxyl groups chemisorb to the Fe–OH molecules presence on the IONPs surface by forming a carboxylate group. This process exposes one or two negatively charged carboxyl groups that can prevent agglomeration, making the nanomaterials hydrophilic, and introduces functional groups as attachment sites for biomolecules (Cheraghipour, 2012; Srivastava *et al.*, 2011).

In recent years, few scholars have explored the stable colloidal IONPs citrate functionalized for sensor applications (Sharma *et al.*, 2015a; Wu *et al.*, 2017). Sharma *et al.* (2015b) fabricated excellent immunosensors for pathogen detection (Vibrio cholerae) by modifying ITO electrodes with IONPs–CA. IONPs–CA aid in increasing electron transfer rate and prepare biocompatible environment for biomolecule attachment by having a large number of carboxyl (–COOH) functional group. Wu *et al.* (2017) reported the excellent peroxidase-like activity of a metal organic framework

in coating the IONPs (IONPs@MIL-100(Fe)) for colorimetric sensors to detect hydrogen peroxide ($H_2O_2$) and cholesterol. The IONPs were synthesized via solvothermal method and functionalized with CA before further being coated with the metal organic framework. However, there is still no work reported on using stable colloidal IONPs–CA in modification of electrodes for glucose biosensor. Moreover, further study on the optimum CA concentration to functionalize IONPs–CA is needed in order to obtain excellent glucose detection performance.

The limitation of enzymatic glucose biosensor lies on the stability issue because enzymes activity is dependent on temperature, pH, and humidity (Abdul Amir Al-Mokaram *et al.*, 2016; Tian *et al.*, 2014). To overcome this problem, the non-enzymatic glucose biosensor has been investigated by many researchers (Dhara *et al.*, 2015; Shi *et al.*, 2016; Wang *et al.*, 2017). The non-enzymatic glucose biosensor based on iron oxide offer advantages of wide linearity, long-term stability, high sensitivity and the ability to be applied for continuous glucose monitoring. The reason is due to IONPs offers an enzyme-mimetic electrocatalytic activity similar to that found in natural peroxidases. Therefore, IONPs will be able to offer similar benefits of noble metal or enzymes in glucose biosensor applications (Guivar *et al.*, 2015).

Recently, scholars reported on modification of electrode for non-enzymatic glucose biosensor mostly using 1-dimensional iron oxide nanostructured such as nanorods, nanotubes and nanowires (Cao and Wang, 2011; Umar *et al.*, 2014; Zhang *et al.*, 2015a). This is because larger surface area offers by 1-dimensional iron oxide nanostructured and less aggregation/agglomeration in between iron oxide nanostructured. Zhang *et al*. (2015) reported the non-enzymatic glucose biosensor

based on iron oxide nanorod arrays (IONRs-Array/foil) prepared by electrochemical anodization of iron foil, followed by *in situ* annealing under hydrogen flow. The high sensitivity of the IONRs-Array/foil glucose biosensor was developed with linearity of 0.5–3.7 mM and detection limit of 0.1 μM. Chen *et al*. (2017) reported recently on the non-enzymatic glucose biosensor based on iron oxide nanotubes developed on the fluorine doped tin oxide (FTO) electrode (IONTs-Array/FTO) which displayed sensitivity of 673.3 $\mu Acm^{-2}mM^{-1}$, 71.2 $\mu Acm^{-2}mM^{-1}$, and 9.58 $\mu Acm^{-2}mM^{-1}$for glucose linear range of 0.1 μM–0.125 mM, 0.125–1.0 mM, and 1.0–5.0 mM, respectively. Both of reported works on IONRs-Array/foil and IONTs-Array/FTO for non-enzymatic glucose biosensor have limitation in terms of small range of glucose linearity and suffer small interference from ascorbic acid.

The drawbacks of the application of 1-dimensional iron oxide nanostructured in electrode modification is in the synthesis process of 1-dimensional iron oxide nanostructured which are complicated and required high temperature. In order to overcome the problems of non-enzymatic glucose biosensor, which are lacked of glucose biosensor selectivity and limited number of systems that are applicable to physiological pH. Therefore, in this work, the non-enzymatic glucose biosensor based on IONPs–CA and Nafion layer protected were developed. Introducing Nafion layer on the non-enzymatic glucose biosensor was believed able to prevent interference and improve sensitivity.

