ENZYME IMMOBILIZATION AND CHARACTERIZATIONS OF GLUCOSE OXIDASE (GOx) ON MOF-199 AND SiO_2 FOR GLUCOSE BIOSENSING APPLICATIONS BY ELECTROSTATIC ADSORPTION

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November 2019

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A Thesis Submitted to the College of Graduate Studies, Universiti Tenaga Nasional in Fulfilment of the Requirements for the Degree of

Master of Mechanical Engineering

NOVEMBER 2019

DECLARATION

I hereby declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and not concurrently submitted for any other degree at Universiti Tenaga Nasional or any other institutions. This thesis may be made available within the university library and may be photocopies and loaned to other libraries for the purpose of consultation.

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NURULAINA FASYA BT. SAIFUL ANUAR

Date :

ABSTRACT

The glucose oxidase (GOx) was immobilized on metal orgnaic framework 199 (MOF-199) and silica spheres (SiO_2/GOx) by electrostatic adsorption. The characterizations analyses were conducted to analyze the morphological analysis, the optimization studies and chemical properties of GOx between before and after immobilization of GOx on MOF-199 and SiO_2. The activities of GOx were measured using optical and amperometric measurements for both MOF-199/GOx and SiO_2/GOx. In addition, The imit of detection (LOD) and stability studies forimmobilized GOx were also studied. Based on the morphological results, both of MOF-199/GOx and SiO_2/GOx nanostructured composites observed with the presence of GOx particles attached to both of MOF-199 and SiO_2 respectively. Both of MOF-199/GOx and SiO_2/GOx reacted optimally at pH and temperature of 7.0 and 50°C (313 K) accordingly. UV-Vis analysis results exhibited the presence of a new broad peak for SiO_2/GOx sample at 250 nm to 280 nm and one narrow peak around MOF-199/GOx suggesting the presence of amino group from GOx. The FT-IR analysis showed that both MOF-199/GOx and SiO_2 exhibited one new absorbance peak at ca. 1720 cm^{-1} and 1722 cm^{-1} attributed to the bending vibration (νbend) of C=O aldehyde obtained from GOx particles. The increase of glucose concentration from 1.0 mM to 5.0 mM resulted to the increment of absorbance value (UV-Vis) and current (mA) for both MOF-199/GOx and SiO_2/GOx suggesting successful immobilization of GOx on MOF-199 and SiO_2. From the limit of detection (LOD) analysis, both MOF-199/GOx and SiO_2/GOx exhibited LOD of 0.2 mM and 0.3 mM respectively. The half lives of MOF-199/GOx and SiO_2/GOx were 23 and 21 days. Meanwhile, GOx's half life was 11 days before losing enzymatic activities.The stabilization study also demonstrated higher stability (days) for MOF-199/GOx and SiO_2/GOx reacted with glucose as compared to actual GOx. These results suggested successful immobilization method using new method (electrostatic adsorption) which was more facile than current immobilization method which was also providing high LOD for glucose sensing and could sustain the activities of GOx for the application of glucose biosensors. Therefore, these results may provide towards the initial step to non-invasive glucose measurement practices particularly in salivary glucose detection.

ACKNOWLEDGEMENTS

I would to express my gratitude to Allah S.W.T for helping me passing through all the obstacles and hardship in completing this study. I would also like to thank my master supervisor, Assoc. Professor Dr. Halina Misran for always motivating and inspiring me to complete my research studies. The door to Dr. Halina office was always open whenever I ran into a trouble spot or had a question about my research or writing. She consistently allowed this paper to be my own work, but steered me in the right the direction whenever he thought I needed it.

I would also like to acknowledge my laboratory members Musdalilah Ahmad Salim and Nurfatihah Mahadi for always supporting me and helped me in conducting experiemnents. I would also like to thank Mdm. Siti Zubaidah for helping some of in experimentations as well as motivating me in completing my studies. I would also express my gratitude toward my former lab members, Nurfanizan and Siti Nurul Akmal for their endless stimulating discussions and motivations which had helped me to validate this research project: Without their passionate participation and input, the validation survey could not have been successfully conducted.

Last but not least, I would like to express my gratitude to Yayasan Tenaga Nasional (YTN) for sponsoring my master studies. I would also would like to acknowldge COGS, MIMOS and Nanoarchitectonic Lab and all individuals who had helped me in completing this study. My sincere thank to all.

DEDICATION

I would also like to deeply dedicate thank my parents; Saiful Anuar Safian and Kasmah Sumani for giving me infinite supports and motivations to handle all the obstacles and throughout my life. Despite of their unstable health conditions, they were still supporting me with their hearts and souls. Without them, I could not imagine on how to tackle all the hardships in completing this study.

I would also would like to express my deep gratitude to my supervisor, siblings and my close friends on their continuous trusts and supports throughout this research period.

Finally, I would dedicate this research thesis to Allah S.W.T for providing me with endless guidance and support throughout my entire life.

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LIST OF ABBREVIATION

EDX	Energy Dispersive X-Ray
FAD	Flavin adenine dinucleotide
FESEM	Field Emission Scanning Electron Microscope
FT-IR	Fourier Transform Infrared Spectroscopy
GDH	Glucose dehyrogenase
GDH-PQQ	Glucose dehydrogenase pyrroloquinoline quinone
GDH-FAD	Glucose dehydrogenase flavin adenine dinucleotide
GOx	Glucose oxidase
LOD	Limit of detection
MOF-199	Metal Organic Framework 199
PB	Phosphate buffer
PODFA	Palm-Oil Derived Fatty Alcohol
PQQ	Pyrrolquinoline quinone
SiO_2	Silica Oxide (Spheres)
UV-Vis	Ultraviolet Visible Spectroscopy

LIST OF PUBLICATIONS

N.F.S.Anuar, H. Misran, M.A. Salim , S.Z. Othman (2016) Facile Method of Enzyme Immobilization on Silica Quasi-Nanospheres (SiO2) Synthesized Using PODFA for Biosensing Applications, *Key Engineering Materials*, Vol. 694, pp 67-71

N.F.S.Anuar, H. Misran, S. Z. Othman, A.Manap (2014) Review on Immobilization of Glucose Oxidase (GOx) on Nanoparticles for Fabrication of Glucose Biosensor Applied *Mechanics and Materials*, Vols. 773-774, pp 720- 724

N.F.S.Anuar, H. Misran, A.N.A. Manap, S.Z. Othman, M.A. Salim, N.N.H. Shah and A.A. Razak, N. Mahadi (2014) Fabrication of Glucose Biosensor by Glucose Oxidase (GOx) Immobilization on Nanoparticles (Review), *NATGRAD 2014* (UNITEN Proceeding)

N. Mahadi, H. Misran, S. Z. Othman, A.Manap, M.A.Salim, N.F.S.Anuar (2014) Hydrothermal Synthesis and Characterizations of MOF-199 using Renewable Template, *Applied Mechanics and Materials*, Vols. 773-774, pp 226-231

CHAPTER 1

INTRODUCTION

1.1 Background

In this modern living lifestyle, over-eating and lack of exercises could lead to many serious health problems. One of the main leading health diseases is diabetes. Diabetes usually occurs in high glucose concentration that may result in metabolic dysfunction due to inability to produce enough insulin (Yoo *et al.,* 2010). Insulin is the corresponding enzyme to regulate concentration blood sugar in the body where it breaks down excess glucose molecule to glycogen to be stored in liver (Duarte *et al.,* 2012).

Diabetes has also been the main precursor to many other severe diseases including heart attack, kidney failure and eyes damage (Duarte *et al.,* 2012). Diabetes could be categorized into two types; type 1 diabetes and type 2 diabetes which were resulted from the inability to produce enough insulin (Assal *et al.,* 1999); Yoo *et al.,* 2010).

Referring to the International Diabetes Federation (IDF), the normal blood glucose level is ranging from 70 mg/dl to 110 mg/dl or 3.9 mM to 6.1 mM (fasting condition) respectively (Roglic *et al.,* 2016). In summary, higher or lower glucose concentration in blood can be one of the roots of other diseases. Therefore, constant glucose measurement is very crucial in order to provide prevention steps to non diabetic patients as well as actual prescription towards diabetic patients.

1.2 Biosensors

The term of biosensor is the short term of ‘biological sensor’. In specific, biosensor is a device that uses biomolecule or living organism such as bacteria or enzymes in order to detect the presence of chemical or biological substrate (Koyun *et al.,* 2012). There are two main compartments of biosensor; bioreceptor and a transducer. Bioreceptors are biological elements that synthesize biochemical reaction of any

chemical or biological substrate (eg: enzyme, cell, receptors, molecule) in order to produce desired produc (Koyun *et al.,* 2012). Meanwhile, the transducer acts as converter to transmit signal of biochemical reaction into measurable and readable output (eg: electrode, rod, nanorod) (Koyun *et al.,* 2012).

1.2.1 Glucose Biosensor

Glucose biosensor is a device used to measure human blood glucose level in order to provide a prescription and preliminary report towards human sugar level. Current body glucose measurement is through blood that requires a direct blood withdrawal from human body (Wang *et al.,* 2001). Glucose was initially measured through urine however the procedure of execution time consuming and lack of accuracy for wide range of glucose concentrations (Lorenz *et al.,* 2003: Wang *et al.,* 2001). This is because, urine consists of other chemical wastes that may interrupt the reading of actual glucose concentrations (Lorenz *et al.,* 2003). Blood glucose concentration could provide better measurement with minimal interruption (Wang *et al.,* 2001). Nonetheless, the method of blood withdrawal can be very invasive since diabetic patients have very low immune systems. Thus, to minimize this complication, scientists had came out with a device that require a finger prick technique to withdraw blood from diabetic patients. These methods have helped to reduce the effect such as severe wounds and bruises (Wang *et al.,* 2001). However, these method is still not effective to prevent the outcome of blood withdrawal for severe diabetic patients since severe diabetic patients need to measure their blood level from 5 to 7 times daily. Therefore, the researchers are still working on the safe, non-invasive glucose sensors that can be applied to all diabetic patients without any unwanted concent to all non medical practitioners.

Similar to biosensors definition, glucose biosensors do require both biological component to detect the presence of glucose as well as the transducer in order to produce more readable output (Koyun *et al.,* 2012). There are two types of common enzymes used for glucose biosensors which are glucose oxidase (GOx) and glucose-1-dehydrogenase (GDH) (Zhou *et al.,* 2010). Both of enzymes differ in term of redox reaction, turnover rate, cofactors and affinity of glucose binding. GOx is

widely employed for glucose biosensors because GOx favors the direct reaction (high rate reaction) of glucose synthesize compared to GDH that favors the reversed reaction (slow rate reaction) of glucose synthesize (Zhou *et al.,* 2010). In addition to that, GOx are cheaper, easy to obtain, and more stabilize towards extreme pH and have greater ionic strength as compared to GDH (Zhou *et al.,* 2010)

1.3 Nanostructured Materials as Supports for High Sensitivity Biosensors

Nanomaterials have gained many attentions especially in fabrication of biosensor. A good biosensor requires high enzyme loading: surface area in order to maximize the enzyme loading onto support. This is important in order to provide higher sensitivity of biosensor as well as could increase the limit of detection of biosenso (Datta *et al.,* 2013). Nanomaterials offered small size template which is suitable for maximizing the load of enzyme onto it. In this research, the metal organic framework-199 (MOF-199) were used as metal nanomaterial and Silica spheres (SiO_2) (nonporous) for non metal nanomaterial. These materials would provide as the support for glucose oxidase (GOx) in attempt for fabrication of high sensitivity glucose biosensing materials.

1.4 Glucose Concentration Measurements and GOx Stability

There were two major types of glucose concentration measurement methods; light absorbance measurement (spectrophotometer) (Shih *et al.,* 2009) and amperometric measurement (Kong *et al.,* 2009). Current glucose biosensors are applied using blood plasma as medium of glucose reading. However, conventional glucose sensors could only detect from 0.56 mM sensitivity (Yoo et al., 2010; Zhang *et al.,* 2015). Meanwhile, from from Agrawal *et al.,* 2013, the mean glucose concentration for normal condition and diabetic melitus were 1.23 mM and 4.22 mM respectively (Agrawal *et al.,* 2013) . Thus, current glucose biosensors are not possible to be used for salivary glucose measurement.

In order to produce a very good biosensing material, the immobilized enzyme should be able to last react more stable and has longer half life compared to stand-alone

enzyme. Enzyme is generally very sensitive and has a very low half- life. Multiple reaction may result to the alteration of its shape thus will disrupt the active site of the enzyme. Thus, it will result to enzyme denaturation and dysfunctionility. Therefore, the enzyme stabilities are measured in order to determine the half life of the enzyme (in days) between before and after immobilization process.

1.5 Problem Statement

Diabetes has led to many severe diseases including heart attack, kidney failure, high blood pressure and in the worst scenarios it could lead towards the amputation of body parts and fatality (Bajaj *et al.*, 2013).

Therefore, constant glucose measurement is very important in order avoid further health complications. However, current glucose biosensors require a finger prick technique or blood withdrawal (Agrawal *et al.,* 2013). Particularly, these methods could be very invasive especially for severe diabetic patients that need to measure their glucose concentration constantly (Agrawal *et al.,* 2013).

Nonetheless, the alternative method for non-invasive glucose sensors could be attempted by measuring glucose concentration using saliva (Agrawal *et al.,* 2013). Yet, the concentration of glucose is very small in saliva which could be as low as 0.11 mM to 0.45 mM (Carda *et al.,* 2006; Agrawal *et al.,* 2013; Gopal et al., 2007). Current glucose biosensors have low limit of detection (LOD) of approximately 0.6 mM which are higher than salivary glucose concentration (Yoo *et al.,* 2010).

Current glucose biosensors offer complicated method for enzyme immobilization including covalent bonding, cross-linking and gel entrapment. The usage of covalent bonding and cross- linking immobilization could alter the enzyme catalytic activities with the formation of strong bonds to the enzyme's active sites (Mohamad *et al.,* 2015). This outcome could lead to the major loss in enzymatic activities on which will require enzymes to be extracted or purchased again (Mission *et al.,* 2015; Mohamad et al., 2015; Homaei *et al.,* 2017). Furthermore, another immobilization procedure needed to be repeated all over again. These processes were highly time

consuming and not cost effective as enzymes are very expensive which might affect the overall productivity of glucose biosensors production. Meanwhile, the usage of gel entrapment in glucose biosensors would restrict the diffusion of glucose to the immobilized enzyme (Homaei *et al.,* 2017). In addition, this process could limit the enzyme movement due to the limitation of the pore sizes (Zucca *et al.,* 2014). Therefore,the reaction between enzymes and glucose would be limited and was not suitable for constant glucose measurement (Homaei *et al.,* 2017). Electrostatic adsorption is a simple method that will not produce any strong bond between the enzymes and supports (Brena *et al.,* 2013; Homaei *et al.,* 2017). Therefore, the enzymes catalytic activities will not be disturbed by this immobilization method. This method is also easy and safe to employ and which will reduce the time for mass production of glucose biosensors. Despite the weak bond formation between the enzyme and supports, this method allow huge enzyme loading with via electrostatic interaction between both of the components (Brena *et al.,* 2013). Besides, the attachment of enzyme on the surface of the support will allow the diffusion of glucose (substrate) for glucose measurement (Homaei *et al.,* 2017). The simple method that would not disturb the enzyme catalytic activities and not time consuming could overcome the limitation from the current methods.

Existing glucose biosensors use enzyme, glucose oxidase (GOx), a specific enzyme that most commonly employed for glucose biosensors. GOx is expensive yet sensitive and has short shelf life for multiple reactions with glucose (Datta *et al.,* 2013). Multiple reaction may result to the alteration of its shape thus will disrupt the active site of the enzyme. Thus, it will result to enzyme denaturation and dysfunctionility (Datta *et al.,* 2013). Therefore, to avoid the complication of GOx denaturation, current glucose biosensors are not made reusable, which can only detect one measurement per usage (Kim *et al.,* 2015). The lack of GOx stability in current glucose biosensors has also increased the cost and production time to produce glucose biosensors. The better stability of GOx would result in cheaper cost and better production time of glucose biosensors.

1.7 Research Objectives

The objectives of this research are:

- To immobilize glucose oxidase (GOx) on MOF-199 and SiO_2 nanomaterials using electrostatic adsorption.

- To characterize the morphology, the optimum pH and temperature and chemical properties between before and after immobilization of GOx on MOF-199 and SiO_2.

- To measure the activities of immobilized GOx and the limit of detection (LOD) on MOF-199 and SiO_2 for glucose sensing applications.

