FUNCTIONALIZATION OF ZINC OXIDE NANORODS FOR GLUCOSE BIOSENSOR APPLICATION

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FUNCTIONALIZATION OF ZINC OXIDE NANORODS FOR GLUCOSE BIOSENSOR APPLICATION

by

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
APS	Aminopropylmethyldiethoxysilane
APTES	Aminopropyltriethoxysilane
AuNPs	Gold nanoparticles
CE	Counter electrode
CV	Cyclic voltammetry
DET	Direct electron transfer
EDX	Energy Dispersive X-ray Spectroscopy
FAD	Flavin adenine dinucleotide
$FADH_2$	Reduced form of FAD
FESEM	Field Emission Scanning Microscope
FWHM	Full width at half maximum
GCE	Glass carbon electrode
GOx	Glucose oxidase
HMT	Hexamethylene-tetramine
IEP	Isoelectric point
IHOAM	Incipient hydrous oxide adatom mediator
IPA	Isopropyl alcohol
ITO	Indium tin oxide
IUPAC	International Union of Pure and Applied Chemistry
LOD	Limit of detection
MNPs	Metal nanoparticles
mPMs	Methoxy phenazine methsulfate

-OH	Hydroxyl
PBS	Phosphate buffer solution
PEI	Polyethyleneimine
PET	Polyethyleneterephthalate
PQ	Phenanthroline quinone
PTFE	Poly(tetrafluoroethylene)
PtNDs	Platinum nanodendrites
RE	Reference electrode
RSD	Relative standard deviation
TEM	Transmission Electron Microscope
UA	Uric acid
WE	Working electrode
XRD	X-Ray Diffraction

LIST OF SYMBOLS

2θ	Diffraction angle
A_e	Effective surface area
D	Diffusion coefficient
F	Faraday constant
K_m	Michaelis-Menten constant
rpm	Rotations per minute
T	Temperature
V	Voltage
Γ^*	Surface amount of electroactive enzyme

ROD NANO ZINK OKSIDA DIFUNGSIKAN UNTUK APLIKASI PENGESAN GLUKOSA

ABSTRAK

Pengesan glukosa berasaskan kaedah elektrokimia mendapat perhatian kerana kesederhanaannya, mudah alih, kos rendah dan tidak memerlukan pengendali khusus. Walau bagaimanapun, jenis pengesan ini mengalami sensitiviti yang rendah disebabkan pemindahan elektron tidak langsung dan penurunan dalam kestabilan jangka panjang disebabkan denaturasi enzim. Untuk mengatasai masalah ini, pengubahsuaian elektrod untuk pengesan glukosa berasaskan kaedah elektrokimia adalah perlu. Elektrod nanostruktur dapat meningkatkan prestasi pengesan glukosa kerana kawasan permukaan yang tinggi dan biokompatibiliti dengan enzim glukosa oksida (GOx). Dalam kajian ini, rod nano zink oksida (ZnO NRs) telah berjaya disintesis menggunakan kaedah hidrotermal pada substrat benih indium tin oksida (ITO) yang kemudiannya digunakan sebagai elektrod untuk pengesan glukosa. Mikroskop Imbasan Elektron (FESEM) dan Teknik Pembelauan Sinar X (XRD) digunakan untuk menganalisa sifat morfologi dan struktur ZnO NR yang disintesis. ZnO NRs dengan ketumpatan homogen dan sejajar yang baik diperolehi apabila benih filem telah pada suhu 500 °C dan hidroterma selama 4 jam. Ciri-ciri elektrokimia elektrod yang telah diubahsuai dianalisis menggunakan voltammetrik kitaran (CV) dan analisa amperometrik. Parameter yang mempengaruhi aktiviti enzim dan prestasi elektrod yang diubah suai telah dikaji: pH elektrolit, suhu elektrolit, kepekatan Nafion dan kepekatan enzim GOx. Elektrod yang telah diubahsuai ditetapkan sebagai Nafion/GOx/ZnONRs/ZnO/ITO. GOx dengan

kepekatan 5 mg/mL telah dipilih sebagai kepekatan optimum untuk immobilisasi pada elektrod ZnO NRs dengan sensitiviti yang tinggi iaitu 23.772 μA/mM.cm^2. Prestasi elektrod apabila dua teknik immobilisasi yang berbeza (penjerapan fizikal dan penyambungan silang) digunakan dan dibandingkan. Permukaan ZnO NRs diubahsuai dengan glutaraldehid (penyambungan silang reagen) terlebih dahulu sebelum GOx diimmobilisasi dan ditetapkan sebagai elektrod Nafion/GOx-glutaraldehyde/ZnONRs/ZnO/ITO. Penyambungan silang elektrod Nafion/GOx-glutaraldehyde/ZnONRs/ZnO/ITO menunjukkan prestasi terbaik dengan sensitiviti 32.24 berbanding hanya 23.77 μA /mM.cm2 untuk elektrod tanpa penyambung silang. Elektrod ZnONRs/ITO juga dihiasi dengan nanopartikel emas (AuNPs) dan nanodendrit platinum (PtNDs). AuNPs dengan diameter ~40 nm dan PtNDs dengan diameter telah berjaya dihiasi pada ZnO NRs melalui kaedah titisan. Sensitiviti bagi Nafion/GOx/ZnONRs/ZnO/ITO, Nafion/GOx/AuNPs/ZnONRs/ZnO/ITO dan Nafion/GOx/PtNDs/ZnONRs/ZnO/ITO elektrod masing-masing ialah 32.24, 54.51 and 98.34 μA/mM.cm^2. Sensitiviti yang tinggi bagi Nafion/GOx/PtNDs/ZnONRs/ZnO/ITO elektrod adalah disebabkan oleh sifat pemangkin logam nanopartikel. Dengan sensitiviti yang tinggi 98.34 μA/mM.cm^2 dan LOD yang rendah 0.03 mM bagi Nafion/GOx/PtNDs/ZnONRs/ZnO/ITO elektrod telah dipilih sebagai elektrod terbaik bagi pengesan glukosa. Elektrod yang telah dihasilkan dan diubahsuai menunjukkan prestasi cemerlang ke atas sampel darah manusia.