## 1.3 Objectives:

i. To determine the effect of CA concentration for functionalization of IONPs colloidal solution for glucose biosensor
ii. To evaluate the applicability and performance of IONPs–CA in glucose biosensor
iii. To optimize conditions for fabrication of SPCE and ITO working electrodes modification for glucose biosensor
iv. To evaluate the effect of enzymatic and non-enzymatic glucose biosensors based on IONPs–CA

## 1.4 Scopes of study

In this work, IONPs were prepared using the precipitation method and functionalized with CA. The optimization of CA concentration to functionalize IONPs and forming stable colloidal IONPs in water was studied. The crystallinity and morphology of the IONPs–CA in water were observed using X-ray Diffractometer (XRD) and Transmission Electron Microscope (TEM). The stable colloidal IONPs–CA in water was then applied for fabrication of glucose biosensor.

Two types of electrodes were used to develop glucose biosensor, which were ITO glass electrode and SPCE. The electrochemical enzymatic and non-enzymatic glucose biosensors were developed using the drop casting method. As for the enzymatic glucose biosensor, the SPCE and ITO electrodes were modified with IONPs–CA, GOx enzymes and Nafion layer. For the non-enzymatic glucose

biosensor, the SPCE electrode was modified with IONPs–CA and Nafion layer. The optimization of fabrication for enzymatic and non-enzymatic glucose biosensor was conducted, such as the effect of IONPs concentration, effect of enzyme loading concentration, effect of operating potential, effect of buffer solution pH and effect of operating temperature. The electrochemical characterization was performed using a portable Bipotentiostat/Galvanostat µSTAT 400 (DropSens, Asturias, Spain). Cyclic voltammetry (CV) testing was conducted to observe the electrochemical and electrocatalytic performance. The sensitivity of the glucose biosensor was tested in amperometric analytical method by adding glucose standard solution into buffer solution under constant stirring. The current-time (i–t curve) method was used for quantitative analysis of glucose.

## 1.5 Thesis outline

This thesis is organized into five chapters. In Chapter 1, the introduction, motivation of study and objectives are stated. In Chapter 2, the literature review of types, mechanism and performance of glucose biosensor were discussed. Research methodology, parameters conducted and the characterization are presented in Chapter 3. While in Chapter 4, results and discussion are presented. The conclusions and recommendation for future works are described in Chapter 5.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Biosensor

Biosensor is a self-contained integrated device that capable to provide specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) that is in direct contact with a transducer element as proposed by International Union of Pure and Applied Chemistry (IUPAC). Clark and Lyons (1962), who are the fathers of the biosensor concept were the first to perform a research on glucose quantification through glucose oxidase (GOx) entrapment with a dialysis membrane on the oxygen electrode surface. Glucose amount was estimated based on the reduction in dissolved oxygen concentration (Turner, 2013). Since then, studies on glucose biosensors have been investigated using amperometric, potentiometric, impedimetric, or conductometric glucose biosensors based on GOx, which catalyzes the oxidation of glucose into gluconic acid (Baghayeri *et al.*, 2017; Khun *et al.*, 2012; Nouira *et al.*, 2013; Xu *et al.*, 2014b).

Biosensors often consist of three-elements system; a bioreceptor, a transducer, and a signal-processing unit as shown in Figure 2.1. A bioreceptor is a biomolecule that specifically recognize the target analyte and translates the information from the biochemical domain, usually an analyte concentration, into a chemical or physical output signal (Kissinger, 2005). A sensitive biological element such as enzyme, deoxyribonucleic acid (DNA) probe or antibody is immobilized on the bioreceptor in

order to recognize the analyte (e.g. enzyme substrate, complementary DNA and antigen).

A transducer part of the sensor functions to convert the biochemical signal into an electronic signal resulting from the interaction of the analyte with the bioreceptor. The intensity of generated signal is directly or inversely proportional to the analyte concentration. In most develop biosensors, electrochemical transducers are commonly used. The transducer part of a sensor can also be called a detector, sensor or electrode.