1.8 Scopes of Works

The scopes of work in this research are:

- Materials and experimental set up for GOx immobilization on MOF-199 and SiO_2 nanoparticles using electrostatic adsorption.

- Parameter characterizations for MOF-199/GOx and SiO_2/GOx optimum operating conditions, morphological, chemical and mechanical characterizations between before and after electrostatic adsorption on MOF-199 and SiO_2 nanomaterials.

- Conducting glucose concentration measurement using both UV-Vis (light absorbance) and amperometric (current measurement) on both MOF-199/GOx and SiO_2/GOx in different low concentration of glucose solutions, simulating the salivary glucose concentrations. From the glucose measurement, the calibration curve will be drawn to provide actual glucose measurement reading based on absorbance or current obtained.

- Preparing the enzyme stability studies by measuring the amperometric measurement of glucose within 30 days in order to measure both MOF-199/GOx and SiO_2/GOx stability and reusability as compared to actual GOx. In addition, the stability and reusability between MOF-199/GOx and SiO_2/GOx are compared accordingly.

using immobilized nanomaterials with GOx and measured using varied glucose concentrations, simulating the salivary glucose concentrations.

1.9 Organization of the thesis

Chapter 1: Introduction

Chapter 1 provides the brief information regarding diabetes and current measurement techniques. In addition, component of glucose biosensors which include the introduction of nanostructured materials in biosensors application, the methods of enzyme immobilization methods for fabrication of biosensors, methods of glucose measurement using biosensors as well as enzyme activities are explained briefly in this chapters. This chapter also discusses problem statements of this research, research objectives as well as the research gap encountered in this research.

Chapter 2: Literature review

This chapter discusses about the history of diabetes as well as the innovations for glucose measurement methods. This chapter also discusses the development of sensitivity and limit of detection (LOD) of previous sensors. Furthermore, this chapter will be explaining towards the development of glucose sensors and the innovation of enzymatic glucose biosensors for highly sensitive glucose biosensors. In addition, the previous materials applied for glucose biosensors are explained in details including the application of nanomaterials for the new, innovation of glucose biosensors.

This chapter discusses on the immobilization methods of enzyme. Therefore, this

chapter will also be focusing on electrostatic adsorption, a new enzyme immobilization method and the advantages of this method. Furthermore, the enzyme measurement methods, parameter studies for optimum performances of glucose sensing materials as well enzyme stability studies are discussed in details in this chapter.

Chapter 3: Methodology

The synthesis method was focusing on the method of immobilization. As discussed the literature review, covalent bonding, gel entrapment and cross-linking immobilization were the prominent methods applied in the production of glucose biosensor. Therefore, in this research, electrostatic adsorption which is a simpler and non-conventional method was introduced for immobilization procedure. Furthermore, in this study, two different materials were applied for GOx immobilization method; silica quasi nanopsheres (SiO_2) and metal organic framework 199 (MOF-199). The SiO_2 was selected as the inorganic, non-porous material meanwhile the MOF-199 was introduced in this study as a new, high porosity material for GOx immobilization. These materials were selected in order was to validate the compatibility of electrostatic adsorption on both porous and non porous materials.

The second part of this research is to provide characterization procedures in order investigate as well as to make comparison on the morphological, physical, and chemical properties of the synthesized materials between before and after immobilization of GOx on both MOF-199 and SiO_2.

The last part of this research covered the procedures for proposed application of this research. One of the objectives of this research is to provide glucose sensing application on the immobilized nanomaterials. Therefore, in this part, the GOx activities on glucose were studied in order to proof the validity for glucose sensing application. The GOx activities studies also focuses on the measurement of low glucose concentration which is suitable for production of highly sensitive glucose biosensor, targeting the noninvasive, salivary glucose measurement.

Chapter 4: Results and Discussions: Characterizations

This chapter discussed on the results obtained from structural and morphology characterizations including X-Ray Diffraction (XRD) and Field Emission Microscopy (FESEM). This chapter also discusses on parameter analyses studies on varied pH and varied temperature using Ultraviolet- Visual Spectrophotometer (UV-Vis). Chemical properties characterizations using Energy Dispersive X-ray (EDX) and Fourier Transform Infrared Spectroscopy (FT-IR) are also explained. between before and after immobilization methods.

Chapter 5: Results and Discussions: Glucose Oxidase (GOx) Activities

This chapter discusses discusses about the optical measurement of glucose using both of the nanocomposites. The measurement of glucose using optical was conducted using UV-Vis analysis on actual GOx, MOF-199/GOx and SiO_2/GOx in varied glucose concentrations. Meanwhile, chapter 5.3 discusses about the amperometric measurement of glucose using both MOF-199/GOx and SiO_2/GOx nanocomposites. The amperometric measurements were then conducted using electrochemical cell analysis on actual GOx, MOF-199/GOx and SiO_2/GOx in varied glucose concentrations. In chapter 5.4, the limit of detection (LOD) analysis was conducted on both MOF-199/GOx and SiO_2/GOx nano composites. This analysis was important in order to measure the sensitivity of both nanocomposites for detection of low glucose concentration particularly for salivary glucose measurement. Finally the chapter 5.5 discusses about GOx stability and reusability. The GOx half life were measured on both MOF-199/GOx and SiO_2/GOx and actual GOx within 30 days when reacted to glucose. The GOx stability and reusability were measured amperometically.

Chapter 6: Conclusions and Future Works Recommendation

This chapter provides the conclusions based on the research objectives and the recommendation for possible future works that enhancing the current research.

CHAPTER 2

LITERATURE REVIEW

2.1 Background

Determination of body glucose level is very important due its clinical and industrial purposes. Glucose biosensors has received many interests especially towards among the researchers nowadays. The development of glucose biosensors has been crucial in order to provide the innovation in medical approach of glucose measurement practices. The production of cost effective, simple, accurate and portable glucose sensors are socially prominent due to the diabetics internationally prevalence that affect 8.5 % of the world's population (Roglic *et al.,* 2016). These innovations are very crucial as to provide the stepping stone towards the fabrication of highly sensitive glucose.

2.2 Diabetes

Diabetes is one of the most fatal diseases which accumulates of 8.5% of total world population. Reportedly the number of diabetic patients has increased tremendously from 108 million in 1980 to 422 million in 2014 (Roglic *et al.,* 2016). Diabetes prevalence has increased significantly especially in middle income and low income countries (Guariguata *et al.,* 2014). These would result towards massive supply in medical applications as well as medications. As discussed, diabetes could result in major metabolic dysfunctions (Hameed *et al.,* 2015; American Diabetes Association *et al.,* 2010). If diabetes are not medically controlled, it will result to other severe diseases including blindness, kidney failure, heart attacks, stroke and lower limb amputation (Hameed *et al.,* 2015; Roglic *et al.,* 2016). These complications may result in disability and death. They have a direct impact on the health care system, families and loved ones. It was estimated that 1.5 million deaths reportedly were directly caused by diabetes and 2.2 million deaths were attributable to high concentration of body glucose level (Roglic *et al.,* 2016). World Health of Organization estimates that diabetes will be the 7th leading cause of fatality by the

year of 2030. There are three main categories of diabetes; type I diabetes, type II diabetes, and gestational diabetes. While type I and type II diabetes are more serious, yet the pre-diabetes and gestational diabetes are equally crucial to allow better prevention steps (Marchetti *et al.,* 2016).

2.2.1 Type I Diabetes

Type I, also known as juvenile diabetes or insulin-dependent diabetes, resulted in immunity disorder that cause deficient insulin production. In type 1 diabetes, the patient's lymphocytes (white cells) attack the insulin-producing cells in the pancreas, causing the major disruption towards the ability on insulin productionn (Ozougwu *et al.,* 2016). Majority of diabetic patients with type I diabetes are diagnosed during very young age. There is still unknown cause as well as prevention steps of type I diabetes (Ozougwu *et al.,* 2016). Thus, patients who are diagnosed with type I diabetes are not able to be cured and must be supplied with insulin in order to live (Chiang *et al.,* 2016). Type I diabetic patients need a constant glucose measurement to allow accurate insulin intake to avoid any shortage or excessive of insulin intake (Rewers *et al.,* 2014).

2.2.2 Type II Diabetes

Type II diabetes has been known as non-insulin dependent. This type of diabetes results from the ineffectiveness of body cells to react with normal amount of insulin supplied (Peyser *et al.,* 2014). This type of diabetes cause the robust production of insulin from pancreas in order to reach the optimum level of needed in blood glucose hormone (homeostasis) (Röder *et al.,* 2016). In the continuous time, the insulin-producing cells in the pancreas can be self-ruptured due to excessive production. The inability to produce enough pancreatic insulin begins the diabetic patients with type II diabetes to take insulin medication (Röder *et al.,* 2016). This type of diabetes comprises the majority of diabetic patients worldwide. This type of diabetes might have been resulted from unhealthy food intake, excess body weight and physical inactivity (Hu *et al.,* 2011). Therefore, this illness are usually diagnosed in adults since it can only be diagnosed several years after onset (pre-diabetes).

However, nowadays this type of diabetes is occurring among kids due to unhealthy lifestyles (Forouhi *et al.,* 2010). Nonetheless, unlike type I diabetes, diabetes type II could be prevented and be managed properly by taking the prevention steps such as healthy food intake, good weight management, exercises and managing healthy lifestyle (Asif *et al.,* 2014).

2.2.3 Gestational Diabetes

This type of diabetes is a a condition when blood glucose concentration is above the normal level but below the diagnose of diabetes during pregnancy period (Buchanan *et al.,* 2012). The main cause of gestational diabetes is still not fully understood (Buchanan *et al.,* 2012). However, the general relationship between pregnancy and diabetes can be observed through the connection of pregnant women's bloodstream to placenta of the fetus (Vambergue *et al.,* 2011). Generally, insulin that is produced in pancreas is needed to breakdown glucose from food intake to release energy (Magon *et al.,* 2011). However, as the fetus grows, many more hormones are produced and released from placenta to the bloodstream, causing some inhibition towards insulin. This will result towards inability to breakdown glucose and causing high blood glucose level for pregnant women (Magon *et al.,* 2011). However, this kind diabetes can be controlled through healthy food intake. The risk factor is higher if a pregnant woman has body mass index (BMI) of more than 30 prior to the pregnancy, pregnant after the age of 25 and a has a pre-diabetic or family historical background of having diabetes (Buchanan *et al.,* 2012). Nevertheless, this type of diabetes is usually consists during pregnancy period and sometimes can appear after giving birth. Often glucose checking is very crucial to monitor the glucose level accurately in order to manage the glucose level throughout the pregnancy period.

2.3 Biosensors

Biosensors have been developed from time to time in various field of interests. The development of biosensors have gained many interests from researchers since the combination of this technologies are fairly important especially towards the development of new research interests as well as in industrial applications. Potential

applications of biosensors embrace literally every possible analytical task, ranging from medical diagnostics through drug discovery, food industry, environmental process monitoring, to security applications (Ahmad *et al.*, 2015). Biosensor is actually an acronym of biological sensor. This term is often applied to describe the analytical sensors devices, incorporating with biological element in order to detect the actual presence or concentrations of any substances or chemicals (Ahmad *et al.*, 2015).

The concept of biosensor was first initiated by Leyland C. Clark in 1962 (Setford *et al.,* 2005; Turner *et al.,* 2013). Clark first came out with his idea of "enzyme electrode" by his invention of oxygen electrode. The electrode was used to measure the electrochemical detection of glucose and hydrogen peroxides that incorporated with enzyme immobilization method (Setford *et al.,* 2005; Turner *et al.,* 2005).

These concepts were applied to the very first glucose biosensor, by attaching the glucose oxidase (GOx) to platinum electrode. The functionalized platinum electrodes were then applied to a better analytical device for human body glucose level monitoring (Harper *et al.,* 2010). Two decades later, the first optical transducers were harnessed in conjunction with antobodies to fabricate real time bioaffinity monitors (Harper *et al.,* 2010). The bioaffinity sensor was pioneered by Ingemar Lundström and his team at Linköping University together with the BIAcore™ company which later owned by Pharmacia and currently owned by GE (Turner *et al.,* 2013).

Enzyme based biosensors is one of particular interests, as they exhibit exceptional specificity to particular substrate or chemical in which desirable feature as a sensor. Enzyme in specific is globular proteins built up from amino acid residues ranging from few dozens to few thousands residues (Lakhtakia *et al.,* 1990). The biological enzymes act in lowering activation energy (E_a), catalyzing the chemical or biochemical reactions (Lakhtakia *et al.,* 1990). An enzyme comprises of an active site, in which substrate is attached to, and a shell surrounding. The process of enzyme reaction with substrate is defined as 'lock and key' process (Johnson *et al.,* 2008). The substrate will fit into the enzyme's active site to allow the catalyzation process (Johnson *et al.,* 2008). Therefore, the active site's shape is very important to

allow only specific substrate to be ‘fit’ into. This reaction provides high selectivity and sensitivity towards biosensing application as it will reduce tremendously the chance for contaminant measurement (Johnson *et al.,* 2008).

There are two main components of biosensor in general; biomolecules and the transducers:

2.3.1 Biomolecules

Biomolecules are molecules which are engaged in the maintenance and metabolic processes of living organisms (Avenas *et al.*, 2012).. These non-living molecules are the actual foot-soldiers of the battle of sustenance of life. The main component of biomolecules is carbon (C) They range from small molecules such as primary and secondary metabolites and hormones to large macromolecules like proteins, nucleic acids, carbohydrates, lipids (Avenas *et al.*, 2012).. The biological component of biosensor can include macromolecules, enzymes, microorganisms and tissues, antibodies, and nucleic acids (Sharma *et al.,* 2003).

They are collectively called as saccharides (Flitsch *et al.,* 2003). Depending on the number of constituting sugar units obtained upon hydrolysis, they are classified as monosaccharides which contain only one unit of sugar, oligosaccharides which contain two to eight units and polysaccharides (more than 10 units) (Avenas *et al.*, 2012). They have multiple functions as they are abundant dietary source of energy; they are structurally very important for many living organisms as they form a major structural component, e.g. cellulose is an important structural fiber for plants (Avenas *et al.*, 2012).

Proteins are another class of indispensable biomolecules which make up around 50% of the cellular dry weight. Proteins are polymers of amino acids arranged in the form of polypeptide chains. The structure of proteins is classified as primary, secondary, tertiary and quaternary in some cases. These structures are based on the level of complexity of the folding of a polypeptide chain. Most enzymes are proteinaceous in nature (Sharma *et al.,* 2003). Enzymes are extensively used as biomaterials for the

development of biosensors such as glucose oxidase in glucose sensor. Immobilized enzymes, together with electrochemical sensors, are used in several instruments available commercially (Sharma *et al.,* 2003). However, sometimes enzyme-based sensors are hampered due to their stability at desired temperature (Sharma *et al.,* 2003). Besides, some enzymes even require cofactors for their optimum activity (Sharma *et al.,* 2003; Arnold *et al.,* 1987). Enzymatic amperometric glucose biosensors were the most common devices commercially available, and have been widely studied over the last few decades (Yoo *et al.,* 2010).

Nucleic acids refer to the genetic material found in the cell that carries all the hereditary information from parents to progeny (Du *et al.,* 2016). There are two types of nucleic acids namely, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Teles *et al.,* 2008). The main function of nucleic acid is the transfer of genetic information and synthesis of proteins by processes known as translation and transcription (Teles *et al.,* 2008). DNA based biosensors, also named as DNA sensors or genosensors, are adoptable in inspecting individual genomic or genetic details of a patient or nucleic acid sequences for pathogens invasion (Drummond *et al.,* 2003). Genomic sequences analysis has provided a sensitive technological foundation for quantitatively detect infectious disease pathogens and genetic variations (Du *et al.,* 2016)

Microorganisms could also act as the biomolecules for biosensors (Park *et al.,* 2013). Whole cells could substitute any specific protein or enzyme since they have the benefits of low cost and improved stability comparing to enzymes or other proteins (Park *et al.,* 2013). Enzyme or proteins purification could be avoided and cells can be massively produced through a simple cell culturing step and the necessary cofactors are already present inside the cells (Su *et al.,* 2011). Moreover, microorganisms are easy to manipulate and have better stability under harsh environments. Microbial auxotrophy have been used to monitor growth-limiting small molecules (Du *et al.,* 2016). For instance, *E. coli* was used as microbioal biomolecule strain for the detection and quantification of mevalonate, which is important secondary metabolites that includes flavor, fragrance, anti-oxidants, steroids, and the anti-malarial drug (Pfleger *et al.,* 2007). Amperometric sensors monitor currents

generated when electrons are exchanged either directly or indirectly between a biological system and an electrode (Yoo *et al.,* 2010).