FUNCTIONALIZATION OF ZINC OXIDE NANORODS FOR GLUCOSE BIOSENSOR APPLICATION

ABSTRACT

Electrochemical based glucose biosensors are of interest owing to its simplicity, portable, low cost and does not require specialize personnel. However, this type of biosensors suffers from low sensitivity owing to indirect electron transfer and decrease in long term stability due to enzyme denaturation. Therefore, modification of electrode for electrochemical based glucose biosensors could overcome these problems. Nanostructure electrodes could enhance the performance of glucose biosensors owing to high surface area and biocompatibility with glucose oxidase (GOx) enzyme. In this work, zinc oxide nanorods (ZnO NRs) was successfully synthesized by hydrothermal method on indium tin oxide (ITO) seeded substrates which was further used as electrodes for glucose biosensor. Field emission scanning electron microscope (FESEM) and X-ray diffractometer (XRD) were used to analyze the morphology and structural properties of synthesized ZnO NRs. Homogeneous density and well-aligned of ZnO NRs was obtained when seed films were annealed at 500 °C and hydrothermally grown for 4 hours. The electrochemical properties of modified electrodes were studied by cyclic voltammetry (CV) and amperometric analysis. The parameters influencing the enzyme activity and modified electrodes performance were studied: electrolyte pH, electrolyte temperature, Nafion concentration and GOx enzyme concentration. The modified electrode was designated as Nafion/GOx/ZnONRs/ZnO/ITO. 5 mg/mL of GOx concentration was chosen as the optimum concentration for immobilization on ZnO NRs electrode with

high sensitivity of 23.772 μA/mM.cm^2. The performance of the prepared electrode when two different immobilization techniques (physical adsorption and cross linking) was employed and compared. ZnO NRs surface was functionalized with glutaraldehyde (cross linking reagent) first before GOx was immobilized and was designated as Nafion/GOx-glutaraldehyde/ZnONRs/ZnO/ITO electrode. Cross-linked GOx electrode showed the best performance with the sensitivity of 32.24 compared to only 23.77 μA/mM.cm^2 for physical adsorption GOx electrode. The produced ZnONRs/ITO electrode was also decorated with gold nanoparticles (AuNPs) and platinum nanodendrites (PtNDs). An average diameter of ~40 nm and ~42 nm of AuNPs and PtNDs, respectively, were successfully decorated on ZnO NRs via drop casting method. The sensitivity of Nafion/GOx/ZnONRs/ZnO/ITO, Nafion/GOx/AuNPs/ZnONRs/ZnO/ITO and Nafion/GOx/PtNDs/ZnONRs/ZnO/ITO electrodes was 32.24, 54.51 and 98.34 μA/mM.cm^2, respectively. High sensitivity of Nafion/GOx/PtNDs/ZnONRs/ZnO/ITO electrode was due to properties of catalytic properties metallic nanoparticle. With high sensitivity of 98.34 μA/mM.cm^2 and low LOD of 0.03 mM Nafion/GOx/PtNDs/ZnONRs/ZnO/ITO electrode was chosen as the best electrode for glucose biosensor. The produced modified electrodes showed excellent performance in human blood samples.

CHAPTER ONE

INTRODUCTION

1.1 Research background

Diabetes mellitus is an incurable disease that resulted from insufficient or failure in producing insulin to regulate the blood glucose level appropriately. The disorder of insulin production can cause hypoglycemia (low levels of blood glucose) or hyperglycemia (high level of blood glucose) (Coster *et al.*, 2000). Diabetic patients are exposed to several medical conditions that slowly damage both small and large blood vessel in body. As a result several complications arise such as heart disease, adult blindness, coeliac diseases and tuberculosis (Ahmad *et al.*, 2017, Bruen *et al.*, 2017, Newman and Turner, 2005). A diabetic patient blood glucose concentrations can vary between 1 to 30mM compared to normal person which range between 3.5 to 6.5 mM (Miller, 2003). Frequent monitoring of physiological blood glucose levels is vital to avoid any hyperglycemia or hypoglycemia, and for an effective treatment. For this, at present, most diabetes patients rely on the glucose strips along with hand-held glucose meter (Vaddiraju *et al.*, 2010, Vashist, 2013). In diagnosis using a hand-held glucose meter a lancet is used to prick a clean finger for a small drop of blood. The blood is placed onto a glucose strip and analyze by glucometer. Several commercial glucose biosensors are available in the market, which only difference in respective performance and cost. Thus, developing a fast, sensitive and reliable biosensor to detect glucose is necessary.

Majority of the commercial glucose biosensors are based on electrochemical type. This is due to the excellent sensitivity, reproducibility easy maintenance as well

as low cost (Yoo and Lee, 2010, Rahman *et al.*, 2010). Electrochemical glucose biosensor operates through an electrode serves as signal transducer. The output signal generated by electrode transducer can be measured by potentiometric (measuring the change in electrode potential), conductometric (measuring the change in charge transfer resistance) and amperometric (measuring the currents produced) (Rahman *et al.*, 2010). Generally electrochemical glucose biosensor can be divided into two categories: enzymatic and non-enzymatic glucose biosensors. The enzymatic based glucose biosensor is the most common devices commercially available in the market where two types of enzymes were used; glucose oxidase (GOx) and glucose-1-dehydrogenase (GDH). However, GOx enzyme is the standard and popular enzyme used since it has a relatively higher selectivity for glucose. Furthermore, GOx enzyme is easy to obtain low cost and less stringent conditions during biosensor fabrication compared to GDH enzyme (Yoo and Lee, 2010). However, challenges in using enzyme-based glucose biosensors remain due to the possible stability and denaturation of GOx enzyme after immobilization.

Most commercially available electrochemical enzyme glucose strips use an artificial electron mediator to transfer electrons generated from the active sites of GOx enzyme to the electrode. The most common mediators use including potassium ferricyanide, methoxy phenazine methosulfate (mPMS) and phenanthroline quinone (PQ) (Abbott Diabetes Care Inc., 2015, Loew *et al.*, 2017). Mediators were used to lower the redox potential which avoids oxidation of interfering species. However due to its small and diffusive molecules, it is also possible for mediators to react with interfering species present which affect the efficiency and accuracy of glucose analysis (Toghill and Compton, 2010). Eventually, this decreases the operational lifetime and efficiency of modified electrode. To overcome this problem, many

researchers have focused on utilizing nanomaterials with different structures modified on working electrode which serve as an electrode in producing a direct electron transfer between GOx enzymes and electrode (Cash and Clark, 2010, Atan *et al.*, 2014, Nirmal and Swapan, 2013).

Electrode is solid supports that holds the sensing biomolecule (enzymes) which enhances the signal transduction and retains the activity of immobilize biomolecule (Arya *et al.*, 2012). Recently metal oxide nanomaterials have gained much attention as a suitable electrode for glucose detection. Metal oxide nanomaterials were less toxic, good catalytic and enhanced electron-transfer kinetics properties (Solanki *et al.*, 2011). Theses nanomaterial also exhibits a strong adsorption capability and high isoelectric point (IEP). Thus provide a suitable environment and reliable surface for immobilization of GOx enzymes (IEP of ~4.2) via electrostatic adsorption. Several high-IEP metal oxide nanomaterials used in glucose detection are zinc oxide (ZnO) (Atan *et al.*, 2014, Ma and Nakazato, 2014), titanium dioxide (TiO_2) (Haghighi *et al.*, 2017b, Yang *et al.*, 2015), copper oxide (CuO) (Wang *et al.*, 2013, Xu *et al.*, 2014), cerium oxide (CeO_2) (Patil *et al.*, 2012b, Saha *et al.*, 2009a) and zirconia (ZrO_2) (Cai *et al.*, 2012, Vilian *et al.*, 2014).