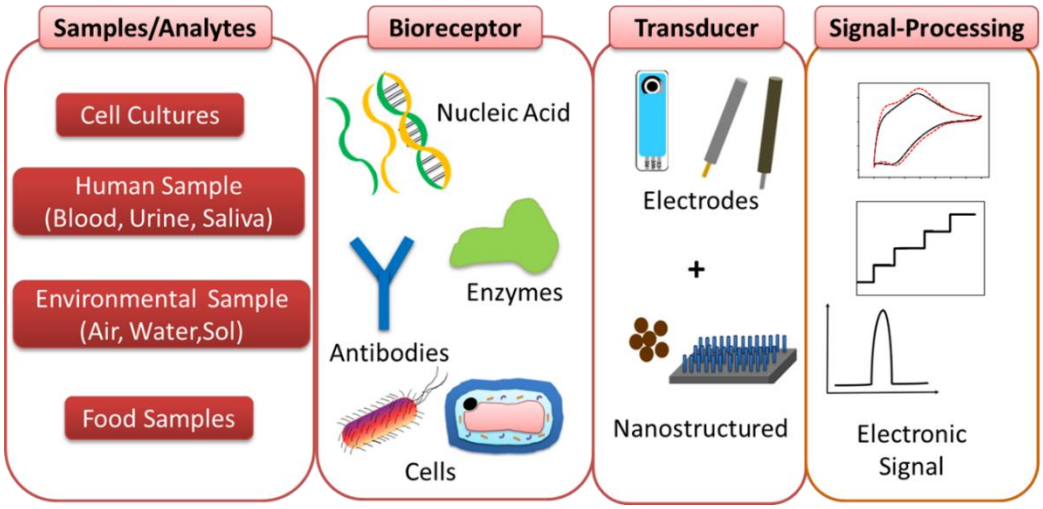


Figure 2.1: Elements and selected components in a typical biosensor (Kafashan and Azadshahraki, 2016)

### 2.1.1 Classification of biosensor

Biosensors can be classified into two types; based on the transduction mechanism (Transducer) or based on the biorecognization elements (Bioreceptor). The classification is shown in the Figure 2.2 (Goode *et al.*, 2014). In optical transduction method, the physical or chemical change produces by the biorecognition process induces a change in the phase, amplitude, polarization, or frequency of the input light.

Biosensors can be based on fluorescence, chemiluminescence, bioluminescence and surface plasmon resonance optical diffraction (Dey and Goswami, 2011). In electrochemical transduction, chemical reactions between immobilized biomolecule and target analyte can either produce or consume electrons thus determine a measurable electrical properties of the solution, such as an electric current or potential (Grieshaber *et al.*, 2008).

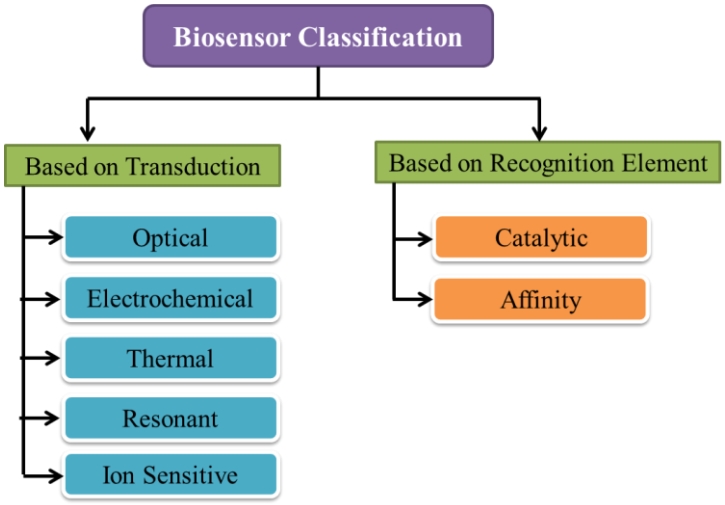


Figure 2.2: Classification of biosensors (Goode *et al.*, 2014)

Thermal transduction measures heat produces from the biological interaction, which in turn changes the temperature. The measurement is performed by thermistor known as 'enzyme thermistor'. Thermal transduction is commonly used for the detection of pesticides and pathogenic bacteria. Biosensors based on ion-selective field-effect transistors (ISFETs) is earlier considered as a category of potentiometric sensor. The surface electrical potential changes due to the interaction between ions and the semiconductor. This change in the potential can be subsequently measured (Monošík *et al.*, 2012).