2.3.2 Transducers

In the presence of target analytes, biological events are converted into an electrical or optical signal in proportion to the target concentration (Park *et al.,* 2013). A transducer converted the biomolecule event into a measurable signal and a signal processing system that converts the signal into a readable form.The five principal transducer classes are electrochemical, optical, thermometric, piezoelectric, and magnetic (Yoo *et al.,* 2010; Wang *et al.,* 2008; Sergey *et al.,* 2008; Walton *et al.,* 1993). The transducer that is applied for biosensor is called as biotransducer (Wang *et al.,* 2008). There are two most common biotransducers which include electrochemical and optical transducers (Wang *et al.,* 2008; Sergey *et al.,* 2008).

Electrochemical biotransducers covert the biomolecule element that selectively reacts with the target analyte to produce an electrical signal that is proportional to the analyte concentration (Wang *et al.,* 2008). Electrochemical biotransducers include amperometry, potentiometry and conductometry (Wang *et al.,* 2008). A potentiometric biotransducer is a type biotransducer that is used to determine the analytical concentration of some components of the analyte by measuring the electrical potential produced by the reaction between biorecognition and analyte (Yoo *et al.,* 2010). Meanwhile, conductometric biotransducer is a measurement of electrolytic conductivity to monitor a progress of chemical reaction between biorecognition and analyte (Lubert *et al.,* 2010). It is an old method to measure the concentration of an analyte using alternating currents which was first introduced in 18th century (Lubert *et al.,* 2010). The most common biotransducer for electrochemical biosensors is the amperometric type, because of their better sensitivity, reproducibility, easy maintenance and low cost (Ahmad *et al.,* 2015). Amperometric biotransducers detect the reaction between biomolecule and targetted analyte from oxidation or reduction process on which result in electron movement (Wang *et al.,* 2008; Kong *et al.*, 2009). The movement of electrons during this redox reaction would produce current which is the direct measure of electron transfer

(Wang *et al.,* 2008; Kong *et al.*, 2009). Typically, biomolecules are immobilized on supports that act as working electrode for electrochemical cell analysis (Ahmad *et al.,* 2015). The amperometrical glucose biosensor was first introduced by Clark and Lyons (Wang *et al.,* 2008; Wang *et al.,* 2001). This first glucose biosensor used oxygen electrode to measure the redox reaction and electron movement between glucose and GOx (Wang *et al.,* 2008; Wang *et al.,* 2001). The concept for amperometric glucose biosensors have been employed in the next generations of glucose biosensors as this concept provided the best and fast measurement for body glucose level (Yoo *et al.,* 2010).

Optical biotransducers are thee most common type of biosensor (Damborsky *et al.,* 2016). Optical detection is conducted by measuring the reaction between the optical field with a biorecognition element (Damborsky *et al.,* 2016). Optical biosensing can be classified into two types: label-free and label-based. Generally, a label-free mode, the detected signal is generated directly by the interaction of the analysed material with the transducer (Damborsky *et al.,* 2016; Sergey *et al.,* 2008). Nonetheless, label-based sensing involves the use of a label and the optical signal is then generated by a colorimetric, fluorescent or luminescent method (Damborsky *et al.,* 2016). Optical biotransducers are employed in optical biosensors for signal transduction. This type of tranducer uses photons in order to collect information about analyte (Sergey *et al.,* 2008). An optical biosensor is a compact analytical device containing a biorecognition sensing element integrated with an optical transducer system. The basic objective of an optical biosensor is to produce a signal which is proportionate to the concentration of a measured analyte (Thient *et al.,* 2018). This method follows the actual optical absorbance law known as Beer's Law (Mohamad *et al.,* 2015). Beer's Law, states that the optical absorbance of a chromophore (molecule) in a transparent solvent varies linearly with both the sample cell pathlength and the chromophore concentration, therefore the increase of the analyte's concentration will increase the absorbance of the solvent (Mohamad *et al.,* 2015). Simple molecules such as glucose can be measured by enzymatic oxidation using label-assisted sensing (Damborsky *et al.,* 2016). The blood glucose analysis is the most commercially successful application of a glucose biosensor biosensor,on which applied in clinical glucose meter for clinical glucose measurement (Damborsky *et al.,* 2016; Yoo *et al.,* 2010). The optical transducers were also

harnessed in conjunction with antibodies to create real-time bioaffinity monitors. These immunosensors laid the foundation for the second major evolutionary line of biosensing instrumentation (Ahmad *et al.,* 2015; Yoo *et al.,* 2010).

2.4 Glucose Biosensor

Body glucose measurement for monitoring diabetes comprises approximately 85% of the world market for biosensors. The significant success demonstrated the utility of the technology and sparks the questions among researchers on whether glucose biosensor is the only field that subsequently worth pursing. Hence, what has made the glucose sensors were among the most essential demand in industry? The extraordinary demands came from diabetic patients as well as awareness on about the dangerous of diabetes.In 1993, Diabetes Control and Complications Trial (1993) published an article that published an unequivocal proof that careful monitoring and control of blood sugar could reduce the horrific side effects associated with chronic diabetes including blindness, amputation, kidney and organ failure (Diabetes Control and Complications Trial Research Group *et al.,* 1993). As a result, this article had increased major awareness about diabetes to many people. Moreover, some of severe diabetic patients require to measure their daily glucose level several times a day. In addition, this medical self-monitor device has helped in reducing the number of hospital loads (Diabetes Control and Complications Trial Research Group *et al.,* 1993).

The development of glucose biosensors kept on rising and considered as one of mature products by many companies. The development of glucose biosensor has offered more opportunities not only in chemical and biochemical research and development (RnD) sectors, but in engineering sectors as well such as integration of the sampling, sensing and data processing elements of these devices. Researchers have developed many more glucose biosensors especially to reduce the pain associated with the blood withdrawal procedures (Bruen *et al.,* 2017). The current state-of-the-art relies on being able to detect corresponding glucose on very small blood samples (1–3 μL) (Bruen *et al.,* 2017). Nevertheless, the after effect of continuous blood withdrawal is still concerned as it may become too invasive

towards severe diabetic patients particularly for young children and elder patients.

2.4.1 First Generation of Glucose Biosensor

The first generation of glucose biosensor was proposed by Clark and Lyons idea that was first applied at the Children Hospital of Cinnati (Wang *et al.,* 2001). The glucose sensors were composed of oxygen electrode with semi-permeable thin layer of GOx covered with dialysis membrane at for the outest layer (Wang *et al.,* 2001). The GOx was immobilized on the electrochemical detector in order to detect the corresponding oxygen concentration. The glucose corresponding glucose concentrations were measured by measuring the concentration of absorbed oxygen (O_2). The decrease of O_2 was proportional to the glucose concentration (Yoo *et al.,* 2010). The first official model of glucose biosensor that applied Clark's technology was manufactured by Yellow Springs Instrument Company (Model 23A YSI Analyzer) (Wang *et al.,* 2001). Nonetheless, these biosensors were only used in clinical laboratories as the devices were very expensive since they consisted of platinum rod (Pt) and required proper handlings of execution (Wang *et al.,* 2001). These devices were still catagorized in first generation of glucose biosensors as the detection of glucose was still based on the reduction of O_2 (Bruen et al., 2017). Although hydrogen peroxides (H_2O_2) was produced through the reaction, the detection of H_2O_2 required special condition and high selectivity (Bruen et al., 2017). The first glucose biosensor that was based on amperometric measurement was then that manifactured by Genetics International and later MediSense, which was incorporated in Boston, USA with lab that was initially based at Cranfield, UK (Turner *et al.,* 2013)

2.4.2 Second Generation of Glucose Biosensors

The first generation of glucose biosensors were innovated using the second generation of 'mediated' glucose biosensors. The improvement made was by replacing O_2 electrode with nonphysiological electron acceptors named as redox mediators. Reduced mediators were formed instead of H_2O_2 and the reoxidized at the electrode (Schuhman *et al.,* 1991). The typical mediators used in second of glucose biosensors including ferrocene, ferrycyanide, quinnine and tethrafulvalene (TTF)

(Yoo et al., 2010). Cranfield and Oxford Universities were then collaborated to bring ferrocene-mediated electrochemistry of GOx. He technology was then sold to Abbott, USA on 1996. The Boehringer Mannheim (latterly Roche) bought the technology of ferricyanide-mediated GOx glucose biosensor from a small company called as Tall Oaks (Turner *et al.,* 2013).

2.4.3 Third Generation of Glucose Biosensor

The medical demands of glucose biosensor were enormously rising until the researchers came out with the third generation of glucose biosensor. Unlike first generation and second generation of glucose biosensors, the third generation of glucose biosensors did apply the direct electron transfer between enzyme and electrode without any mediator (Turner *et al.,* 2013). The main reason of this new approach was because, mediators could be very toxic and require specific execution of glucose measurement (laboratory testing) (Turner *et al.,* 2013). The researches found that high conductivity electrodes were able to directly receive electrons using organic conditioning materials such as enzymes (Grieshabe *et al.,* 2008). The pioneer The Johnson & Johnson Lifescan was the pioneer for the third generation of glucose biosensor (Bollella *et al.,* 2017; Yoo *et al.,* 2010). The Johnson & Johnson Lifescan bought the technology of ferrocene-mediated GOx sensor with capillary fill for capillary prick glucose biosensor from SME, Kyoto Daiichi Kagaku that is known as Akray now. These technology were then improvised to non mediated electrode glucose biosensors. Therefore, the 'big four' companies (Bayer, Roche, Abbot, Johnson & Johnson Lifescan) dominated about 90% of sales in glucose sensing market (Bollella *et al.,* 2017; Yoo *et al.,* 2010). These glucose biosensors have been improvised and innovated to the current type of glucose biosensors which are mainly by needle type from high conductivity material such as gold, polypyrole and boron doped diamond for continuous in vivo monitoring glucose concentration (Turner *et al.,* 2013).

The table 2.1 provided the data for current conventional glucose biosensors from the thrid geeneration fo glucose biosensors. Based on the table, the highest limit of detection (LOD) for current glucose biosensor was recorded at approximately 0.6

mM by Akray, Bayer and Roche (Yoo *et al.,* 2010). The LOD recorded were still high to be applied for salivary glucose measurement since the concentration of glucose in saliva can be as low as 0.11 to 1.23 mM (nondiabetic and up to 4.22 mM (diabetic) patients (Agrawal et *al.,* 2013).

Table 2.1: The performance of different glucose biosensors (Yoo *et al.,* 2010).

Manufacturer	**Brand**	**Assay method**	**Minimal sample volume (uL)**	**Assay range (mg/dL)**	**LOD (mM)**
Abbott	FreeStyle Freedom Lite	GDH-PQQ	0.3	20–500	1.11
AgaMatrix	WaveSense KeyNote	GOx	0.5	20–600	1.11
Arkray	Glucocard X-meter	GDH	0.3	10–600	0.56
Bayer	Ascensia Contour	GDH-FAD	0.6	10–600	0.56
Bionime	Rightest GM300	GOx	1.4	20–600	1.11
Diabestic Supply of Suncoast	Advocate Redi-Code	GOx	0.7	20–600	1.11
Diagnostic Devices	Prodigy Autocode	GOx	0.6	20–600	1.11
LifeScan	OneTouch UltraLink	GOx	1.0	20–600	1.11
Nova Biomedical	Nova Max	GOx	0.3	20–600	1.11
Roche	Accu-Chek Aviva	GDH-PQQ	0.6	10–600	0.56

***** GDH-PQQ: Glucose dehydrogenase pyrroloquinoline quinone

***** GDH-FAD: Glucose dehydrogenase flavin adenine dinucleotide (FAD)

2.5 Drawbacks of Current Glucose Biosensors

Based on current development of glucose biosensors (third generation of glucose biosensors), the method that officially approved clinically was based on finger prick technique to allow minimal blood withdrawal from human body (Roglic *et al.,* 2016). This method is essential to minimize the after effect complication or blood withdrawal including bruises or wounds that can be invasive towards patients. However, these practices could still be painful especially towards children and elder severe diabetic patients (Agrawal *et al.,* 2013). Even though finger prick technique has been attempted to reduce massive pain, the continuous measurement or malpractice of these methods would still lead towards uncomfortable, messy and often has to be repeated several times every day (Agrawal *et al.,* 2013).

Salivary glucose measurement can be attempted with high sensitivity fglucose sensing material (Agrawal *et al.,* 2013). Nonetheless,current glucose biosensors could only detect at the minimum glucose concentration of 10 mg/mL (0.56 mM) as shown in table 2.1. The limit of detection (LOD) was not able to detect the salivary glucose concentrations which ranging from 0. 11mM to 0.42 mM in fasting salivary glucose (FSG) (Gupta *et al.,* 2017).

Furthermore, current glucose biosensors are not reusable and have low stability due to sensitivity of enzymes eg. GOx towards surrounding (Roglic *et al.,* 2016). GOx was immobilized in different supports to avoid any enzyme loss (House *et al.,* 2007). The repetitive reaction of GOx with glucose could alter the GOx active site (Moehlenbrock *et al.*, 2011). Therefore, the whole process of fabrication of glucose biosensors require more GOx and supports for immobilization which can be very costly (Wang *et al.,* 2001). Thus, the higher stability of glucose biosensing materials could reduce the labor cost for production of current glucose biosensors.

To add, GOx immobilization methods have gained many interests by researchers in order to avoid enzyme loss on the support (House *et al.,* 2007). Therefore, many immobilization procedures involving GOx have been attempted in previous studies including covalent bonding, cross-linking, physical adsorption and gel entrapment

methods. However, due to complex methods and expensive materials (gold, platinum, inert proteins and high conductivity electrodes) in previous studies, none of these methods and materials have been employed in marketed glucose biosensors (Homaei *et al.,* 2017; Yoo *et al.,* 2010; House *et al.,* 2007; Mohamad *et al.,* 2015; Wang *et al.,* 2001). Therefore, facile and direct method of immobilization on support would be suitable for industrial production of glucose biosensors.

2.6 Enzyme Immobilization Methods for Biosensing Application

In order to produce a good biosensor, enzymes needed to be attached properly on the surface of the support without losing the enzyme catalytic activity. The process of enzyme attachment is called as immobilization (Walcarius *et al.,* 2013) . Enzymes were needed to be optimally loaded on the surface of the support in order to increase the sensitivity as well as the limit of detection (LOD) of biosensors. Enzymes are very sensitive towards certain ambiences; therefore enzymes were needed to be appropriately attached in certain conditions to avoid any denaturation to occur (Zhou *et al.,* 2010). There were several procedures that can be applied for immobilization of enzymes. The immobilization procedure was chosen due to many considerations for example type of transducer, physiochemical of the analyte, and the presence of material (Zhou *et al.,* 2010).

One of the most prominent roles of enzymes as natural biocatalysts is their ability to increase the rate of chemical reactions within a cell. The rate of the reactions are increased tremendously up to over a million-fold, thus the reaction of catalysis can now occur within fractions of a seconds, without taking years in absence of enzymes (Mäntsälä *et al.,* 2009). Therefore, most biochemical reactions are significantly slowed down in the absence of enzymes thus, that they would no longer be able to sustain complex life (Mäntsälä *et al.,* 2009). Enzymes react with specific substances without being permanently altered after each reaction (Wei *et al.,* 2014). They also increase the rate of reaction without changing the equilibrium state between both reactants and products. Nonetheless, the use of enzyme is usually related with other drawbacks resulting from sensitivity towards ambiances such as low stability towards temperature, pH and concentrations that may result towards the the loss of

enzyme's activities (Collins *et al.,* 2003).

The majority of enzymes are unstable towards mild to extreme ambiances and industrial application is often affected by a lack of long-term enzymatic stability as well as challenging recovery process and reuse of the enzyme (Collins *et al.,* 2003).

In order to increase the enzyme's usage industrial purposes, the bioengineering methods are utilized to provide more cost effective and durability to these enzymes. One of the methods is by enzyme immobilization process.

The process of enzyme immobilization can be defined as the attachment of enzyme in a distinct solid support (Husain *et al.,* 2017). The supports of immobilized enzyme shall allow the exchange medium containing enzyme to react with its specific substrate or chemical in order to produce desired reaction (Husain *et al.,* 2017; Homaei *et al.,* 2013). This process is very important in the production of biosensor mainly in the applications of glucose biosensors. There are advantages of enzyme immobilization as well as the drawback of these methods as in Table 2.2.