ZnO is a promising electrode material used to fabricate electrochemical glucose biosensors because of its biocompatibility and excellent properties, such as low toxicity, high electron mobility and easy fabrication (Zhao et al., 2009). *In vitro* analysis by Sahu et al. (2013) showed that 50 nm of ZnO nanoparticles with concentration of 25µg/mL could induce cytoxicity in cultured human lung epithelial cells (L-132) (Sahu et al., 2013). Oxidative stress was a common mechanism for cell damages either by apoptosis or necrosis which was induced by ZnO nanoparticles.

Cytoxicity of human lung epithelial cells (L-132) after 50 nm of ZnO nanoparticles exposure was evaluated by DNA damage analysis and Hoechst staining.

ZnO also possesses an IEP of ~9.5 which is suitable for adsorption of low-IEP enzymes, particularly GOx (IEP: ~4.2–4.5), at the physiological pH of 7.4 through electrostatic attraction (Aydoğdu *et al.*, 2013, Huh *et al.*, 2012). One of the limitations in enzymatic glucose biosensor is the insulated redox centre of enzyme which makes the enzyme redox capabilities to be delayed. Thus, electron transfer does not occur directly if no redox mediator is used. This problem can be resolved with the use of ZnO nanostructures. ZnO can provide a direct electron transfer (DET) without using a redox mediator because the electrode and enzyme operate in a small potential window closer to the redox potential of the enzyme itself, thereby causing the biosensor to be less prone to other interfering biomolecules (Arya *et al.*, 2012). Various morphologies of ZnO e.g. nanoparticles (NPs) (Hu *et al.*, 2011), nanorods (NRs) (Bhattacharya *et al.*, 2012), nanotubes (NTs) (Yang *et al.*, 2009), nanowires (Miao *et al.*, 2016) and nanosheets (Ahmad *et al.*, 2016) have been investigated as electrode as this significantly affects the glucose detection performance. A difference in nanostructure morphology significantly affects the glucose detection performance due to the difference in surface-area-to-volume ratio, which affects the number of enzymes immobilization. Subsequently, nanostructure ZnO provides a responsive microenvironment for stabilising and preventing the leakage of immobilized enzymes (Cash and Clark, 2010). In this work, ZnO nanorods (NRs) were used as electrode in glucose biosensor since nanorods morphologies can provide efficient electron transfer compared to nanoparticles and nanofilms. Moreover ZnO NRs offer a high surface area which ensures a high loading of enzyme immobilization.

Chu et al. (2012) developed the glucose biosensor where the ZnO NRs powder prepared by hydrothermal method was re-dispersed and sonicated in double distilled water to get a uniform suspension. The ZnO NRs suspension was coated on the surface of pretreated Pt electrode and left too dry in air. The sensor has a high sensitivity of 61.78 μA/mM.cm^2 and the detection limit of the sensor was determined to be 2.50x10^{-6} mM. More works on the use of ZnO NRs grown through hydrothermal for glucose biosensor have been reported (Lei *et al.*, 2011, Karuppiah *et al.*, 2015, Ma and Nakazato, 2014, Atan *et al.*, 2014). All the aforementioned works offer a good surface of ZnO NRs for enzyme immobilization but show rather poor stability due to easy removal of ZnO NRs from the modified electrode during functionalization (Rahman *et al.*, 2010). Adherence of ZnO NRs can be improved by growing ZnO NRs directly on substrates rather than transferring loose nanorods onto substrates. Therefore, in this work, ZnO NRs was grown directly on the substrates to provide a good stability as the process is chemically and mechanically robust. Numerous approaches are available for the growth of ZnO NRs. However, hydrothermal method is of interest for its simple process, low temperature and low cost process. Hydrothermal method was able to provide a good control over the morphology of grown nanorods (Baruah and Dutta, 2009). To ensure aligned and homogeneous growth of ZnO NRs, Indium tin oxide (ITO) glass was coated with sol-gel ZnO films. Sol-gel method is a simple, economic and effective process in producing a good quality of ZnO seed layer for further hydrothermal growth. Seeded substrates are used during hydrothermal growth of ZnO NRs to achieve a well-controlled and perpendicular aligned of rods. ITO glass substrates was used as electrode, as it is more economic, has low resistivity and can be easily shaped and trimmed to facilitate the fabrication (Liu *et al.*, 2009b).

Other challenges associated with electrochemical glucose biosensors is the enzymes immobilization methods used which may affect the stability of enzymes activity after immobilizes onto desired electrode. However, enzymatic glucose biosensor ensures specificity towards glucose and selectivity against interfering species such as ascorbic acid and uric acid presence in blood (Vaddiraju *et al.*, 2010, Si *et al.*, 2013). Therefore it is crucial that the immobilization process need to retain as much enzymes while maintaining the biological structure and catalytic activity (Sassolas *et al.*, 2012). Most of the reported works used physical adsorption methods to immobilize GOx onto ZnO NRs surface (Zhao *et al.*, 2016, Miao *et al.*, 2016, Ahmad *et al.*, 2012, Asif *et al.*, 2010, Bhattacharya *et al.*, 2014) . This is due to the high IEP of ZnO (~9.5) which makes the adsorption of GOx by electrostatic interaction available. However the limitation of physical adsorption immobilization method was the possible leaching of GOx over period of time. To overcome this, covalent binding and crosslinking immobilization method can be used. (Jung and Lim (2013) presented the effect of different coupling agent from aminosilanes (AS) group for covalent binding immobilization such as APTMS, 3-aminoporpyltriethoxysilane (APTES) and 3-aminopropylmethyldiethoxysilane (APS). They found that APS gave the highest sensitivity of 17.72 $\mu Acm^{-2}mM^{-1}$ compared to other samples due to its diethoxyl groups which has low chance of excessively crosslink with GOx which caused change in GOx biological structure further hinder efficient electrocatalytic activity with glucose (Jung and Lim, 2013). Although covalent binding offer a minimal leaching of GOx as it is tightly bound to ZnO NRs but the process is more expensive due to its complexity as reactions need to execute in low temperature and electrode support needs to be activated prior immobilization (Ramon-Marquez *et al.*, 2015, Mulchandani, 1998).