Biosensors can also be classified based on the biorecognition elements which are catalytic biosensors and affinity biosensors. In catalytic biosensors, enzymes function as a bioreceptor to recognize and catalyst the analyte conversion into detectable signal such as current, potential or resistance (Grieshaber *et al.*, 2008). The reaction process of the biocatalysis can be monitored by measuring the formation rate of a product, the disappearance of a reactant, or the inhibition of the reaction (Goode *et al.*, 2014; Patel *et al.*, 2010). In affinity biosensors, the analyte binds with the biological material presence on the biosensor e.g., antibodies, receptors, DNA and nucleic acids. Thus, affinity biosensors are based on affinity interactions by separating an individual or selected range of components from complex mixtures of biomolecules. There are vast potential application areas for affinity-based biosensor techniques such as in clinical/diagnostics, food processing, military/antiterrorism, and environmental monitoring (Pandey *et al.*, 2017; Rogers, 2000; Saber-Tehrani *et al.*, 2013).

## 2.2 Glucose biosensor

Glucose biosensor that is capable to provide fast and quantitative determination is important in the field of clinical chemistry, and food analysis (Arduini *et al.*, 2016; Ozdemir *et al.*, 2012). Glucose biosensors based on commercial strips for self-monitoring of blood glucose have long been developed. Until now, three generations of glucose biosensors using (i) natural oxygen co-substrate generation and detection of hydrogen peroxide, (ii) synthetic electron mediators, and (iii) direct electron transfer between enzymes and the electrode, have been reported. The detection signal of glucose biosensors comes directly from glucose or by promoting conversion of glucose into other determinable electroactive species. So far, both enzymatic and non-

enzymatic glucose biosensors have been employed for determination of glucose. Over the past years, glucose biosensors have principally evolved as in Table 2.1.

Table 2.1: History of glucose biosensors (Yoo and Lee, 2010)

| Year | Event | Generation of Glucose Biosensor |
|---|---|---|
| 1962 | Introduction of a biosensor concept by Clark and Lyons | First generation |
| 1967 | Launch of first practical enzymatic electrode by Updike and Hicks | First generation |
| 1973 | Development of enzymatic glucose electrode based on hydrogen peroxide detection | First generation |
| 1975 | Introducing of the first commercial biosensor, i.e., YSI analyzer | First generation |
| 1976 | Launching of bedside-type artificial endocrine pancreas (Miles) | First generation |
| 1982 | Launching needle-type enzymatic electrode for hypodermic implantation by Shichiri | First generation |
| 1984 | Development of amperometric glucose biosensor based on mediator (ferrocene) | Second generation |
| 1987 | Launching of home personal blood glucose meter (MediSense ExacTech) | Second generation |
| 1999 | Launching of a commercial *in vivo* glucose biosensor (MiniMed) | Third generation |
| 2000 | Launching of a wearable non-invasive glucose monitoring (GlucoWatch) | Third generation |

## 2.3 Component of glucose biosensor

### 2.3.1 Glucose

Glucose with the molecular formula of $C_6H_{12}O_6$ is a monosaccharide containing six carbon atoms and an aldehyde group, which is referred to as an aldohexose (Joseph, 1998). The naturally occurring form of glucose is D-glucose, which is also known as dextrose. The intramolecular reaction between the alcohol group and the aldehyde group of glucose molecule forms an intramolecular hemiacetal (Figure 2.3 (a)). The glucose molecule can exist in an open-chain (acyclic) and ring

(cyclic) form, as a result of an intramolecular reaction. Figure 2.3 (b) shows the linear form of D-glucose undergoes an intramolecular reaction to form a cyclic hemiacetal. In solid form, glucose usually presence as a monohydrate with a closed pyran ring (dextrose hydrate). In aqueous solution, D-glucose has an open-chain to a small extent and is present predominantly as α- or β-glucopyranose, which commonly merge by mutarotation as shown in Figure 2.3 (b). In body, glucose is one of preferred sources of energy in the form of carbohydrates. Our body processes glucose multiple times a day. Glucose is obtained by enzymes breakdown process with help from pancreas. Glucose is also called blood sugar as it circulates in the blood at a concentration of 65-110 mg/dL (4-6 mM) of blood.