Table 2.2: The advantages and disadvantages of enzyme immobilization (Husain *et al.,* 2017; Homaei *et al.,* 2013; Brena *et al.,* 2013)

Advantages of Enzyme Immobilization	Disadvantages of Enzyme Immobilization
● Functional efficiency of enzyme can increased. ● The reproducibility of enzyme is enhanced ● Reusability of the enzyme is increased ● Providing less labor input in the process ● Provide minimum reaction time compared to conventional method ● Minimal risk of contamination in products ● Providing a high enzyme-substrate ratio.	● The processes of enzyme isolation, purification and recovery of active enzyme are very time consuming and very costly. ● Industrial applications are limited and only very few industries are using immobilized enzymes or immobilized whole cells. ● Catalytic properties of some enzymes can be reduced or completely lost after their immobilization on support or carrier. ● Some enzymes lost its stability after immobilization. ● Enzymes can be easily denature (dysfunction) in extreme temperature and pH.

Therefore, the enzyme immobilization has been improvised and provided by many kind of approaches to optimize the advantages and reduce its disadvantages. The main problems encountered are the cost and time involved in the enzyme purification

(Datta *et al.,* 2013). The biochemical method of producing enzyme are expensive and time consuming; hence immobilization procedures are the option to reduce the cost and time (Datta *et al.,* 2013). Furthermore, enzymes have many other limitations such as low stability, highly sensitive to the process conditions and these problems can be overcome by the immobilization techniques (Husain *et al.,* 2017; Homaei *et al.,* 2013). The immobilization process may cause the disruption in enzyme catalytic activities as reported (Zdarta *et al.,* 2018). Therefore, safe and facile method of enzyme immobilization is important to minimalize the disadvantage as well as maximizing the advantages of these processes. There are many types of enzyme immobilization methods including cross-linking, covalent- bonding, gel entrapment, and physical adsorption.

Apart of providing better stability for enzyme, immobilization of enzyme should be able to allow facile separation of enzyme from the product in order to reduce the contamination of both enzyme and chemicals. Besides, the enzyme immobilization procedure should be cost effective and able to provide better reusability for biochemical process that reduces the enzyme purification costs (Ahmad *et al.,* 2015).

Immobilization of enzymes to the solid support will benefit the enzyme catalytic activities which include increment to rate of reaction by removal of the enzyme from the reaction solution and improvement of enzyme stability against ambiances such as extreme temperature and pH (Guzik *et al.,* 2014). It also helps for efficient recovery and reusabilty of expensive enzymes (Guzik *et al.,* 2014; Homaei *et al.,* 2013). Therefore, it is possible to conclude that good enzyme immobilization increases the productivity of enzymes and reactant, making them more attractive for diverse applications particularly in biosensing.

The enzymes can be attached by interactions ranging from reversible physical adsorption, chemical bonding, covalent bonding and gel entrapment (Mohamad *et al.,* 2015).

Basically, there are four types of immobilizations procedures which include physical adsorption, chemical bonding immobilization, cross-linking immobilization and gel entrapment.

2.6.1 Cross-Linking

Cross-linking is the process when biomaterials are chemically bonded to cross-linking agent or other matrices and solid support (Davidenko *et al.,* 2015; Shao *et al.,* 2010). Enzyme can be cross-linked with each other or with functionally inert protein to provide strong support among them and provide more biological environment among the enzymes and cross-linker (Zdarta *et al.,* 2018; Homaei *et al.,* 2015). This method was widely employed due to its simplicity and strong chemical binding formed. However, this method can lead to the massive losses of enzyme catalytic activities due to major alteration of enzyme conformation that might affect the enzyme catalytic function (Zdarta *et al.,* 2018; Homaei *et al.,* 2015).

There are few main procedures involved in enzyme cross linking. Firstly, the enzyme is attached to a solid support via a cleavable linkage before the enzyme is cross-linked with itself. The last step is the release of the cross-linked monomer (Zdarta *et al.,* 2018; Homaei *et al.,* 2015) .

Cross-linking is a irreversible method of enzyme immobilization that does not require a solid support to prevent enzyme loss into the substrate solution (Mohamed *et al.*, 2015). This is due to enzymes are cross linked towards each other in order to sustain the activities. The method is also called carrier-free immobilization where the enzyme acts as its own carrier. The involvement of carriers will result in tremendous increment in cost (Mohamad *et al.*, 2015).

Cross-linking of enzymes to nanomaterials have proven better residual activity. This was due to increased surface area and porosity.

2.6.2 Covalent Bonding

Covalent bonding immobilization was the most studied immobilization method. In this method, ezymes are covalently attached to a surface or support matrix (Shao *et al.,* 2010). Immobilization of enzyme or biomolecule by covalent-bonding is one of the widely employed for enzyme attachment methods (Yoo *et al.,* 2010). An advantage of using this was providing strong covalent bonds between enzyme and support that would avoid enzyme leaching (Yoo *et al.,* 2010). Reportedly, enzymes were attached to support *via* functional groups that not crucial for enzyme catalytic activity (Zhou *et al.,* 2010). The most frequently used covalent bonding formation involved the following side chains of the amino acids; lysine (aimno group) and aspartic and glutamic acid (carboxylic group) (Zhou *et al.,* 2010). Even though this method was easy to apply, this method could bond to enzyme's active site which could result in the disruption of enzyme's activities.

This immobilization method could provide strong bonds between the enzyme and its carrier matrix, allow its reuse more often than with other available immobilization methods and prevent enzyme release into the reaction environment. The method also increases half-life and thermal stability of enzymes when coupled with different supports.

This method has been used in many fabrication of biosensors. This method was based on the attachment of the enzymes to solid supports by covalent bonds that requires the formation of bond between enzyme and the carriers as well as the bond between carriers to the solid support (Mohamad *et al.,* 2015). Thus, the covalent bonding will be formed between both enzyme to the biological carriers to the carrier of the solid support. The outcome of this immobilization is a stable and a strong bond between enzyme and solid support (Homaei *et al.,* 2013; Brena *et al.,* 2013).

Nonetheless, covalent bonding immobilization requires different reactions depending on the type of given enzyme or carriers used in the immobilization process. The choice of reactions is limited by two aspects: the binding reaction must be performed under specific enzyme conditions that do not cause loss of enzymatic activity, and

the active site of the enzyme must not be involved in the covalent bonding process (Brena *et al.,* 2013).

The conditions for covalent bonding immobilization are more complicated as compared to cross-linking and physical adsorption methods. This method provides high binding force between enzyme and support that may avoid any leakage of enzymes even in the presence of substrate of solution in high ionic strength. However, due to the strong covalent bond formation between enzymes and solid carriers, this method may result to the alteration of enzyme's conformational structure as well as the active center of the enzyme, resulting in major loss of activity and changes of the substrate (Mohamad *et al.,* 2015).

Covalent bonding is one of the most widely employed methods for irreversible enzyme immobilization. The functional group that involves in the immobilization of the enzyme usually involves the chains of lysine (ϵ-amino group), cysteine (thiol group) and aspartic and glutamic acids (carboxylic group) and imidazole and phenolic groups which are not essential for the catalytic activity of enzyme (Mohamad *et al.,* 2015).

Even though covalent bonding immobilization provides a strong bond between enzyme and support, this method is very sensitive. The direction of enzyme binding is prominent for covalent bonding attachment in order to provide optimum stability as well as highest activity (Hoarau *et al.,* 2017). The direction of the enzyme binding is a crucial factor that determines its stability. It has been reported that the highest enzyme activity level is achieved when the active centre of amino acid is not involved in the binding with the support (Hoarau *et al.,* 2017). The improper attachment of enzyme covalent bonding immobilization will result in total loss in enzyme catalytic activities as the result of covalent attachment to the enzyme catalytic active site (Mohamad *et al.,* 2015).

Furthermore, the another drawback of enzyme covalent bonding immobilization is the procedure of covalent bonding attachment requires a carrier to link the enzyme to the solid supports (Mohamad *et al.,* 2015). As discussed before, the presence of

carriers will result in major decrement of enzyme rate of reaction (Homaei *et al.,* 2013). In addition to that, the carrier is usually expensive such as inert proteins (Homaei *et al.,* 2013). This will result to the increase of immobilization cost as well as time consuming.

2.6.3 Gel Entrapment

The entrapment method is based on the entrapment of an enzyme within gel lattices that that allows the movement of enzyme through the gel lattices (Mohamad *et al.,* 2015). The gel lattices are the polymer that hold the enzymes in order to limit the enzymes activities not to be lost to the surrounding. The practical use of these methods is limited by mass transfer limitations through membranes or gels.

Entrapment is an enzyme immobilization method which provide an irreversible method where enzymes are entrapped in a support or lattices. The support or lattice must be facile to allow the movement of enzyme through it. Therefore, entrapment is a method that provide mechanical support and restriction for enzymes from leaching out. The solid support must nit be chemically reacted with the enzymes to avoid extra loss of the enzymes (Mohamad *et al.*, 2015).

The entrapment also enables the ability to created optimal condition towards fpr emzymes to react (Mohamad *et al.*, 2015). The optimum condtions for enzymes are including optimal pH, polarity and affinity (Zucca *et al.,* 2014). Thus, the selection of polymers, lattices or supports are crucial to optimize the micro ambiances for enzymes thus maintaining the enzymes catalytic activities.

Nonetheless, the entrapment method provides major drawbacks to the withdrawal of enzyme from the support. The pore size of support materials are very crucial in order to allow the adsorption of the enzymes off the support (Zucca *et al.,* 2014). The adsorption could not be possible of the pores of the support are too small or the extra withdrawal of enzymes could be resulted from large pores of the solid support (Zucca *et al.,* 2014). The simplest methods and materials that are facile for enzyme entrapment polyanionic or polycationic polymers by the addition of multivalent

counter-ions. The usage of following polymers as a matrix: alginate, collagen, carrageenan, gelatin, silicon rubber, polyacrylamide, polyurethane and polyvinyl alcohol with styrylpyridium group. Alginates are commonly used polymers due to their mild gelling properties and non-toxicity. However, the practical use of these polymers were limited as one can provide major withdrawal of enzymes causing extra loss of enzyme catalytic activities of substrate or analyte to the enzyme active site (Mohamad *et al.,* 2015). The other disadvantage of this method is the possibility of the leakage of enzymes which can occur if the pores are too huge (Homaei *et al.,* 2017). This method could also lead towards the deactivation of enzyme during immobilization. In addition to that, if the pores from the gel are too small the enzyme withdrawal will be too small and could not react with glucose sufficiently (Zucca *et al.,* 2014). Thereforore, it will provide longer reaction between enzyme and chemicals (substrate). This was due to the limitation of enzyme mobility when entrapped in the gel (Mohamad *et al.*, 2015). Furthermore, the ratio of immobilized particle size to the support material pore size is a significant factor to be considered for the usability of ready probes. Hence, this method would slow the process of reaction between enzyme and glucose.

2.6.4 Physical Adsorption

Adsorption was the simplest immobilization procedure for biosensor fabrication. There were two frequent adsorption methods used for enzyme immobilization process which were physical adsorption and electrostatic interaction. Physical adsorption is basically a simple deposition of an enzyme to a surface and allows attachment through weak bonds (Zhou *et al.,* 2010). The physical adsorption method can be defined as one of the most direct methods of which allow easy and reversible immobilization (Anuar *et al.,* 2015). This method involves physical adsorption of enzymes to attach the enzyme onto any support material. The process of adsorption could occur with physical bonds such as *van der Waals*, hydrophobic interactions and hydrogen bonds (Dhotel *et al.,* 2015). Meanwhile, physical adsorption method could also lead towards the production of ionic bonding in which enzymes are attached through salt linkages with the treatment of buffer or salt solution (Brena *et al.,* 2013).

Meanwhile electrostatic interaction indicated the method where enzyme was attached electrostatically onto charged surface. Usually, the surface of the support was treated with buffer solution to create a hydrophilic surface in order attract the negatively charged biomaterial (Zhou *et al.,* 2010). Regardless to its simplicity, electrostatic adsorption has not been applied for enzyme immobilization particularly in fabrication of glucose biosensor. Therefore this method was attempted and studied in this research.

This method could also provide a better reversible interaction between enzyme and supports. The immobilized enzymes can be discarded from the supports by some chemical interactions and when the attached enzymes have fully decayed and not functioning (Misson *et al.,* 2015). Therefore, this method is highly attractive as when the enzymatic activity has decayed, the support material can be reused and be attached back with fresh enzyme (Misson *et al.,* 2015). This is a huge advantage as the reusability will provide primary factor in the whole cost of immobilized enzymes. This is prominent aspect to provide an easy and cost effective method for biosensor's fabrication.

2.7 Support Materials for Glucose Oxidase (GOx) Immobilization

Nanomaterials are basically microscopy elements that can be measured in nanosize (nm) (Husain *et al.,* 2017; Datta *et al.,* 2013). The small sizes provide high surface area to volume (Datta *et al.,* 2013). Reduction of size of enzyme-carrier could improve the immobilization of enzyme thus could enhance the higher enzyme loading onto the surface. This unique property also allows the tremendous diffusion rate that is essential as support for stabilization of enzyme (Datta *et al.,* 2013). These properties may provide the best properties for enzyme immobilization therefore would increase the sensitivity of glucose biosensors. There are two types of nanoparticles used in this research which were metal organic framework 199 (MOF-199) (porous) and silica spheres (SiO_2) (nonporous).

2.7.1 Metal Organic Framework-199 (MOF-199)

Metal organic frameworks (MOFs) are rising materials, comprise of metal clusters that play role as inorganic joints to connect the organic linker together. MOF-199 provide unique properties such as versatile structures, tunable porosity interesting physicochemical properties, and chemical sensing (Senkovska *et al.,* 2014). The coordination of metal clusters with the organic linker formed three-dimensional (3D) MOFs by repeating the coordination entities (Senkovska *et al.,* 2014). MOF-199 materials have unique properties such as high porosity, high flexibility but crystalline framework, low density high surface area and pore volume. When these organic linkers that consists of ditopic or polytopic carboxylates, linked to metals, would produce the architecturally robust crystalline MOF structures with high porosity of greater than 50% of the MOF crystal volume (Senkovska *et al.,* 2014). The MOF-199 surface area ranging from 1000 to 10,000 m^2/g, thus exceeding those of current porous materials including zeolites and carbons (Mahadi *et al.,* 2015; Senkovska *et al.,* 2014). There are several types of MOFs that have been successfully synthesized . These MOFs are differed based on the type of metal that acts as base in producing MOFs including MOF-5, MOF-199 and MOF-74 (Misran *et al.,* 2013). MOF-199 in particular is the most stable in aqueous solution and has higher porosities compared to other MOFs (Misran *et al.,* 2013). MOF-199 has orthorombic shape as shown in Figure 2.1 (Thi *et al*., 2013; Mahadi *et al.,* 2015). These properties are positive for enzyme immobilization process for fabrication of biosensor. High porosity and low density of MOF-199 could allow maximum adsorption of enzyme onto it. In addition MOF-199 could act as the material for supporting the enzyme shape, hence stabilizing the enzyme's structure. The high loading of enzyme could tremendously increase the sensitivity of the biosensor. MOF pore structure can also be functionalized that would allow the electron transfer that is essential for conductivity.

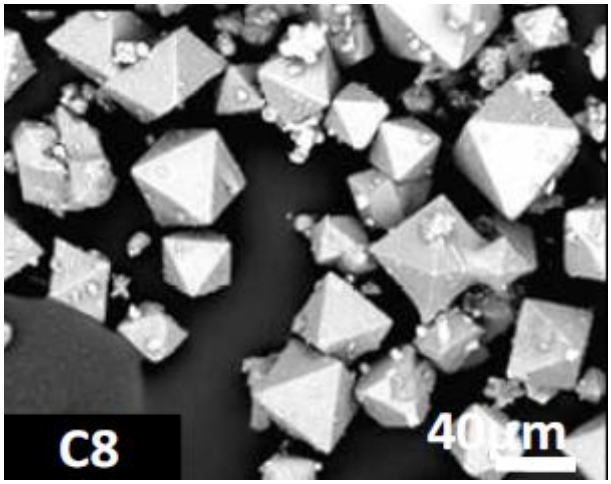


Figure 2.1**:** Scanning Electron Microscope (SEM) image for MOF-199 (Mahadi *et al.,* 2015)

2.7.2 Silica Spheres Nanoparticles (SiO_2)

Silica based organic and inorganic nanocomposites have high potentials as electrode materials since they provide biological-friendly environment for enzyme attachment with a more enhanced electrochemical stability (Rahman *et al.,* 2010). Silica Spheres nanostrutured materials (SiO_2) in particular are a non-toxic materials which could provide the 'cell-like' ambiance for enzyme immobilization and favorable conductivity for biosensor application. SiO_2 particles have sphere in shape and nonporous in morphology shown in Figure 2.2 (Zhu *et al*., 2015; Misran *et al.,* 2013). Furthermore, SiO_2 could as chemical inert to enzyme thus promote the functionalization by its chemical conjugate (organosilane) (Anuar *et al.,* 2014).