Recently, hybrid electrode nanostructures offer an opportunity to amplify the intensity of analytical signal produce from electrochemical reaction. This led to the development of ultrasensitive and low detection limit (LOD) of glucose biosensor. Several studies reported on ZnO hybrid with carbon-based materials (Hwa and Subramani, 2014, Karuppiah *et al.*, 2014), metal-transition based materials (Ma and Nakazato, 2014, Yang *et al.*, 2016), and metal based materials (Chou *et al.*, 2015, Tian *et al.*, 2015). Among all, metal based materials such as gold (Au) and platinum (Pt) offers variety of advantages. By incorporating metal nanoparticles with ZnO NRs for electrode can significantly increase glucose biosensor performance, due to the excellent catalytic properties of metal nanoparticles to more efficiently catalyze the glucose electrode (Saei *et al.*, 2013). Anusha et al. (2014) developed a simple construction of glucose biosensor by dispersing platinum (Pt) nanoparticles over ZnO nanopores. A high sensitivity of 62.14 μA/mM.cm^2 and low detection limit of 16.6 μM was obtained. The presence of PtNPs increased the magnitude and background current response compared to the absence of PtNPs. This result is due to the behavior of PtNPs, which accepted more electrons during reoxidation of GOx that resulted in the formation of electron-transfer accelerating layer (Anusha *et al.*, 2014). This finding motivated the researcher to develop a hybrid nanostructure electrode by employing a combination of ZnO NRs with AuNPs and PtNDs for a high performance glucose biosensor. In this work, well-aligned ZnO NRs showed an immense capability to hold lots of AuNPs and PtNDs due to their nanostructure morphology and high surface area of nanorod arrays. Several works have been reported, but there are more room for developing a stable and active catalyst of MNPs and ZnO NRs hybrid electrode for the application of glucose biosensor.

1.2 Problem statement

To date commercial glucose strip uses mediator which induces the electron transfer generated from the active sites of GOx enzyme to the working electrode. However mediators tend to react with interfering species presence in blood which affect the efficiency and accuracy of glucose analysis due to its small and diffusive molecules (Toghill and Compton, 2010). In response to this problem, this study proposes to investigate another possibility in replacing the artificial mediators in glucose strip. Zinc oxide nanorods (ZnO NRs) are directly grown on a substrate which serves as an electrode to enhance direct electron transfer between GOx enzymes and the substrate. Adherence of ZnO NRs to the substrate as a working electrode can be improved by growing ZnO NRs directly on the substrates rather than transferring loose ZnO NRs onto substrates. This ensures a good stability during enzyme immobilization and electrochemical analysis as the process is chemically and mechanically robust. The choice of immobilization technique plays a crucial role in the performance of enzymatic bionsensor as it is directly affects the enzyme loading which leads to the improvement of biosensor performance. Thus in this work crosslinking and physical adsorption immobilization methods were introduced in which the sensitivity and stability of enzyme immobilized on electrode over time were compared. Recently, hybrid nanostructures demonstrated a remarkable enhanced functions and improved performance in glucose biosensor. This finding motivated the researcher to develop a hybrid nanostructure electrode by employing a combination of ZnO NRs with metal nanoparticles for a high performance glucose biosensor. To date, limited work based on decorated ZnO NRs with metal nanoparticles for glucose biosensor electrode was reported. Therefore in this work, gold nanoparticles (AuNPs) and platinum nanodendrites (PtNDs) were decorated

separately on ZnONRs as glucose biosensor electrodes. This work aimed to develop a glucose biosensor with a good selectivity against interfering species presence in blood and longer stability and lifetime of ZnO NRs electrode which could retain the biological properties of GOx immobilized over time.

1.3 Research objectives

i. To obtain optimum conditions to produce ZnO NRs via hydrothermal method for glucose biosensor
ii. To study the applicability and performance of ZnO NRs in glucose biosensor
iii. To investigate the glucose biosensor performance using two methods; physical adsorption and crosslinking
iv. To investigate the performance of decorated ZnO NRs with AuNPs and PtNDs in glucose biosensor.

1.5. Scopes of study

In this work, ZnO NRs were prepared by hydrothermal method on seeded ITO substrates. The optimization of grown ZnO NRs was studied by varying the number of seed layers deposition (1 to 5 layers), seed annealed temperature (300 to 600 °C) and hydrothermal growth duration (1 to 6 hours). The crystallinity and morphology of produced ZnO NRs were observed with X-ray diffractometer (XRD) and field emission scanning electron microscope (FESEM). In this work, grown ZnO NRs were used to develop an enzymatic glucose biosensor. The optimization of glucose biosensor was studied by varying parameters: effect of supporting electrolyte's pH solution (pH 4 to 8), operating temperature (10 to 60 °C), GOx enzyme concentration (1 to 10 mg/mL) and Nafion concentration (1 to 5 wt%).

Electrochemical measurement was performed using Portable Bipotentiostat/Galvanostat μSTAT 400 from DropSens (Asturias, Spain) with a three-electrode system. The sensitivity of glucose biosensor was analyzed via amperometric analytical method where glucose standard solution was added gradually into buffer solution under constant stirring. The influence of different immobilization technique: physical adsorption and glutaraldehyde crosslinking where different concentration of glutaraldehyde was studied 0.5 to 4 wt% on the performance as well as shelf life of glucose biosensor was studied. The performance of ZnO NRs hybrid with AuNPs and PtNDs on glucose detection was carried out. AuNPs was synthesized via seeding growth method while PtNDs was prepared via chemical reduction method. Drop casting method was used to decorate ZnO NRs with respective metal nanomaterials and were denoted as Naf/GOx/AuNPs/ZnONRs/ITO and Naf/GOx/PtNDs/ZnONRs/ITO. The applicability of Naf/GOx/ZnONRs/ITO, Naf/GOx/PtNDs/ZnONRs/ITO and Naf/GOx/AuNPs/ZnONRs/ITO was further evaluated using human blood samples and compared with commercial standard glucometer (Accu-Check Active Model GB).

1.6. Thesis outline

This thesis is organized and divided into five chapters. Chapter 1 comprises of overall introduction to this thesis. Chapter 2 covers the literature review of the related works including background and fundamental of electrochemical glucose biosensors, enzyme immobilization methods, glucose biosensor performances based on ZnO and ZnO hybrid nanostructures. Additionally, general properties of ZnO, fabrication method of ZnO seeds layer and ZnO NRs with various synthesis methods

are presented. Chapter 3 describes the equipment and general experimental procedures that are used in this study, which include fabrication technique of seeded substrates, hydrothermal growth of ZnO NRs, chemical preparation and electrochemical analysis used in glucose detection. Chapter 4 consists of results and discussions of ZnO NRs formed via hydrothermal method on seeded substrates. The discussion of the glucose sensor using ZnO NRs electrode, AuNPs and PtNDs decorated ZnO NRs electrode, glutaraldehyde crosslinking immobilization as well as the reliability of produced electrodes in real sample are presented. Chapter 5 contains the conclusion and recommendations for future works.