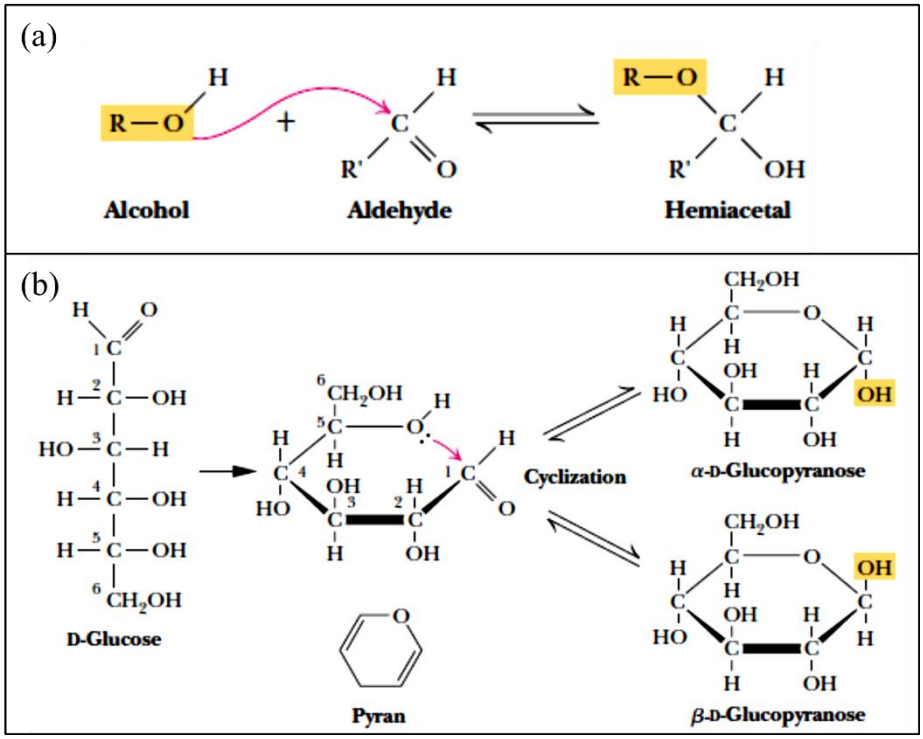


Figure 2.3: (a) Intramolecular reaction between alchohol and aldehyde group to form an intramolecular hemiacetal, (b) The linear form of D-glucose undergoes an intramolecular reaction to form a cyclic hemiacetal (Garrett and Grisham, 2010)

### 2.3.2 Enzymes

Two types of enzymes commonly use in glucose biosensor as the catalyst for the glucose redox reaction are glucose oxidase (GOx) and glucose dehydrogenese (GDH). These enzymes differ in redox potential, cofactors, and selectivity for D-glucose (Narla *et al.*, 2016). GOx is a homodimer comprises of two identical subunits and two non-covalently bound flavin adenine dinucleotides (FAD) as shown in Figure 2.4 (Ferri *et al.*, 2011). The FAD is a redox cofactor (coenzyme) that bound deep inside the enzyme, which utilizes oxygen as the external electron acceptor, liberating hydrogen peroxide ($H_2O_2$) and acts as an electron carrier during catalysis (Wilson and Turner, 1992). GDH is a monomer comprises of two domains, which is a central nucleotide as the binding domain and flanked by the catalytic domain. The GDH may contain one of three cofactors: pyrroloquinoline quinone (PQQ), nicotinamide adenine dinucleotide (NAD), and flavin adenine dinucleotide (FAD) (John *et al.*, 1994).

In glucose biosensor application, GOx enzyme offers advantages of low in cost and good stability, but suffer limitation of dependency to the oxygen concentration presence in the measuring media (Raba and Mottola, 1995). Alternatively, the GDH enzyme does not utilize oxygen as the electron acceptor and instead transfer electrons to various natural and artificial electron acceptors. However, the GDH enzyme limitations depend on each of their cofactor. FAD-GDH is costly and requires complex preparation process, while PQQ-GDH has poor selectivity due to susceptible interference from variety of saccharides. The NAD-GDH exhibits higher selectivity and stability, but limitations in finding a match with mediator properties.