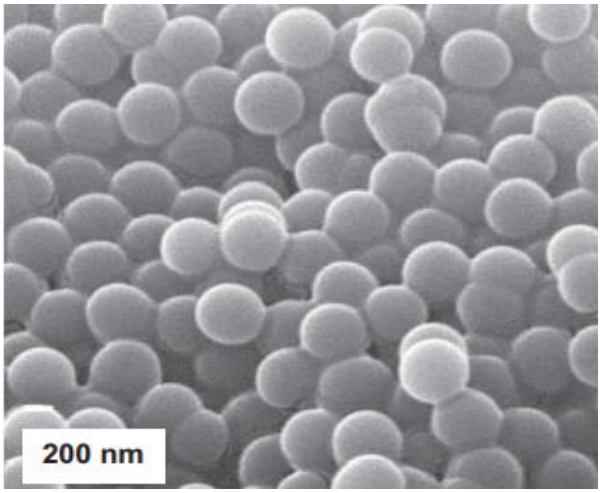


Figure 2.2: FESEM image for Silica Spheres (SiO_2) (Misran *et al.,* 2013)

2.8 Properties of Glucose Oxidase (GOx)

Glucose oxidase (GOx) is an enzyme that oxidizes glucose to gluconic acid. This enzyme was found by Muller in 1928-1936 in Aspergillus niger and Penicillium Glaucum. GOx was also found to catalyze galactose and mannose and not other sugar (Keilin *et al.,* 1948; Turner *et al.,* 2013).

2.8.1 Physical Properties of GOx

GOx is an enzyme thus it is made of protein. It has amorphous shape (Altun *et., al* 2015) (Figure. 2.3). Therefore, just like other enzymes, GOx can be very sensitive towards extreme ambiances. This property is important in order to make sure that enzyme works in the optimum ambience by following the homeostasis within organism. From previous studies, GOx was found to work optimally at pH 7.0 (Keilin *et al.,* 1948; Kong *et al.,* 2009; Turner *et al.,* 2013). The too extreme acidic or basic condition will lessening the activity of the enzyme. GOx works optimally at temperature of 50°C (313 K) (Keilin *et al.,* 1948; Kong *et al.,* 2009; Turner *et al.,* 2013). The increment of temperature from 0°C to 50°C increased the activity of GOx. However GOx activities slowed down after the temperature of 50°C showing that GOx denatured in high temperature (Kong *et al.,* 2009).

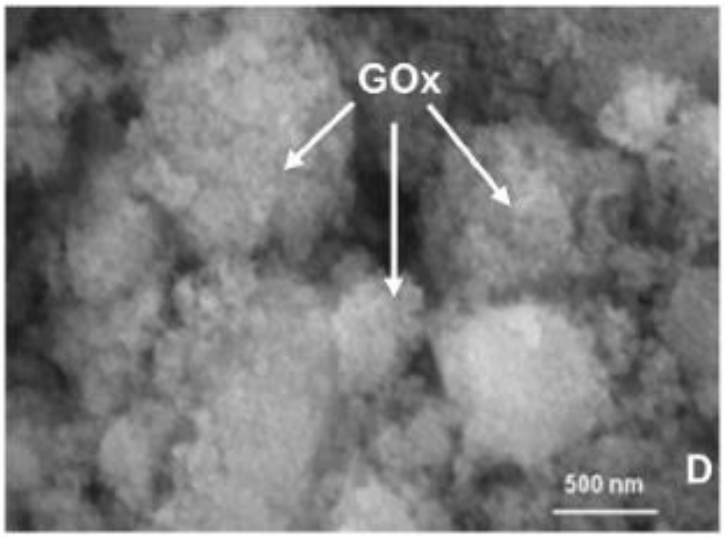


Figure 2.3: FESEM image for GOx (Altun *et., al* 2015)

2.8.2 Chemical Properties of GOx

Glucose oxidase (GOX) from Aspergillus niger is a glycoprotein which consists two identical 80 kilodaltons (kDa) subunits with two flavin adenine dinucleotides (FAD) co-enzymes bound (Keilin *et al.,* 1948, Luca *et al.,* 2007; Wong *et al.,* 2008).GOX catalyses the oxidation of D-glucose ($C_6H_{12}O_6$) to D-gluconolactone ($C_6H_{10}O_6$) and hydrogen peroxide (Keilin *et al.,* 1948, Luca *et al.,* 2007; Wong *et al.,* 2008). GOx exhibited absorbance peak at 280, 380 and 450 nm using UV-Vis spectrophotometer (Wong *et al.,* 2008; Luca *et al.*, 2007; Mohamad *et al.,* 2015).The GOx contains about 20 wt.% amino sugar and about 16–19 wt.% carbohydrate in which 80 wt.% is Nitrogen (N) or O-glycosidically linked mannose molecules (Wong *et al.,* 2008). Therefore, GOx also exhibited N spectra in EDX as it contained amino group (NH) (Balashanmugam *et al.,* 2015). The presence of N element would provide evidence of the presence of GOx. The existence of carbohydrate contribute towards the formation of aldehyde and ketone sugars in GOx (Avenas *et al.,* 2012).

2.8.3 Glucose Oxidase (GOx) Activities

There are two major types of glucose concentration measurement methods; light absorbance measurement (spectrophotometer) (Shih *et al.,* 2009) and amperometric

measurement (Kong *et al.,* 2009). Current glucose biosensors are applied using blood plasma as medium of glucose reading. However, conventional glucose sensors could only detect down to 10 mg/mL (0.56 mM) sensitivity (Zhang *et al.,* 2015). Glucose oxidase activities were recorded in order to measure the capability of biosensors. From previous studies, the glucose measurements were often calculated in mg/dL. Therefore the converstion from mg/dL to mmol/L (mM) is glucose level in mg/dL is divided by 18 (Chase *et al.,* 2000). From table 2.1, the current glucose biosensor produced by current companies including Roche, Lifespan and Nova could only detect as low as 10 mg/dL to 20 mg/dL which are 0.56 mM to 1.1 mM of sugar. These values are sufficient in order to provide good blood glucose measurement.

From the previous studies, the salivary glucose concentrations were measured two main controls; controlled fasting blood glucose (FBG), controlled fasting salivary glucose (FSG), uncontrolled BG (UBG), uncontrolled SG (USG) and salivary glucose for non diabetic patients. The controlled patients needed to fast upon the collection for both FBG and FSG. On the other hand, the UBG and USG groups were not required of fasting.

From the previous results obtained, the average mean for FBG and FSG were 121.53 ± 34.24 mg/dL (6.75 mM) and 4.86 ± 1.37 mg/dL (0.27 mM). (Gupta *et al.,* 2017). The other studies had recorded that the FSG were 7.64 mg/ mL (0.42 mM) (Panchbai *et al.,* 2010), and 5.91 mg/dL (0.33 mM) (Soares M.S *et al.,* 2009). Meanwhile, the SGL recorded were as low as 0.75-2 mg/dL (0.11 mM). (Gupta *et al.,* 2017; Viswanath *et al.,* 2017; Wang *et al.,* 2017). Furthermore, the biochemists have suggested that the normal amount of salivary sugar is below 2.0 mg/dL (0.1 mM) (Gupta et al., 2017).

Meanwhile, the average means obtained for UBG were 10.46 ± 6.50 mg/dL (0.58 mM) (Gopal *et al.,* 2017) and 8.09 ± 6.45 mg/dL (0.45 mM) (Panchbai *et al.,* 2010). In addition to that, the biochemists have concluded that the mean salivary non diabetiv paetients was below 2 mg/mL (0.11 mM) (Carda C. *et al.,* 2006, Gopal et al., 2017). Therefore, the approach of noninvasive glucose biosensor is limited by the current limit of detection.

Moreover, from the studies conducted by Gopal *et al.,* the increase of blood glucose level did increase the salivary glucose level (Gopal *et al.,* 2017) which provided evidence that salivary glucose measurement is relevant and would provide the better innovation towards the fabrication of non-invasive glucose biosensor.

2.9 Summary

This chapter summarizes about the role of glucose biosensor for diabetic patients. Diabetes was discussed thoroughly in this chapter by discussing about type of diabetes and how severe are the implications of diabetes toward people. Drawn by this disease, glucose biosensor was then discussed in order to encounter the the problem faced by diabetic patients. Thus, the history of glucose biosensor was then discussed which involves the definition development and of glucose biosensor; from first generation to third generation and also the drawbacks of current glucose biosensors. Then, enzyme immobilization methods were discussed in order to overcome the drawbacks of current glucose bionsensor's immobilization methods. The materials for enzyme immobilizations which mainly focusing on MOF-199 and SiO_2 were then introduced in this chapter. The glucose measurement's methods used in fabricated glucose sensing material were also discussed in this chapter. Finally, the glucose oxidase (GOx) activities were discussed thoroughly in order to understand the limit of detection (LOD) of FSG and FBG. These aspects are important in order to support this research in fabricating facile and non invasive highly sensitive glucose biosensing material.

CHAPTER 3

METHODOLOGY

3.1 Introduction

In specific, this research is divided into three main parts; synthesis method, characterizations and finally, to provide the experimental procedure in order to validate the application proposed in this research.

Generally this chapter is segregated into 7 sub-chapters. Chapter 3.2 discusses the materials and chemicals used in glucose oxidase (GOx) immobilization on metal organic framework 199 (MOF-199) and silica quasi nanospheres (SiO_2) using electrostatic adsorption. Meanwhile Chapter 3.3 discusses the instruments and machines used for both experimental and characterization in this reseach. Then, the synthesis procedure; the immobilization method of GOx on MOF-199 and SiO_2 using electrostatic adsorption was explained in chapter 3.4. Besides, the coating procedures for fabrication of thin films of MOF-199, SiO_2. MOF-199/GOx and SiO2/GOx were discussed in chapter 3.5. The production of the thin films were essential in order to prepare the materials for for GOx activities analyses. The characterizations of MOF-199/GOx and SiO_2/GOx were discussed in chapter 3.6. The MOF-199/GOx and SiO_2/GOx were characterized for their morphology and chemical properties between before and after immobilization of GOx. Meanwhile, the physical properties were discussed in chapter 3.7 The GOx activities are then discussed in chapter 3.8.

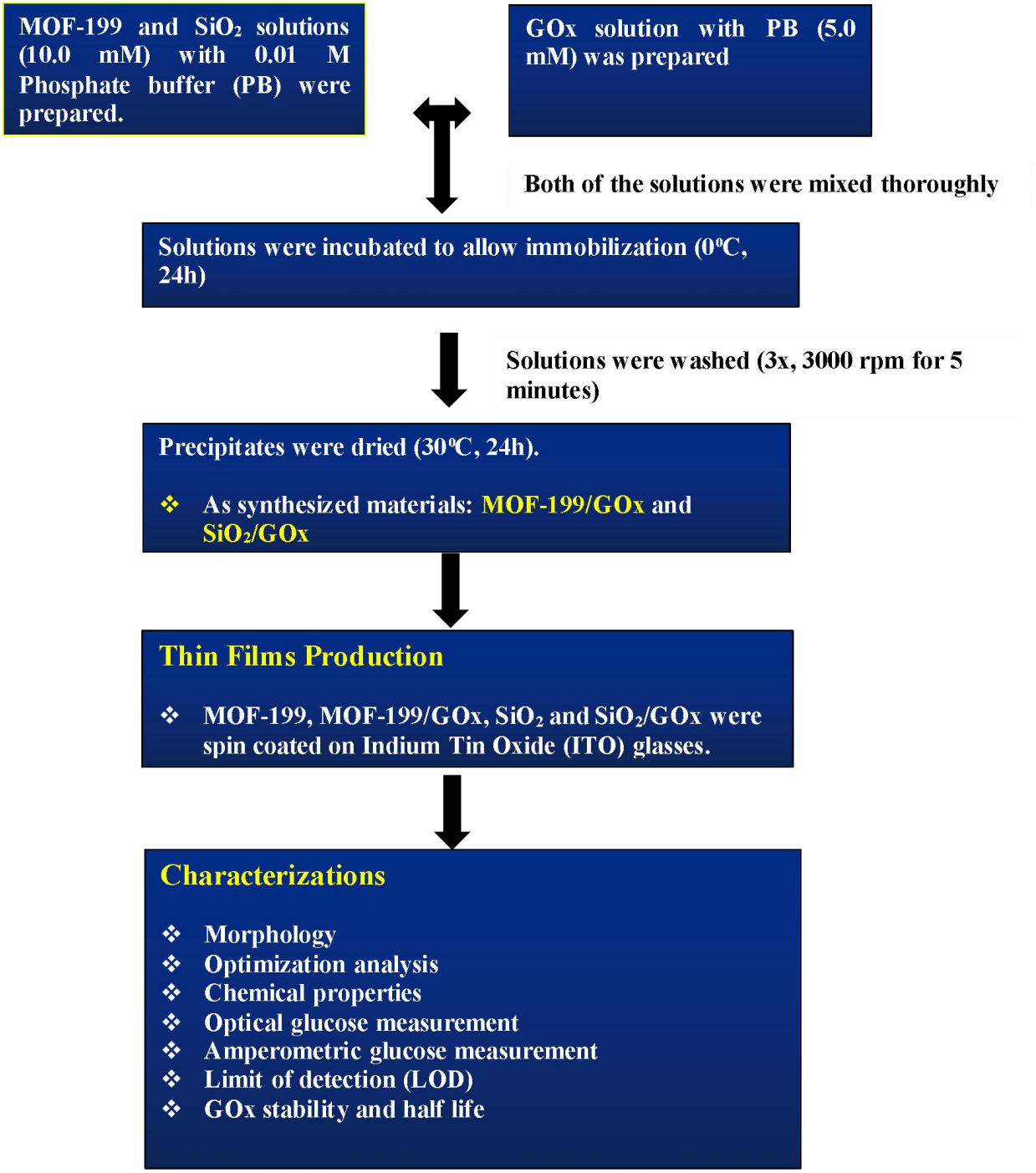


Figure 3.1: Flowchart of synthesis and characterizations of GOx on MOF-199 and SiO_2 using electrostatic adsorption

3.2 Materials

Glucose oxidase (GOx), enzyme activity of 50 KU were purchased from Sigma Aldrich, Metal Organic Framework-199 (MOF-199) and Silica Quasi Nanospheres (SiO_2) were synthesized in-house using palm oil derived fatty alcohol (PODFA) with eight carbon chain, known as octyl alcohol ($C_8H_{17}OH$) and phosphate buffer (PB) 0.1 M was purchased from Energy Oleochemicals. In addition, ethanol (1.0 M) and distilled water were used as solvents well as in cleaning process. All chemical were used without further purification.

3.3 Apparatus

The equipments used for GOx immobilization on both MOF-199 and SiO_2 using electrostatic adsorption were beakers (50.0 mL, 100.0 mL, 250.0 mL and 500.0 mL), spatula, weighing paper, magnetic stirrer, plastic pippete (3.0 mL), micropipette (200.0 uL and 1.0 mL), centrifugation tube (100.0 mL), Indium Tin Oxide (ITO) glass (1.0 cm^2), drying plate, alligator cables, copper probe, weighing scale (Ohaus, PA214), Fisher Scientific UltraSonic bath (FB15051), stirring hot plate (FAVORIT, HS0707V2), spin coater (POLOS^{TM} SPIN150i) and drying oven.

The instruments used in characterizations are including X-Ray Diffraction (6000 Shimadzu), Field Emission Microscopy and Energy Dispersive X-Ray (Hitachi SU8030), Ultra Violet- Visual Spectrophotometer (UV- Vis) (Perkin Elmer Lambda 35), Fourier Transform Infrared Spectroscopy (FT-IR) (Perkin Elmer Spotlight 400 FTIR) and spin coater. The simple electrochemical cell was set up using ammeter (mA), voltaic cell (3V), alligator cables and copper (Cu) electrode as reference electrode in order to measure the glucose concentration for GOx activities measurement.