CHAPTER TWO

LITERATURE REVIEW

2.1 Glucose biosensor

The term biosensor can be defined as a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using biological recognition element which is in direct spatial contact with a transducer element according to International Union of Pure and Applied Chemistry (IUPAC) (Ahmet Koyun *et al.*, 2012). Biosensor consists of three main elements; bioreceptor, transducer and signal processing unit as shown in Figure 2.1. Bioreceptor is a biological element that specifically recognizes and binds to the target analyte. Meanwhile transducer converts the physico-chemical change into an appropriate output signal whereby the intensity of generated signal is directly or inversely proportional to the concentration of analyte (Ahmet Koyun *et al.*, 2012).

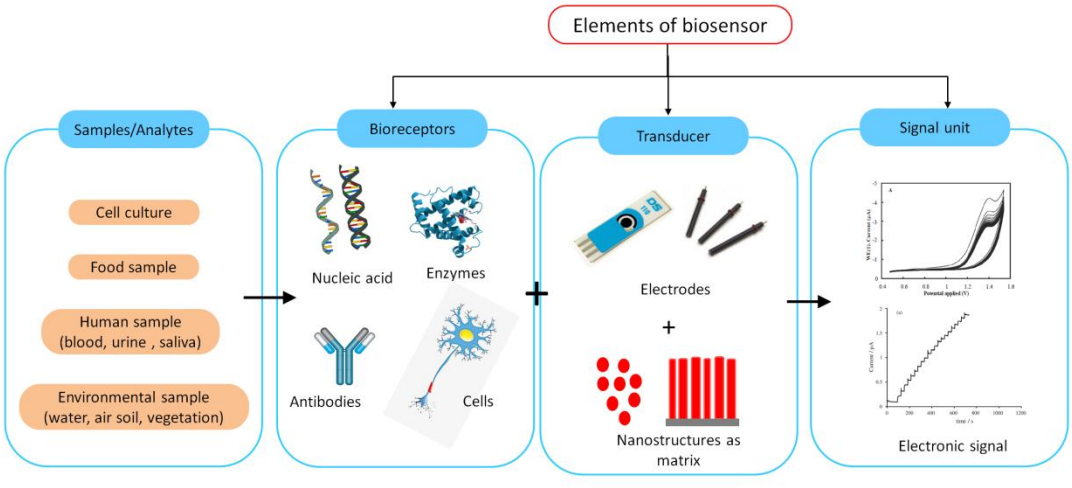

Figure 2.1: Schematic of a typical biosensor (modified and adopted from (Touhami, 2014)

Generally, biosensor can be categorized according to the biological elements and the basic principles of signal transducer. The biological elements can be categorized according to protein types (enzyme or antibody), nucleic acid type (oligonucleotides, aptamers or nucleic acid enzymes), whole cells type or any other biomimetic molecules that may interact with interest analytes (Palchetti and Mascini, 2010). For category based on transducer process, biosensor can be divided into optical, calorimetric, acoustic and electrochemical (Touhami, 2014, Zhiwei and Helong, 2010). Optical biosensor is based on measurement of luminescence or fluorescence as a consequence of biochemical reaction, while calorimetric biosensor is based on change in heat generates or temperature change as biochemical reaction takes place (Prasad and Uda, 2012). On the other hand, acoustic biosensor operates by monitoring the change in amplitude, phase, frequency or time delay obtain from analyte and biological element reaction (Durmuş *et al.*, 2015, Mehrotra, 2016). In electrochemical biosensor, analyte and biological elements react, generate or consume electrons which further produce an electrochemical signal (Chaubey and Malhotra, 2002). Biosensor has been widely applied in various fields such as food industry, environmental monitoring, bioprocess monitoring and health and clinical diagnosis (Zhiwei and Helong, 2010, Ahmet Koyun *et al.*, 2012, Mehrotra, 2016). In health and clinical diagnosis, biosensors also have been utilized in uric acid detection for monitoring and diagnosis of patients with gout, hypertension and myocardial infarction symptoms (Ivekovic *et al.*, 2012, Arslan, 2008). Additionally, cholesterol determination by cholesterol biosensor is widely used in diagnosis and prevention of hypertension, coronary artery diseases and transient heart attacks (Pundir *et al.*, 2012, Pakapongpan *et al.*, 2012, Alagappan *et al.*, 2018). In addition to urea and cholesterol detection, glucose detection is of great importance for clinical diagnosis. Glucose

biosensor offers an early diagnosis and monitoring glucose level for patients with diabetic problem.

Diabetes is one of the most commonly long-life occurring diseases. By definition diabetes mellitus is described as a metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbance fat, protein and carbohydrate metabolism resulting in defects in insulin secretion, insulin action, or both (Alberti and Zimmet, 1998). Blood glucose monitoring at regular intervals is one of the key factors required for diabetes patient as this may cause several complication such as cardiovascular disease, neuropathy, retinopathy, nephropathy and worse amputations (So *et al.*, 2012). As there is no cure for this disease, it is important for diabetic patients to monitor their blood glucose levels to avoid further complications. Therefore, developing a fast, sensitive, and reliable biosensor in detecting glucose is necessary. In general detection signal by glucose biosensors originates directly from glucose or by promoting the conversion of glucose to other determinable electroactive species (Saei *et al.*, 2013). In recent years, many research have been carried out to develop a sensitive and selective glucose detection, including calorimetric approach (Fatoni *et al.*, 2016, Liu *et al.*, 2014b, Xue *et al.*, 2011), acoustic signal transducer (Hu *et al.*, 2012, Luo *et al.*, 2013, Basaeri *et al.*, 2016), optical mechanism (Cummins *et al.*, 2013, Fadhil, 2016), and electrochemical reaction (Anusha *et al.*, 2014, Aydoğdu *et al.*, 2013, Chou *et al.*, 2015). Among these methods, electrochemical analysis offers better sensitivity, easy fabrication, good reproducibility and relatively low cost (Yoo and Lee, 2010, Aydoğdu *et al.*, 2013, Bruen *et al.*, 2017)

2.1.1 Electrochemical glucose biosensor

Electrochemical glucose biosensor was first introduced in 1962 by Clark and Lyons where they used a potentiometric measurement to determine glucose in blood plasma using glucose oxidase (GOx) (Clark and Lyons, 1962). They constructed a thin layer of GOx entrapped over an oxygen electrode via semipermeable dialysis membrane by monitoring the oxygen consumption as enzyme-catalyzed reaction occurs. Then negative potential was applied to the platinum cathode for a reductive detection of oxygen consumption which was used as the signal for glucose determination (Chen *et al.*, 2013, Wang, 2008, Si *et al.*, 2013). Over the past decades the development of electrochemical glucose biosensor has increased and evolved at a large rate.