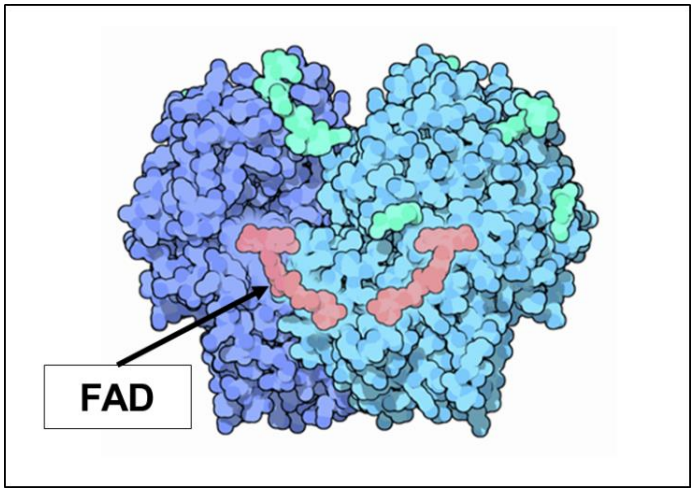


Figure 2.4: GOx enzyme with its two subunits depicted as dark and light blue, while the FAD coenzyme is depicted as pink (Goodsell, 2006).

### 2.3.3 Nafion

Nafion is a copolymer of perfluorinated polymer chain contains Teflon backbone and a sulfonic acid (-$SO_3H$) side chain. In glucose biosensor application, Nafion layer offers semi permeable membrane that has high resistivity to chemical attack, highly conductive to cation, high thermal stability, highly permeable to water and avoid interference from oxidizable species such as ascorbic acid and uric acid (Ensafi *et al.*, 2013; Lee *et al.*, 2007). Choi *et al*., (2011) reported that Nafion film deposited on the glucose biosensor electrode was able to accelerate the electron transfer and minimized the interference effect from ascorbic acid and uric acid. In addition, Ahmad *et al.*, (2012) claimed that Nafion film on the glucose biosensor electrode acted as a protective membrane against degradation and provided a biocompatible environment to enzyme in enzymatic glucose biosensor.

## 2.4 Generations of enzymatic glucose biosensor

### 2.4.1 First generation glucose biosensor

First generation glucose biosensors measures glucose concentration in the sample based on $H_2O_2$ generation or by decreases in oxygen ($O_2$) concentration as a natural co-substrate. The immobilize GOx catalyzes the oxidation of D-glucose ($C_6H_{12}O_6$) into gluconolactone ($C_6H_{10}O_6$) utilizing molecular $O_2$ as an electron acceptor, producing $H_2O_2$ and water as by-product. The gluconolactone ($C_6H_{10}O_6$) further hydralizes and forms gluconic acid ($C_6H_{12}O_7$) (Figure 2.5) (Pluschkell *et al.*, 1996; Taguchi *et al.*, 2014).

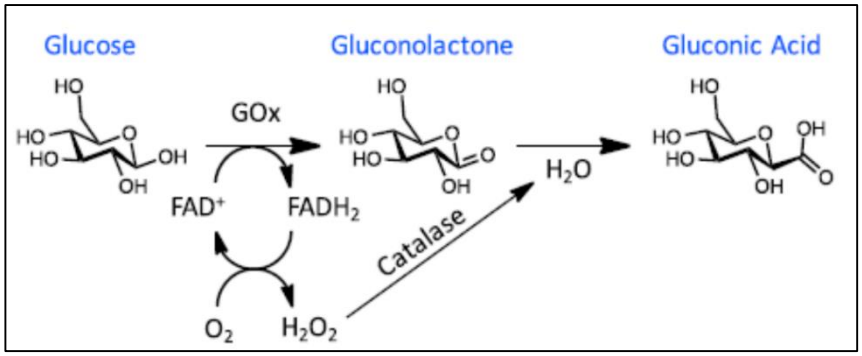


Figure 2.5: Schematic of GOx enzyme catalyst oxidation of D-glucose to gluconic acid (Bruen *et al.*, 2017)