3.4 Immobilization of GOx onto MOF-199 and SiO_2 using Electrostatic Adsorption

3.4.1 Preparation of MOF-199 and SiO_2 Solutions

Phosphate buffer (PB) 1.0 M was diluted to 0.1 M using 1: 100 dilution factor with distilled water. Each of MOF-199 and SiO_2 powders that were synthesized in house using PODFA were diluted differently in PB (0.1M) to produce 10.0 mg/mL of each solution. Both of the solutions were stirred separately until completely dissolved. 1.0 mL of ethanol (EtOH) was added to SiO_2 to allow complete dissolve of the powders into the solution. Paramater of samples preparation followed the previous study (Kong *et al.,* 2009)

3.4.2 Preparation of GOx Solution

The GOx (50KU) was mixed in phosphate buffer (PB) in 0.1 M at pH 7.0 to produce final concentration of 5.0 mg/mL. The GOx solution as mixed using micropipette (suck and release) in isothermal condition (temperature = 0°C) until completely dissolved.

3.4.3 Electrostatic Adsorption Immobilization

The electrostatic immobilization method allows the attachment between nanomaterials and GOx through physical adsorption with an entrancement of electrostatic force between positive and negative charges between the the surfaces of nanomaterials and the enzyme. The treatment of both nanomaterials and GOx with PB enables the formation of charges on the surface of both materials. Each of MOF-199 and SiO_2 solutions was then mixed with GOx solutions under isothermal condition, mixed until completely dissolved. Both of the solutions were incubated for 24h under in 0°C to allow immobilization process between nanomaterials and GOx. The incubated solutions were then washed using centrifuge machine (3 times, 3000 rpm, 5 minutes) and the the precipitates (MOF-199/GOx and SiO_2/GOx) of both materials were dried using drying oven (30°C, 24h). The dried precipitates were

collected and stored in isothermal condition. The electrostatic interaction method followed the previous studies conducted (Bahrami *et al.,* 2013; Kong *et al.,* 2009). The selection for phosphate buffer (PB) was considered as it contains phosphate element (P) which suits the GOx physiological property (Bankar et al., 2009). The immobilization process was conducted under isothermal condition to avoid any denaturation of GOx in high temperature (Bankar et al., 2009, Turner *et al.,* 2013).

3.5 Coating Method to Fabricate Thin Films

In this research, the MOF-199, SiO_2, MOF-199/GOx and SiO_2/GOx were coated on a substrate to produce thin films for mechanical property and GOx activities characterizations. The Indium Tin Oxide (ITO) glass were used as the substrate for coating.

The MOF-199, SiO_2, MOF-199/GOx and SiO_2/GOx were coated on ITO glass using spin coater machine. The MOF-199, SiO_2, MOF-199/GOx and SiO_2/GOx solutions were prepared by suspending each of the nanomaterial in PB and EtOH using ultrasonic bath until completely dissolved. The sample lid was opened and ITO glass was placed at the center on the sample place inside of the spin coater and the vacuum was turned on. The lid was closed and the MOF-199 solution was dropped 6 times using plastic pipette before the spin coating process began. The spin coating process was conducted using 2000 rpm speed for 5 minutes. In this process, the spin coater was set into three different phases. The first phase was initiated with 300 rpm for 30 second. Then, the second phase began when the speed was increased to 2000 rpm for 4 minute before the coating process entered the third phase, which the speed was decreased to 300 rpm for another 30 seconds. The parameter of spin coating was applied from previous studies (Isherwood *et al.,* 2014; Cho *et al.,* 2011). These procedures were conducted two times using the same solution, similar speed and time for spin coating to allow better coating on the substrate. The parameter for The whole spin coating procedures were repeated using SiO_2, MOF-199/GOx and SiO_2/GOx to fabricate thin films for each of the material. All of the films were stored separately in room temperature.

3.6 Characterizations of Immobilized GOx onto SiO_2 and MOF-199

3.6.1 Field Emission Scanning Electron Microscope (FESEM)

Morphologies of MOF-199, SiO_2, MOF-199/GOx and SiO_2/GOx were identified using Field Emission Scanning Electron Microscope (FESEM) Hitachi SU8030 with acceleration voltage from 1 kV to 5 kV. A very small amount of MOF-199 was attached on the carbon tape (1 cm^2). The carbon tape was then attached to the sample holder before it was positioned in the specimen chamber. The sample was set in vacuum condition and the images were taken in different magnifications. These methods were repeated using SiO_2, MOF-199/GOx and SiO_2/GOx.

3.6.2 Energy Dispersive X-Ray (EDX)

Energy Dispersive X-Ray (EDX) was used in order to compare the chemical composition of elements in nanomaterials between before and after immobilization of GOx. This characterization process is essential in order to proof the presence of GOx on the MOF-199 and SiO_2 to determine the successfulness of electrostatic adsorption immobilization method.The atomic percentages were auto calculated in this analysis. EDX analysis was conducted on MOF-199, SiO_2, MOF-199/GOx and SiO_2/GOx using Hitachi SU8030 with energy dispersive detector. The sample preparation procedures are similar to FESEM's sample preparation methods.

3.6.3 Fourier Transform Infrared Spectroscopy (FT-IR)

Fourier Transform Infrared Spectroscopy (FT-IR) is an instrument that is applied to analytically identify the presence of organic, polymeric as well as inorganic materials. The detection of chemicals is based on Infrared (IR) radiation to the sample used. The IR radiation will pass through the sample and will be either absorbed or transmitted in different wavelength providing the identification of each chemical (Fan *et al.,* 2012) detection of each element will based on absorbance or transmittance peak from a function of wavelength. Therefore, FTIR is able to

identify the presence of unknown chemicals, the consistency of the samples as well as the amounts of the components in mixture.

The main purpose of using FT-IR as one of the characterization methods is to make comparison of chemical compositions of MOF-199 and SiO_2 between before and after immobilization process. Similar to the EDX, this characterization procedure is important to proof the successfulness of electrostatic immobilization by observing the properties of GOx on MOF-199/GOx and SiO_2/GOx.

MOF-199 was mixed with Pottasium Bromide (KBr) with the ratio of approximately 1:10 of sample to Kbr in the mortar and pestle, mixed thoroughly until finely pulverized. Then, the finely pulverized sample was put into a pellet-forming die. A force was applied for several minutes to form transparent pellets. The transparent pellets was applied on FT-IR sample holder to form the analysis. The KBr was pulverized and heated to 110°C for 2h before it was mixed with the sample in order to avoid oxidation. These methods were repeated with SiO_2, MOF-199/GOx and SiO_2/GOx.

3.6.4 Ultraviolet-Visible Spectroscopy (UV-Vis)

The Ultraviolet-Visual Spectrophotometer (UV-Vis) analysis was conducted in order to detect the chemical reactivity in the materials. UV-Vis transmitted the light into the cuvette with specific chemicals thus the lights were absorbed on specific wavelength based on the chemical component in the chemicals. The absorbance or transmittance values in each of the wavelength were referred to the UV-Vis library in order to validate the chemicals in each of the analyzed material.

The glucose optical sensing has been largely studied by researchers for both clinical and industrial applications (Behera *et al.,* 2012). One of the glucose optical sensing methods are by the detection using florescence spectroscopy and Ultraviolet-Visible Spectrophotometer. Ultraviolet-Visible Spectroscopy (UV-Vis) is an instrument that often used to measure the absorptivity, light intensity or light transmission in the ultraviolet-visible (UV) spectral region (Behera *et al.,* 2012). UV-Vis is often used

for analytical chemistry and quantitative detection of chemicals or analytes such as biological molecules, and organic compounds.

Particularly, for organic compound such as glucose and glucose oxidase, the UV- Vis detection is based on either UV or visual light in electromagnetic spectrum. Therefore, the solvent used for organic compound detection is organic soluble compound such as ethanol or aqueous solution.

The detection of specific analyte or chemical solution is based on maximum absorption in function of wavelength (lambda-max). The absorbance is depended on the presence of particular chromophores (light-absorbing groups) in a molecule. Therefore, the absorbance or transmittance peak on specific wavelength will determine the specific compound appear in chemicals or analytes (Jin *et al.,* 2016).

The UV-Vis procedures were conducted on MOF-199, SiO_2, MOF-199/GOx and SiO_2/GOx using the assays as in Table 3.1. The main purpose for this characterization is to determine the chemical compound in each material. Furthermore, this characterization was conducted in order to make comparison study of between before and after immobilization of GOx on MOF-199 and SiO_2, in order to proof the presence of GOx (successfulness of immobilization).

All of the compound's composition were following the UV-Vis assays as in Table 3.1 that include blank (control), MOF-199, SiO_2, MOF-199/GOx and SiO_2/GOx. The UV-Vis was initially set to analyze 4 samples before the analyses began. The analyses were then continued background setting using the blank sample. The blank sample was placed in the quartz cuvette (path length = 1cm) before the cuvette was placed in the cuvette holder 1 in the cuvette holder. The blank was run using UV-Vis until the analysis completed. The with MOF-199 sample was inserted into the second cuvette and was placed in the cuvetter holder 2 before the UV-Vis was run until the analysis completed. The analyses were continued using the SiO_2, MOF-199/GOx and SiO_2/GOx solutions. The data recorded in function of absorbance vs wavelength (lambda).

Table 3.1: The sample assay for UV-Vis analyses.

Sample / Material (mL)	Blank	MOF-199	SiO_2	MOF-199/ GOx	SIO_2/GOx
PB (0.1 M)	1.0	1.0	1.0	1.0	1.0
EtOH (1.0 M)	1.0	1.0	1.0	1.0	1.0
MOF-199 (10 mM)	-	0.5	-	-	-
SiO_2 (10 mM)	-	-	0.5	-	-
MOF-199/GOx (10 mM)	-	-	-	0.5	-
SIO_2/GOx (10 mM)	-	-	-	-	0.5
Glucose (3 mM)	1.0	0.5	0.5	0.5	0.5
Final Volume	3.0	3.0	3.0	3.0	3.0

3.7 Optimization Analysis of MOF-199/GOx and SiO_2/GOx

The physical properties of immobilized GOx were investigated in order to measure the optimum conditions for enzyme performance as well as demonstrate the viability of GOx immobilization method using electrostatic adsorption.

3.7.1 Temperature Effect

The temperature effect analysis were conducted using UV-Vis on MOF-199, SiO_2, MOF-199/GOx and SiO_2/GOx in varied temperature in order investigate and to make comparison of temperature effect between before and after immobilization of GOx. Moreover, this study is also important in order to investigate the optimum working condition of immobilized GOx. The sample preparation of all of the samples did follow the sample preparation method of UV-Vis in section 3.6.4. The varied temperature the samples were 30°C, 40°C, 50°C and 60°C before each of the sample was placed in the UV-Vis for analysis. The data were recorded in absorbance (a.u) vs. temperature (°C). The selection of temperatures were in accordance to previous studies of temperature effect for GOx optimization (Kong *et al.,* 2009; Wong *et al.,* 2008; Yoo *et al.,* 2010).

3.7.2 pH Effect

Similar to temperature effect analysis, the pH effect analyses were also conducted using UV-Vis. The pH were varied in order to in order investigate and to make comparison of the effect on varied pH between before and after immobilization of GOx. Furthermore, study is also essential in order to investigate the optimum condition for immobilized GOx to work. The sample preparation of all of the samples did follow the sample preparation method of UV-Vis in section 3.6.4. The pH were varied to 5.0, 6.0, 7.0, 8.0 and 9.0 before each of the sample was placed in the UV-Vis for analysis. The temperature of the solutions were based on the optimum temperature obtained in 3.7.1. The data were recorded in absorbance (a.u) vs. pH. (Kong *et al.,* 2009; Wong *et al.,* 2008; Yoo *et al.,* 2010). The selection of pH were in following the GOx properties and were also following the previous studies of pH effect for GOx optimization (Kong *et al.,* 2009; Wong *et al.,* 2008; Yoo *et al.,* 2010).

3.8 GOx Activities

3.8.1 Optical Glucose Measurement (UV-Vis Analysis)

The original method for the measurement of glucose concentration is by following the Beer's Law (Palanisamy et al., 2012; Amerov et al., 2004). From the Beer's law (Equation 3.1), absorbance value will be increased with the increment of concentration in the constant path length and molar extinction coefficient (Shih *et al.,* 2009). Therefore, the glucose concentration is measured by the absorbance value from UV-Vis data in function of wavelength (lambda).

Beer's law; $A=ecl$ (Equation 3.1)

Where A= absorbance, e= molar extinction coefficient, l= path length

The optical glucose measurement analyses were conducted using GOx, MOF-199/GOx and SiO_2/GOx in glucose concentration of 1.0 mM following the sample assays as in Table 3.2.

Table 3.2: The sample assays for glucose (1.0 mM) measurement using UV-Vis.

Sample / Material (mL)	Blank	MOF-199	SiO_2	MOF-199/ GOx	SIO_2/GOx
PB (0.1 M)	1.0	1.0	1.0	1.0	1.0
EtOH (1.0 M)	1.0	1.0	1.0	1.0	1.0
Glucose (1 mM)	1.0	0.5	0.5	0.5	0.5
Final Volume	3.0	3.0	3.0	3.0	3.0

The optical glucose measurement was conducted using UV- Vis following the UV-Vis analysis method in section 3.6.4. The pH and temperature of the solvent were selected based on the optimum pH and optimum temperature from 3.7.1 and 3.7.2 respectively. The optical glucose measurement analysis was repeated by following table 3.2 assays using glucose concentration of 2.0, 3.0, 4.0 and 5.0. The data were recorded in function of absorbance (a.u) vs concentration of glucose (mM).

3.8.2 Amperometric Glucose Measurement

In order to fulfill conventional glucose biosensors measurement, glucose solution was also measured using amperometric (electrochemical) measurement. Amperometric measurement is the measurement of current produced by the electron movement which is applied in current glucose biosensor's measurement (Ang *et al.,* 2015; Clarke *et al.,* 2006; Amerov et al., 2004) demonstrated in Equation 3.2.

$$Glucose\ (C_6H_{12}O_6) + O_2 \xrightarrow{GOx} Gluconic\ acid\ (C_6H_{12}O_7) + H_2O_2 \quad \text{(Equation 3.2)}$$

The amperometric measurement was conducted by building a simple electrochemical cell (Kong *et al.,* 2009) as conducted demonstrated in Fig. 3.2. The aim for this method was to measure different glucose concentrations using MOF-199/GOx/ITO and SiO_2/GOx/ITO. The electrochemical cell was built with MOF-199/GOx/ITO thin film produced in 3.5 on anode and copper electrode at cathode in 0.1 M of PB. 1.0 mL of glucose 1.0 mM was then added in the PB in 3V of potential as shown in figure 3.2. The current was then measured. The method of measurement was then repeated with SiO_2/GOx/ITO. The glucose concentrations were then repeated in 2.0-5.0 mM for bot of the nanocomposites. The range selected for glucose concentrations, pH and temperature of the solutions were similar to 3.7.1, 3.7.2 and 3.8.1.

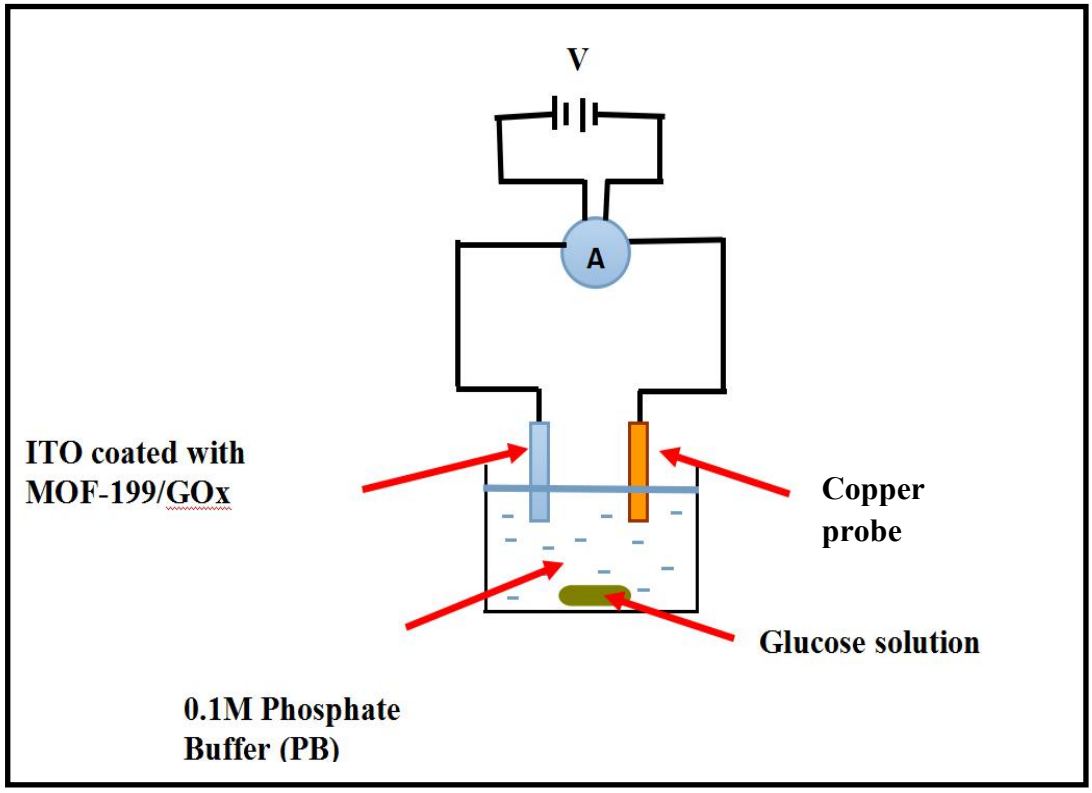


Figure 3.2: Schematic diagram of glucose amperometric measurement

3.8.3 Limit of Detection (LOD) Analysis

In order to reach the objective for salivary glucose detection, the limit of detection (LOD) of MOF-199/GOx and SiO_2/GOx must be analyzed using low concentration of glucose. The LOD analysis was conducted amperometically using GOx, MOF-199/GOx and SiO_2/GOx in 0.0 mM, 0.2 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM and 1.0 mM of glucose concentration. The amperometric measurement was conducted using electrochemical cell, following similar methods and conditions in section 3.8.2. The data were recorded in function of current (mA) vs. concentration of glucose (mM).