Electrochemical glucose biosensor can be subdivided into conductometric, potentiometric and amperometric which are based on their respective detection mechanism (Yoo and Lee, 2010, Ahmet Koyun *et al.*, 2012, Grieshaber *et al.*, 2008). Potentiometric is based on measurement of charge potential accumulation at a working electrode in an electrochemical cell compared to the reference electrode when zero or no significant current flow occurs (Grieshaber *et al.*, 2008). For potentiometric glucose sensor, relationship between glucose concentration and potential is driven by Nernst equation (Ahmet Koyun *et al.*, 2012, Grieshaber *et al.*, 2008). In conductometric, measurement is based on performance of analyte or working electrode to conduct an electrical current in electrochemical cells. Conductometric measurement is used to study GOx enzymatic reaction that produces change in the concentration of analyte solution and also to monitor the change of working electrode conductivity after being immobilize with enzymes onto the

electrode surface (Grieshaber *et al.*, 2008). Amperometric is based on measurement of current change causes by oxidation or reduction reaction of analyte and working electrode while a constant potential is being applied. The current change is proportional to the concentration of analyte (Ahmet Koyun *et al.*, 2012). Since Clark and Lyons introduced electrochemical glucose biosensor concept, many researchers have been working on development of a better glucose biosensor for blood glucose monitoring even though several major companies such as Abbott, Lifescan and Telcare Inc. have commercialized blood glucose monitoring (Ramchandani and Heptulla, 2012). Figure 2.2 shows an evolution of first blood glucose sensor by Clark and Lyons to sophisticated glucose detection strips produced by Abboot.

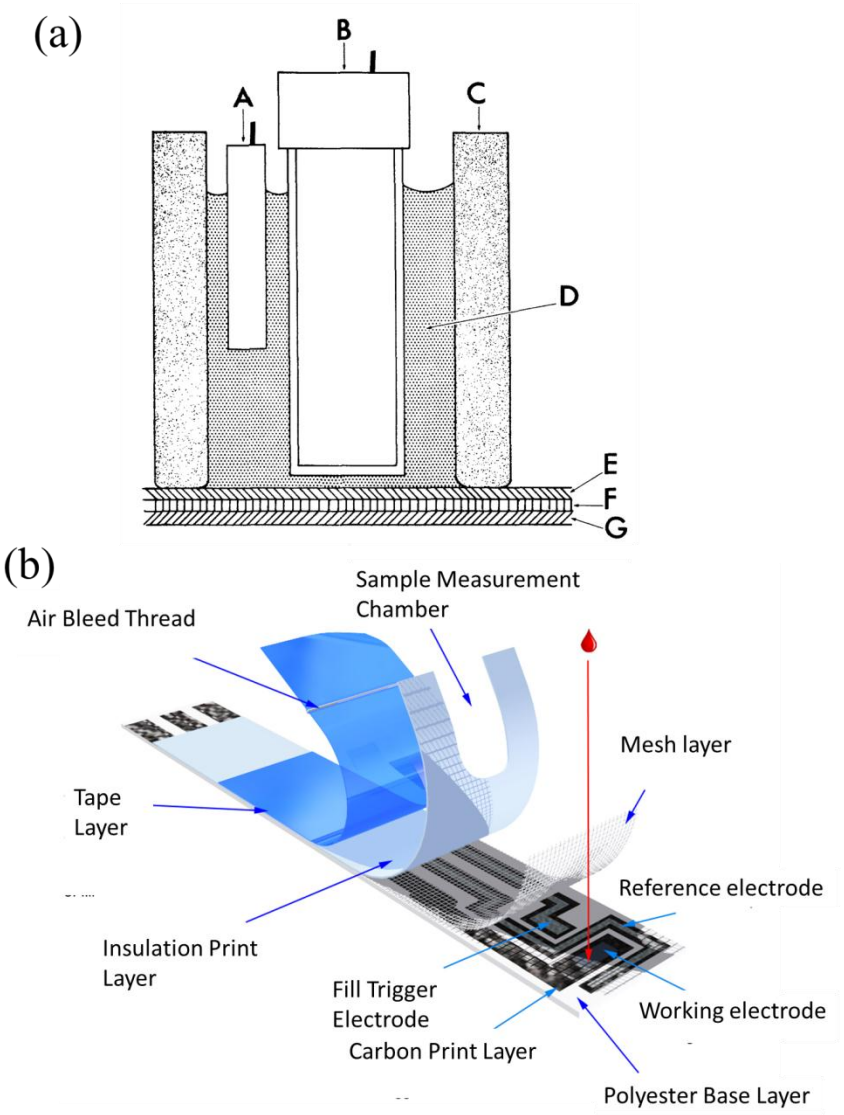

Figure 2.2: (a) Enzyme membrane electrode design consist of (A) reference electrode, (B) sensing electrode, (C) cell consisted of electrolyte, (D) electrolyte contain enzyme, (F) thin layer of concentrated enzyme, (E) and (G) membranes (Clark and Lyons, 1962), (b) Test strip architecture of Abbott Diabetes Care FreeStyle Precision Neo (Abbott Diabetes Care Inc., 2015)

Glucose sensor development can be divided into four primary generations according to their electron transfer mechanism with three generations enzyme-based and one non-enzyme based glucose biosensor as shown in Figure 2.3. The 1st generation of glucose biosensor is based on measurement of hydrogen peroxide produced or depletion of oxygen concentration during the catalytic reaction of

glucose (Yoo and Lee, 2010). In the first generation glucose sensor, GOx enzyme acts as a catalyst for glucose and oxygen reaction. However, this process requires free oxygen to act as mediator for electron to transfer to the electrode (Zhu *et al.*, 2016). Due to the oxygen dependence, the first generation of glucose bionsensor face a problem as there is an oxygen limitation to act as catalytic mediator in real blood sample which causes an error in determining the glucose level correctly (Toghill and Compton, 2010). The reaction could be explained according to equations 2.1 and 2.2 (Chen *et al.*, 2013, Si *et al.*, 2013). The immobilized glucose oxidase enzyme (GOx) catalyzes glucose in the presence of oxygen molecules and produces gluconic acid and hydrogen peroxide (H_2O_2) (equation 2.1). At negative applied potential to the platinum (Pt) electrode then oxidizes H_2O_2 and produces electrons (equation 2.2) (Zhu *et al.*, 2012a). Therefore, the number of electrons produced is proportional to the number of glucose molecules exist.