In order to work as a catalyst, GOx requires a redox co-factor flavin adenine dinucleotide (FAD) that is called active redox centre. FAD plays a role as the initial electron acceptor and is reduced to $FADH_2$ with the presence of glucose (equation 2.1). The re-oxidation of the $FADH_2$ with free oxygen generates the oxidize form of the enzyme FAD (equation 2.2). Basically, the glucose concentration is directly proportional to electrochemical oxidation of the produce $H_2O_2$ (equation 2.3) or electrochemical reduction of $O_2$ (equation 2.4) at the working electrode (Vaddiraju *et al.*, 2010):

$$GOx\ (FAD) + Glucose \rightarrow GOx\ (FADH_2) + Gluconolactone \quad (2.1)$$

$$GOx\ (FADH_2) + O_2 \rightarrow GOx(FAD) + H_2O_2 \quad (2.2)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (2.3)$$

$$2H^+ + O_2 + 2e^- \rightarrow H_2O_2 \quad (2.4)$$

The counter electrode will recognize and collect the electrons transferred, thus the number of glucose molecules presence will be directly proportional to the number of electrons transferred. The advantages of glucose biosensor based on $H_2O_2$ measurements are in miniaturization of the biosensor and a simple design of the devices (Harper and Anderson, 2010; Rahman *et al.*, 2010; Zhu *et al.*, 2012).

The main drawback of the first-generation glucose biosensors is the high operation potential required for amperometric measurement of $H_2O_2$. This high operation potential leads to interference with other various reducing species (electroactive interference), such as ascorbic acid and uric acids and some drugs, like acetaminophen (Yoo and Lee, 2010). Another drawback is oxygen deficit which occurs due to the limited oxygen solubility in biological fluids that produces fluctuation in the oxygen tension (Wang, 2002). The oxygen deficit is then affected the change in sensor response and narrowing the linearity of glucose detection of the biosensor.

Numbers of approaches have been implemented to overcome the drawbacks of first generation glucose biosensor which are interference from electroactive molecules and oxygen deficit. Electrodes were modified using Nafion, polyurethane, polycarbonate or acetate layer as the selective or protective membrane to minimize the interference towards the electrode (Gough *et al.*, 1985; Murphy, 1998). Electrodes also

were modified through co-deposition with metallized material such as ruthenium and rhodium in order to reduce the operation potentials to around 0–0.2 V vs. Ag/AgCl, which was optimum to prevent the interference electroactivity (Wang *et al.*, 1994; Wang and Wu, 1995). Another approach involved the use of oxygen-rich carbon paste enzyme electrodes, that became the internal source of oxygen due to high oxygen solubility (Wang and Lu, 1998).

### 2.4.2 Second generation glucose biosensor

Second generation glucose biosensor involves the artificial redox mediators in replacing the oxygen dependence electrode. GOx unable to transfer electrons directly to electrodes because the FAD active redox centre presents deeply inside a thick protein layer (Harper and Anderson, 2010). Therefore, redox mediator's help to shuttle the electron transfer in between redox centre of the enzyme to the surface of the electrode. This will then reduce the operating potential of the biosensors at the moderate value of redox potentials, thus able to avoid oxidation of other interfering species (Wang *et al.*, 1994). The equations list below show the role of mediators in facilitating electron transfer (Abasıyanık and Şenel, 2010):

$$\text{Glucose} + \text{GOx(FAD)} \rightarrow \text{Gluconolactone} + \text{GOx}(FADH_2) \quad (2.5)$$

$$\text{GOx}(FADH_2) + 2M_{(ox)} \rightarrow \text{GOx(FAD)} + 2M_{(red)} + 2H^+ \quad (2.6)$$

$$2M_{(red)} \rightarrow 2M_{(ox)} + 2e^- \quad (2.7)$$

where, $M_{(red)}$ and $M_{(ox)}$ represents the reduced and oxidized forms of synthetic mediator. Glucose solution diffuses into the active sites of the enzyme and transforms into gluconolactone (equation 2.5). During the glucose conversion, the electrons