3.8.4 Glucose Oxidase (GOx) Stability and Half Life

In order to produce a very good bio-sensing material, the immobilized enzyme should has better stability and longer half life when reacted to chemical substrate compared with stand-alone enzyme. Enzyme is generally very sensitive and has a very low half- life. Multiple reactions may result to the alteration of its shape thus will disrupt the active site of the enzyme. Thus, it will result to enzyme denaturation and dysfunction. Therefore, the enzyme stability are measured in order to determine the half life of the enzyme (in days) between before and after immobilization process.

In this characterization, glucose were measured amperometically using GOx, MOF-199/GOx and SiO_2/GOx in order to measure the stability and to compare of GOx activities between GOx and immobilized GOx on both MOF-199 and SiO_2.

The GOx, MOF-199/GOx and SiO_2/GOx were reacted in 3mM of glucose separately. Then, glucose measurements were taken amperometically for 30 days by following the similar methods and conditions in 3.7.1. All of the GOx, MOF-199/GOx and SiO_2/GOx were stored separately in isothermal condition to avoid contamination. The data were recorded in function of current (mA) vs time (days).

3.9 Summary

Immobilization, characterizations and the GOx activities methods of MOF-199/GOx and SiO_2/GOx were discussed throughout this chapter. In general, the MOF-199/GOx and SiO_2/GOx were synthesized using electrostatic adsorption method with two nanomaterials as support; MOF-199 and SiO_2. All the MOF-199, SiO_2, MOF-199/GOx and SiO_2/GOx were coated on ITO glass using spin coater to create thin films. These thin films were used for hardness testing and GOx activities analyses. Then, these samples were characterized using FESEM, EDX, UV-Vis and FT-IR. The GOx activities were conducted to support the proposed application of fabrication glucose sensing material. The thin films of MOF-199/GOx and SiO_2/GOx were used to measure the corresponding glucose concentrations, measuring the LOD of immobilized GOx as well as for GOx stability analysis.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Introduction

This chapter discusses about results and discussions obtained for this research. In section 4.2, the morphology of MOF-199/GOx and SiO_2/GOx were discussed in order to observe the presence of GOx after immobilization. Meanwhile, section 4.3 discusses the physical properties of MOF-199/GOx and SiO_2/GOx which incudes the effect temperature and pH studies. Section 4.4 then explains the chemical properties of MOF-199/GOx and SiO_2/GOx that includes the elemental analysis, light absorbance and chemical bonding analyses. The third objective in this research is to investigate the activities of GOx in order to fulfill the proposed application of immobilized GOx using electrostatic adsorption. Therefore, in chapter 4.5 and 4.6, optical glucose measurement and amperometric glucose measurement were conducted respectively. In section 4.7, the limit of detection (LOD) analysis was conducted on the nanostructured composites. Finally, GOx stability on both immobilized composites was also analyzed in chapter 4.8. The summary for characterization chapter of synthesized MOF-199/GOx and SiO_2/GOx was presented in chapter 4.9. All of the analyses conducted were investigated and compared with MOF-199 and SiO_2 (before immobilization) in order to provide better comparison and strengthen the findings.

4.2 Morphology Analysis

Morphology or structure of nanocomposites was investigated using Field Emission Scanning Electron Microscope (FESEM) as mentioned in chapter 3. The purpose for this investigation was to investigate the morphology between before and after immobilization of GOx on both MOF-199 and SiO_2.

4.2.1 MOF-199 and MOF-199/GOx

The initial structures of MOF-199 and MOF-199/GOx were observed in Figure 4.1. The structure of MOF-199 that was synthesized in house observed using 1.0 K magnification was clear orthorhombic structure with about 15-30 µm in size. This morphology of MOF-199 did follow the the morphology of MOF-199 from previous study on where the copper metals (Cu) were linked together with organic linker forming a mesophorus orthorhombic framework (Mahadi *et. al.,* 2015). After the immobilization of GOx using electrostatic adsorption, the MOF-199 was observed to be coated with other particles (Fig. 4.1 (b)). The electrostatic forces between the amino group of GOx (NH_4^-) and H^+ from PB would provide the electrostatic attachment between both MOF-199 and GOx thus resulting in the attachment between the surface of both MOF-199 and GOx causing the coating of GOx onto the surface of MOF-199 (Ekanayake *et al.*, 2008). In addition to that, the size of GOx is smaller as compared to MOF-199 which was about 160 kilo dalton (kDa) that is equal to 50 nm (Bankar *et al.,* 2013; Ekanayake *et al.*, 2008). . This could provide better explanation of the coating of GOx onto the MOF-199 (Mahadi *et. al.,* 2015).

In addition, the structure of GOx observed in Fig. 4.1d was similar as reported in (Altun *et al.*, 2015). The results were supported by observing the surface pores of MOF-199 (Fig. 4.1(c)) and MOF-199/GOx (Fig. 4.1 (d)). Both of the images were obtained under 50.0K magnification which have allowed the observation of surface pores of MOF-199. In Fig. 4.1 (c), the surface pores of MOF-199 could be observed as highlighted. However after the immobilization of GOx, the pores of MOF-199 were filled by some particles of GOx (Fig. 4.1 (d)). MOF-199 is a mesoporous nanostructured particles with very high very high porosity. The electrostatical interaction between GOx and MOF-199 have loaded GOx on the surface and also filling the surface pores of MOF-199. Based on the results, it can be concluded that GOx were present by coating and filling the pores of MOF-199 framework thus suggesting that the immobilization of GOx onto MOF-199 was successful using electrostatic adsorption method.

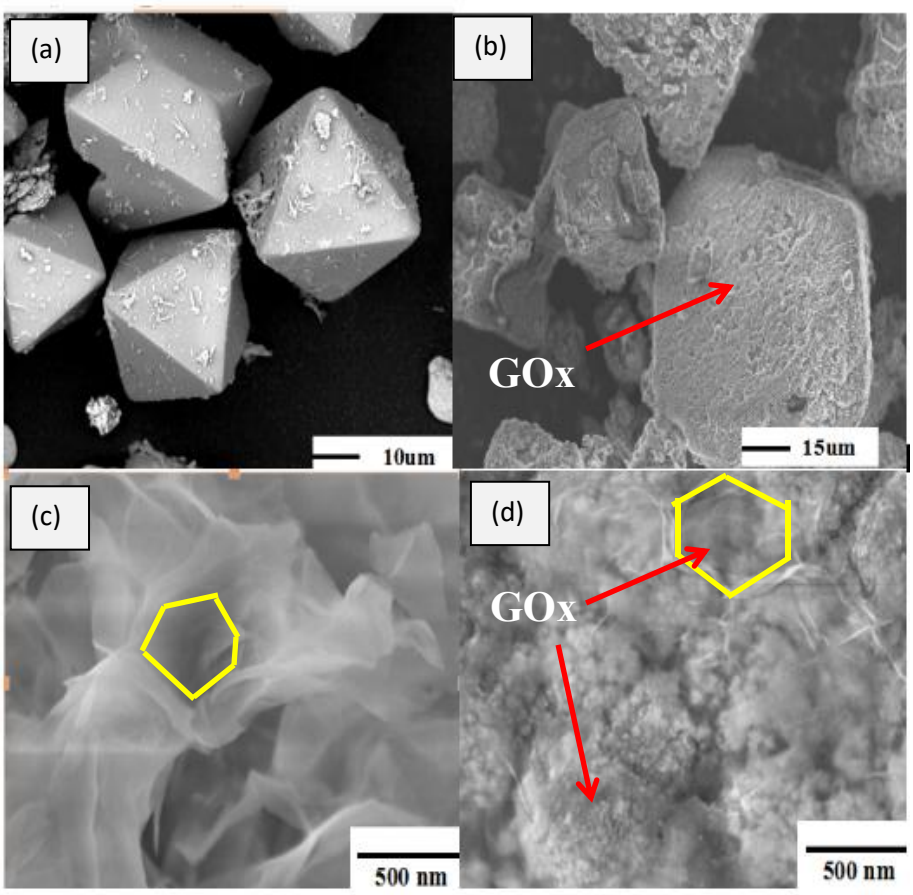


Figure 4.1: FESEM images of MOF-199 (a) and MOF-199/GOx (b) at 1.0K magnification, MOF-199 (c) and MOF-199/GOx (d) at 50K magnification.

4.2.2 SiO_2 and SiO_2/GOx

The structures of SiO_2 and SiO_2/GOx were demonstrated in Figure 4.2. The structure of SiO_2 observed in was spherical in shape (Figure 4.2a). This result supported the morphology of SiO_2 which were non porous and spherical (Salim *et al.,* 2014).

The GOx particles were observed attached to SiO_2 on SiO_2/GOx nanostructured composites as labeled 'X' (Figure 4.2 b) after the immobilization.Therefore, this result provided the initial result for GOx attachment on SiO_2 after the immobilization.

Similar to the interaction between MOF-199 and GOx, the electrostatic forces between amino group (NH_4^-) and hydrogen ion (H^+) from both GOx and PB on SiO_2 surfaces respectively had provided attachment through electrostatic forces (Ekanayake *et al.*, 2008). Meanwhile, unlike MOF-199, SiO_2 particles were nonporous (Salim *et al.,* 2014). The treatment of PB was occurred only on the surfaces of SiO_2. Therefore, the presence of GOx will only occur on the surface of slica. These results supported the attachment between SiO_2 particles and GOx after electrostatic interaction immobilization process. Therefore, the results demonstrated suggested successful immobilization of GOx on SiO_2 particles using electrostatic adsorption method.

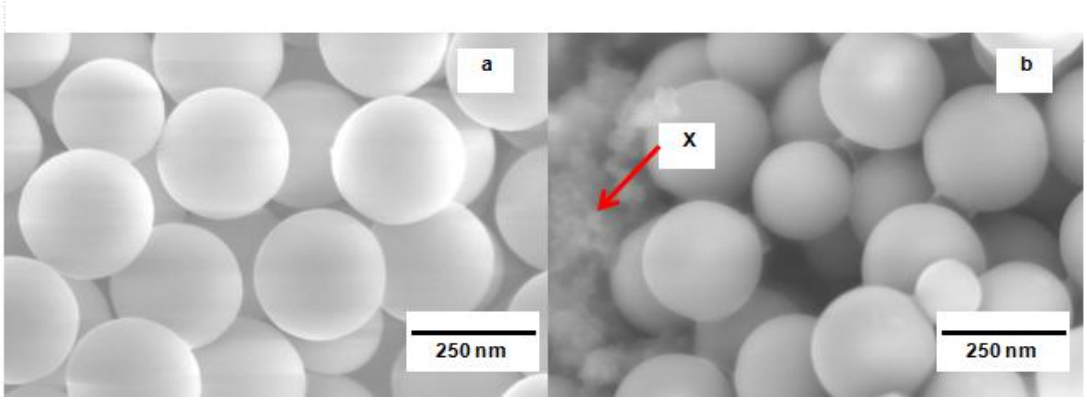


Figure 4.2: FESEM images of SiO_2 (a) and SiO_2/GOx (b) at 50 K magnification,

4.3 Optimization Analyses of MOF-199/GOx and SiO_2/GOx

The optimization analyses were conducted on both MOF-199 and MOF-199/GOx in order to investigate the GOx activities in varied pH and varied temperatures between before and after GOx immobilization. These analyses were also conducted to measure the optimum conditions for enzyme performance after immobilization process.

4.3.1 Effect of Temperature

The effect of temperature on MOF-199, MOF-199/GOx, SiO_2 and SiO_2/GOx was investigated in various temperatures ranging from 30˚C to 60˚C in 3 mM of glucose solution. The result in Fig 4.2 (a) demonstrates no significant change in absorbance for MOF-199 when reacted with glucose in different temperatures. On the other hand, the absorbance values for MOF-199/GOx in 3mM of glucose shows significant increment from 30˚C to 50˚C before it started to decrease at the temperature of 60˚C. From the result, the highest absorbance value of MOF-199/GOx was at 50˚C suggested optimum temperature of MOF-199/GOx at 50˚C.

Similar with MOF-199, SiO_2 demonstrates no significant difference in absorbance when reacted in glucose in different temperatures (Fig.4.2 (b)). Nonetheless, the absorbance values of SiO_2/GOx reacted in glucose were increasing from 30˚C to 50˚C before the absorbance values started to debase at 50˚C to 60˚C. The result obtained for SiO_2/GOx did follow the similar pattern of MOF-199/GOx which react optimally at the temperature of 50˚C.

Based on the results acquired, there was no reaction occurred when both MOF-199 and SiO_2 were reacted in glucose in different temperatures. This result suggested that both nanomaterials could not react with glucose with the increase of temperature. This is due to non reactive surface of both nanomaterials which will not perform any reaction with GOx regardless in varied temperature (Makaram *et al.,* 2014), Nevertheless, there were significant changes occurred in absorbance to MOF-199/GOx and SiO_2/GOx when reacted in glucose in varied temperatures demonstrating the presence of GOx on the surface of both MOF-199 and SiO_2 after the immobilization process (Koyun *et al.,* 2012). GOx is an enzyme which reacts very sensitively towards different temperatures (Koyun *et al.,* 2012). GOx is a biological enzyme which consists of protein that require optimum biological ambiance to maintain the homeostasis of cell within the organism (Sheldon *et al.*, 2011). In this study, the GOx was extracted from *Aspergillus Niger*, a species of fungus that oxidizes the beta-D glucose with a multiple oxidizing agent. Therefore, the ambiances for GOx reaction are within the conditions biological components

including cells and proteins (Ferri *et al.*, 2011; Wohlfahrt *et al.*, 1999). This is crucial in order to maintain the inner cell activities as well for life continuation. (Ferri *et al.*, 2011). The low temperature resulted in less activity GOx to react with GOx. However, with the increase of temperature, the activities of GOx would be increased. The GOx are typically stored in extremely low temperature to avoid any loss in activities. On the other hand, the high temperature leads to the denaturation (loss of activity) of GOx. The GOx reportedly withstood optimally at the temperature of 40˚C and exhibiting half life (decrease of activities) at 50˚C (Wilson *et al.,* 1992; Bankar *et al.,* 2009; Singh *et al.,* 2013).

Based on the result obtained, both of MOF-199/GOx and SiO_2/GOx nanocomposites provided better endurance towards heat as compared to free GOx. Both of the nanocomposites withstood up to 50˚C before experiencing half life at 60˚C thus making the GOx more heat-stable as compared to free GOx. This is due to the physical supports that both of nanomaterials provided to the GOx, providing better endurance towards high temperature (Koyun *et al.,* 2012). The exhibited the reactivity with glucose when reacted in different temperature ranging from 30˚C to 60˚C. In addition, both of the, from the results obtained, both MOF-199/GOx and SiO_2/GOx demonstrated active GOx reaction with glucose as the temperatures were increased from 30˚C to 50˚C before the GOx activities started to decrease at the temperature of 60˚C. Thus, from the results, the optimum temperature for both MOF-199/GOx and SiO_2/GOx was 50˚C when reacted in glucose accordingly.