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconic acid} + H_2O_2 \qquad (2.1)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \qquad (2.2)$$

In order to overcome the dependency on oxygen level, synthetic electron acceptor or mediator was used to ensure electron transfer to be more efficient as in 2nd generation glucose biosensor. Mediator is an artificial electron transfering agent that is small, low molecular weight soluble redox component which shuttle electrons from the redox center of enzyme to the surface of working electrode surface (Chen *et al.*, 2013, Chaubey and Malhotra, 2002, Zhu *et al.*, 2016). Various types of electron mediators that are effective for GOx are ferrocene derivaties, ferricyanide, quinone compounds, conducting polmer salt tetrathiafulvalene-tetracyanoquinodimethane, (TTF-TCNQ), transition-metal complexs and phenothiazine (Chen *et al.*, 2013, Yoo

and Lee, 2010). The mechanism for mediator glucose biosensor is presented in chemical reactions (2.3-2.5) below (Zhu *et al.*, 2016, Rahman *et al.*, 2010). 2nd generation of glucose bionsensor begins with glucose catalyzation by GOx(FAD) enzyme and produces gluconic acid with reduced form of GOx($FADH_2$) (equation 2.3). FAD is flavin adenine dinucleotide, while $FADH_2$ is reduced form of FAD from GOx enzyme (Rahman *et al.*, 2010). Electrons produced from glucose oxidation are collected by mediator (M) and are reduced (M(red)) (equation 2.4). At the same time, the reduction of mediator facilitates the re-oxidation of GOx($FADH_2$) to the GOx(FAD) (equation 2.4). Finally the reduced mediator is oxidized at platinum electrode surface generating two electrons (equation 2.5). The generated electrons are proportional to the glucose concentration level (Si *et al.*, 2013).

$$\text{Glucose} + GO_x(FAD) \rightarrow \text{Gluconic acid} + GO_x(FADH_2) \qquad (2.3)$$

$$GO_x(FADH_2) + 2M(ox) + 2e^- \rightarrow GO_x(FAD) + 2M(red) + 2H^+ \qquad (2.4)$$

$$2M(red) \rightarrow 2M(ox) + 2e^- \qquad (2.5)$$

For 3rd generation glucose biosensor, mediator is removed from associating with enzyme but relies on direct electron transfer (DET) between enzyme and the working electrode. This can be achieved by using nanostructured material in the working electrode to act as matrix which enables GOx to be immobilized directly and in a close proximity. By doing so, enables redox center of GOx to be reduced by glucose and oxidize directly onto the modified electrode. The mechanism of 3rd generation glucose biosensor is presented in chemical reactions (2.6-2.7) (Zhu *et al.*, 2016, Wang, 2008). GOx(FAD) catalyzes the glucose solution and produces gluconic acid with reduced form of GOx($FADH_2$) (equation 2.6). Then the reduced

GOx($FADH_2$) re-oxidize with dissolved oxygen molecules at Pt electrode to produce two electrons (equation 2.7). Subsequently electrons produced is proportional to the glucose concentration presence.

$$\text{Glucose} + GO_x(FAD) \rightarrow \text{Gluconic acid} + GO_x(FADH_2) \quad (2.6)$$

$$GO_x(FADH_2) + O_2 \rightarrow GO_x(FAD) + 2H^+ + 2e^- \quad (2.7)$$

Finally, 4th generation of glucose biosensor or non-enzymatic glucose biosensor involves a direct electron transfer by electro-oxidation of glucose to gluconic acid at nanostructures matrix that possesses high electrocataltic activity (Vaddiraju *et al.*, 2010). In the non-enzymatic glucose biosensor, atoms from nanostructure act as electrocatalyst in glucose reaction (Tian *et al.*, 2014). In this work, the produced glucose biosensor electrode is of the 3rd generation type, where the electrons produced by the the biocatalyst are directly absorbed by the ZnO NRs electrode in the absence of a mediator. The 3rd generation of glucose biosensor offers several advantages such as high selectivity and specificity towards glucose rather than interfering species such as ascorbic acid and uric acid, fast response time and low operating voltage (Zhu *et al.*, 2016, Holade *et al.*, 2017, Bollella *et al.*, 2017). Table 2.1 lists the advantages and disadvantages of all generations of glucose biosensors. Further discussion regarding the non-enzymatic glucose biosensor is discussed in the Section of 2.3.2.

Table 2.1: Comparison of glucose biosensors evolution

Generation	Advantages	Disadvantages	Reference
1st (enzymatic)	• Simple design of biosensor by only measuring H_2O_2 concentration	• Requires high operating voltage (> 1 V) • Restrict to solubility of oxygen in biological fluid • Deactivation of enzyme due to production of H_2O_2	(Putzbach and Ronkainen, 2013)
2nd (enzymatic)	• Low operating voltage (<0.6V) • Enhance electron transfer • Less dependence on oxygen concentration	• Requires extra steps to ensure no leaching in mediator since mediator is small and easy to diffuse • High competition with dissolved O_2, causes faulty result • Easily react with interfering species presence	Bollella et al., 2017, Toghill and Compton, 2010, Chaubey and Malhotra, 2002)
3rd (enzymatic)	• Easy fabrication • High selectivity and specificity • Direct electron transfer • Low operating voltage (<0.6V)	• Requires good conductivity of nanostructure material • Redox co-factor of enzyme buried deep inside the cavity for reaction to occur easily • Enzyme leaching if not properly immobilize onto matrix	Zhu et al., 2016, Holade et al., 2017), (Bollella *et al.*, 2017)
4th (non-enzymatic)	• High stability (>3 month depends on the nature of materials used for electrode) • Low cost in fabrication as no enzyme use	• Influence of specificity and selectivity against interfering species	(Vaddiraju *et al.*, 2010, Si *et al.*, 2013)

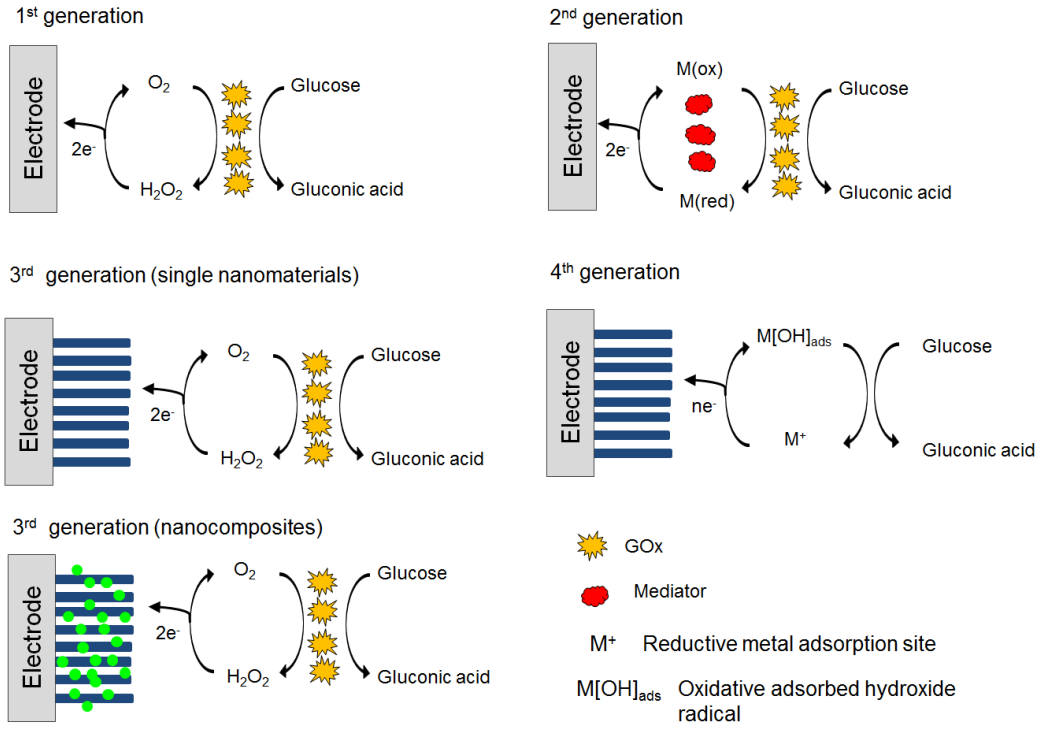

Figure 2.3: Schematic representation of four generations of glucose biosensors

2.1.2 Non-enzymatic glucose biosensor

As mentioned earlier, the enzymatic glucose detection method relies on the catalytic reaction of GOx enzyme to oxidize glucose. Meanwhile non-enzymatic glucose biosensors forgo the use of biological components entirely. In the non-enzymatic glucose biosensor, matrix surface serves as catalyst for the electro-oxidation of glucose to occur. Generally, there are two widely accepted models that explain electro-oxidation of glucose; activated chemisorption model and incipient hydrous oxide adatom mediator (IHOAM) model (Zhu *et al.*, 2016, Si *et al.*, 2013, Toghill and Compton, 2010, Amala and Gowtham, 2017). The activated chemisorption model was proposed by Pletcher (1984) that suggests that electro-oxidation of glucose begins through the adsorption of glucose analyte onto matrix surface which then allows glucose molecules to form bond with matrix surface,

where electrocatalyst aids in the oxidation process. Simultaneously, the hydrogen atom which attach to hemiacetal carbon is removed as shown in Figure 2.4(a). Once removed, this hydrogen atom bonds with matrix surface at a site adjacent to the glucose molecule bond. The hydrogen removal process is considered as the rate-determining step in glucose electro-oxidation and is generally considered to occur concurrently with the glucose analyte chemisorption (Si *et al.*, 2013, Zhu *et al.*, 2016). Therefore a suitable geometry of matrix could contribute to the kinetic enhancement of glucose oxidation process as well-spaced of adsorption sites for electrocatalyst and adsorption of single absorbate each time (Zhu *et al.*, 2016).

The second model, known as IHOAM model was proposed by Burke (1994). The IHOAM model specifies the role of hydroxyl radicals in the electrocatalytic process. This model is based on the presence of active metal atoms which get through a pre-monolayer oxidation step in which an incipient hydrous oxide (OH_{ads}) layer is formed. This layer of OH_{ads} on matrix surface is believed to mediate the oxidation of glucose on the matrix surface (Tian *et al.*, 2014). The chemisorption of OH_{ads} on the reductive metal adsorption sites causes the formation of MOH_{ads}, which is then oxidized glucose (Amala and Gowtham, 2017). Figure 2.4(b) shows an illustration of the IHOAM model.

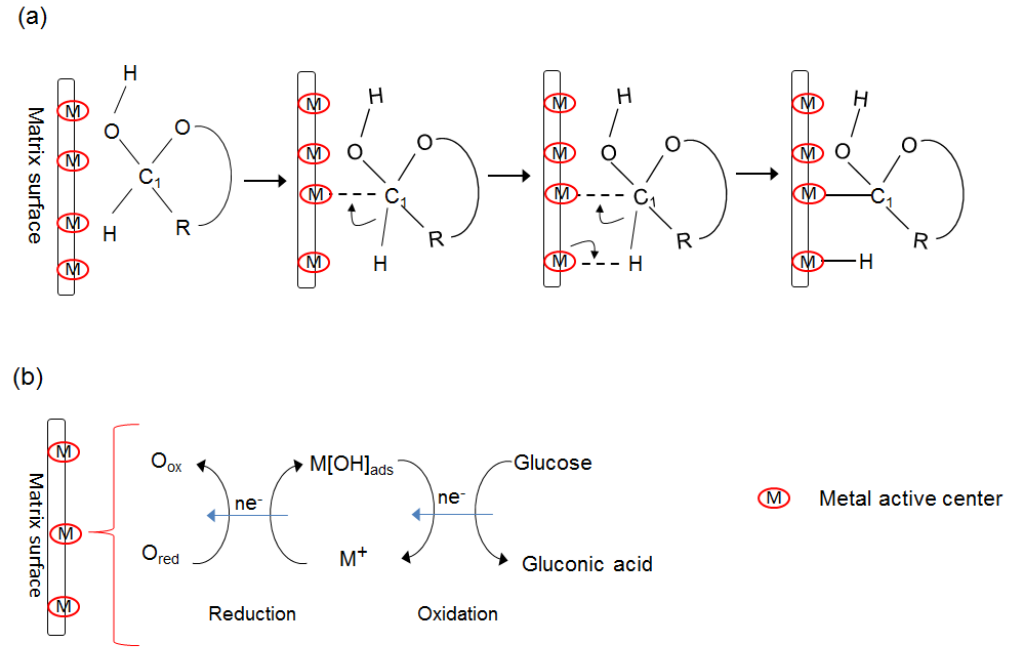

Figure 2.4: (a) The activated chemisorption model with adjacent adsorption sites proposed by (Pletcher, 1984) C1 : hemiacetal carbon atom and R : other part of glucose molecule, (b) IHOAM model proposed by Burke. M+ is the reductive metal adsorption site and M[OH] ads is is the reactive absorbed hydroxyl radical (Amala and Gowtham, 2017, Tian et al., 2014)

2.1.3 Enzymatic glucose biosensor

2.1.3.(a) Glucose oxidase (GOx)

Enzymes are biological catalysts with highly active units rapidly increase and that speed up biochemical reactions (Robinson, 2015). Hexokinase, glucose oxidase (GOx) and glucose dehydrogenases (GDHs) enzymes have been used in enzymatic glucose monitoring system. GOx enzyme is widely explored due to easy handling and has a potential for industrial applications due to high sensitivity and specificity (Yoo and Lee, 2010, Bankar *et al.*, 2009). *Aspergillus niger* fungal is commonly used to produce GOx enzyme due to a very high substrate specificity towards glucose environment (Ferri *et al.*, 2011). GOx enzyme is an oxido-reductase consists of two