These results provided strong parallel evidences for both optimum temperature pH of active GOx with previous studies on which, the both MOF-199/GOx and SiO_2/GOx did provide the similar optimum pH and temperature, suggesting successful immobilization method using electrostatic adsorption (Palanisamy *et al.,* 2012; Amerov *el al.,* 2014).

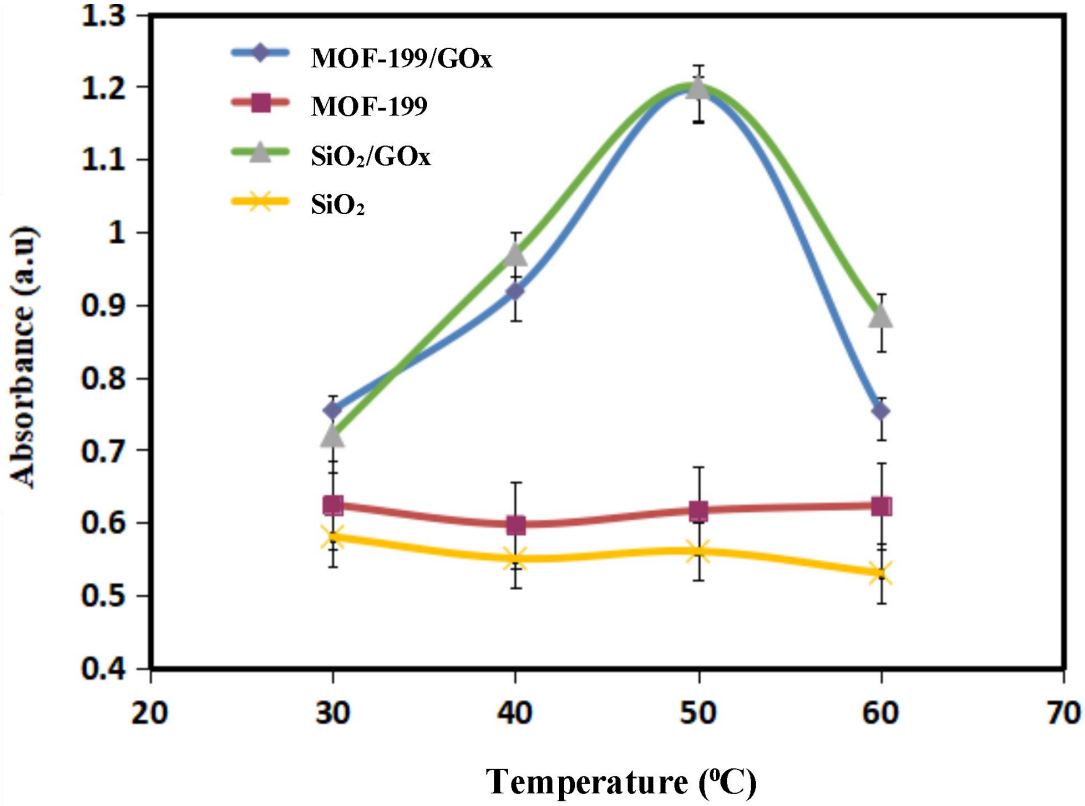


Figure 4.3: Temperature effect of MOF-199, MOf-199/GOx, SiO_2 and SiO_2/GOx in 3 mM glucose at 280 nm wavelength.

4.3.2 Effect of pH

The effect of pH on MOF-199, SiO_2, MOF-199/GOx and SiO_2/GOx were also studied using UV-Vis in the pH range from 5.0 to 9.0 in 3mM of glucose solution. The results in Fig. 4.3 exhibited no significant changes in absorbance value in different pH for bare MOF-199 and SiO_2 when reacted with glucose. Conversely, the absorbance of both MOF-199/GOx and SiO_2/GOx elevated from pH 5.0 to its highest value at pH 7.0 before the absorbance started to decrease at pH 8.0 to 9.0.

Similar to the effect of temperature, GOx did react sensitively in different pH. Similar to temperature, maintaining the optimum pH of GOx is important to prolong the homeostasis regulation of its host organism (Kalogeris, *et al.*, 2014). There was no absorbance change when both MOF-199 and SiO_2 were reacted in glucose in different pH. These results indicated both nanomaterials did not react in specific pH, similar to the reaction toward varied temperatures. GOx, appeared in living cell in

which the optimum reaction occured in neutral pH (Yoo *et al.*, 2010). Maintaining the neutral pH is very optimum in order to reduce the tension between the enzymes and reactants while performing biochemical reactions within the living cells. The condition of being too acidic or basic will disrupt the ambiance of inner cell thus will result in demolition of cell's activities and discontinuation of living.

There were no changes in absorbance when both MOF-199 and SiO_2 were reacted in glucose in different pH. However, the significant changes occurred in absorbance to MOF-199/GOx and SiO_2/GOx when reacted in glucose in varied temperatures demonstrated active GOx activities on both of the nanocomposites. The extreme pH may cause high tension towards GOx thus causing the loss of its activities (denaturation) (Yiu *et al.*, 2012).

GOx reacted optimally of the pH of 7.0 (Wilson *et al.,*1992; Bankar *et al.,* 2009) which is in neutral condition. The immobilization of GOx onto various nanomaterials have been conducted previously. The immobilized GOx onto SiO_2 exhibited the similar optimum pH which was 7.0 (Zhu *et al.*, 2014). Other immobilization of GOx onto metal oxide and inorganic nanomaterials such as graphene, zinc oxide nanotube (ZnONt), nickle oxide (NiO) and zeolite provided the same optimum pH o reaction at 7.0 (Kong *et al.,* 2009; Hahn *et al.,* 2012; Zhou *et al.,* 2012; Tyagi *et al.,* 2013).

Therefore, from the results obtained, both MOF-199/GOx and SiO_2/GOx exhibited active GOx reaction with glucose as the pH were increased from 5.0 to 7.0 before the GOx activities started to decrease at the temperature and experiencing the half life at of 8.0 and decreasing in actvity as the pH increased to 9.0. Thus, from the results, the optimum pH for both MOF-199/GOx and SiO_2/GOx was 7.0. This result followed the previous studies of active GOx activities as well as actual GOx biochemical property on both MOF-199/GOx and SiO_2/GOx. Therefore, the results were, suggesting successful immobilization method using electrostatic adsorption.

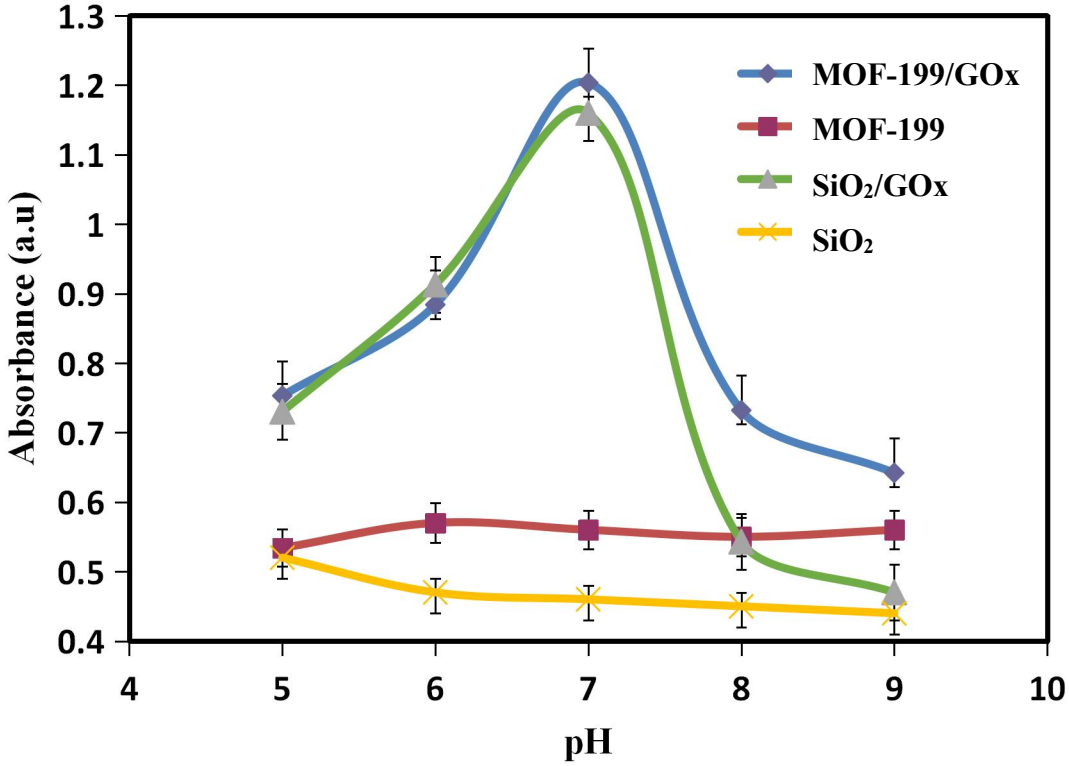


Fig 4.4: UV-Vis spectra of MOF-199 and MOF-199/GOx, SiO_2 and SiO_2/GOx different pH in 3 mM of glucose at 280 nm wavelength.

4.4 Chemical Properties

4.4.1 Elemental Composition

Table 4.1 illustrates the result of elemental composition of MOF-199, SiO_2, MOF-199/GOx and SiO_2/GOx. The results were obtained from EDX analysis that generated the images as well as atomic percentages of analyzed materials. The results were compared between before and after immobilization of GOx in order to provide comparison analyses. From the table, the bare MOF-199 exhibited the atomic percentage ratio of 41: 26: 33 of copper (Cu): oxygen (O): carbon (C) respectively of copper (Cu). The highest percentage of Cu atomic composition observed resulted in MOF-199 was a copper based MOF (Mahadi *et al.*, 2016; Halina *et al.*, 2013). In addition, the presence of carbon (C) from MOF-199 attributed to the C of the benzene rings from the organic linkers that linked the Cu together (Mason *et al.*,

2014). However, the percentage of C in MOF-199/GOx was higher as compared to MOF-199. This is due to added C from GOx that has increased the C percentage after immobilization of GOx. As mentioned in literature review, GOx contains C element (Altun *et al.,* 2015; Keilin *et al.,* 1948). This result the presence of GOx presence after immobilization on MOF-199.

The result of SiO_2 demonstrated the atomic percentage of 24: 64: 12 silica (Si): oxygen (O) and C (carbon). These results supported the previous results of SiO_2 which consists of Si, O and carbon composition of SiO_2 where the molecular ratio of silica: oxygen atoms were 1:2 (Salim *et al.,* 2014). From the elemental composition, the O percentage was more than as twice than Si which supported the composition of SiO_2. Therefore, theoretically the atomic composition of oxygen shall be twice as much as Si (Salim *et al.*, 2014; Cendrowski *et al.*, 2011). However, there was carbon occurance in the SiO_2 sample. The percentage of C however was lower than the percentage of Si and O elements. This was due to the carbon from the carbon tape in the sample preparation procedure (Salim *et al.*, 2014).

Meanwhile, the EDX result observed in MOF-199/GOx exhibited the nitrogen atom (N) at 22%. The SiO_2/GOx nanocomposite did exhibit the N of 1 %. The occurance of N content in both MOF-199/GOx and SiO_2/GOx nanocomposites was due to the GOx after the immobilization process. GOx is a protein that is consists of amino acids (Vashist *et al.*, 2011; Wong *et al.*, 2008; Leskovac *et al.* 2005). These amino acids consist of several elements such as P, N, C=C, OH and many more (Vashist *et al.*, 2011; Wong *et al.*, 2008; Leskovac *et al.* 2005). GOx consists of two most important side chains; carboxylic chain (COOH) and amino chain (R-NH). However the amino chain is the main activation chain for glucose activities thus more abundantly distributed among the Hence. N would be easier to be detected as compared to other elements (Wong *et al.*, 2008; Leskovac *et al.* 2005). The results suggested clear comparison between before and after immobilization of GOx onto both MOF-199 and SiO_2 with the presence of N on both MOF-199/GOx and SiO_2/GOx demonstrated the presence of N from amine group from GOx after the immobilization thus supporting the FESEM's results. These results suggested the

successful immobilization procedure using electrostatic adsorption on both MOF-199 and SiO_2.

Table 4.1: Atomic percentage for MOF-199 and SiO_2 between before and after immobilization of GOx.

Base material	Element	Atomic Percentage (%)	
		Before GOx immobilization	After GOx immobilization
MOF-199	Copper (Cu)	41	23
	Oxygen (O)	26	8
	Carbon (C)	33	47
	Nitrogen (N)	-	22
SiO_2	Silica (Si)	24	27
	Oxygen (O)	64	66
	Carbon (C)	12	6
	Nitrogen (N)	-	1

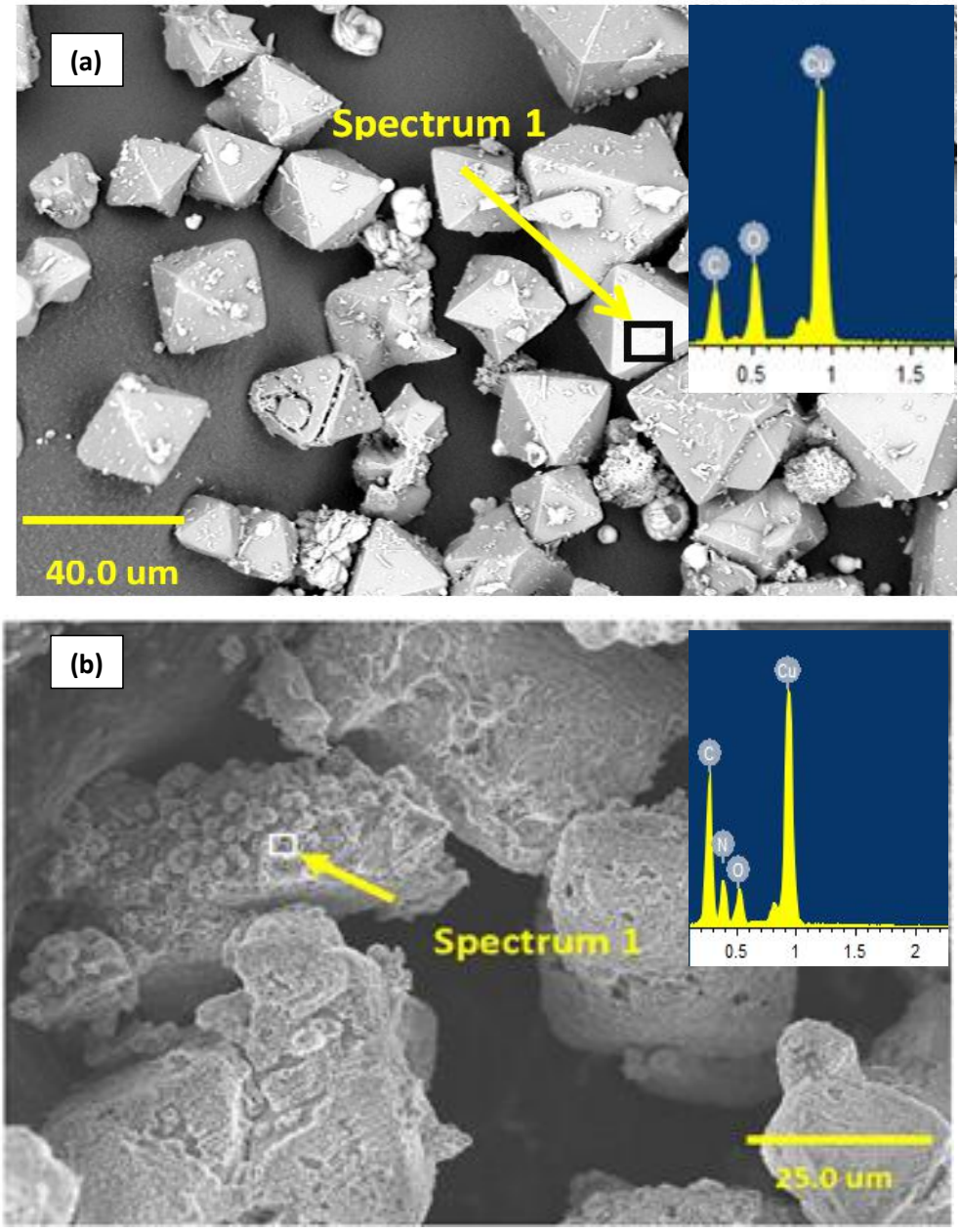


Figure. 4.5: EDX analysis of MOF-199 (a) and MOF-199/GOx (b).

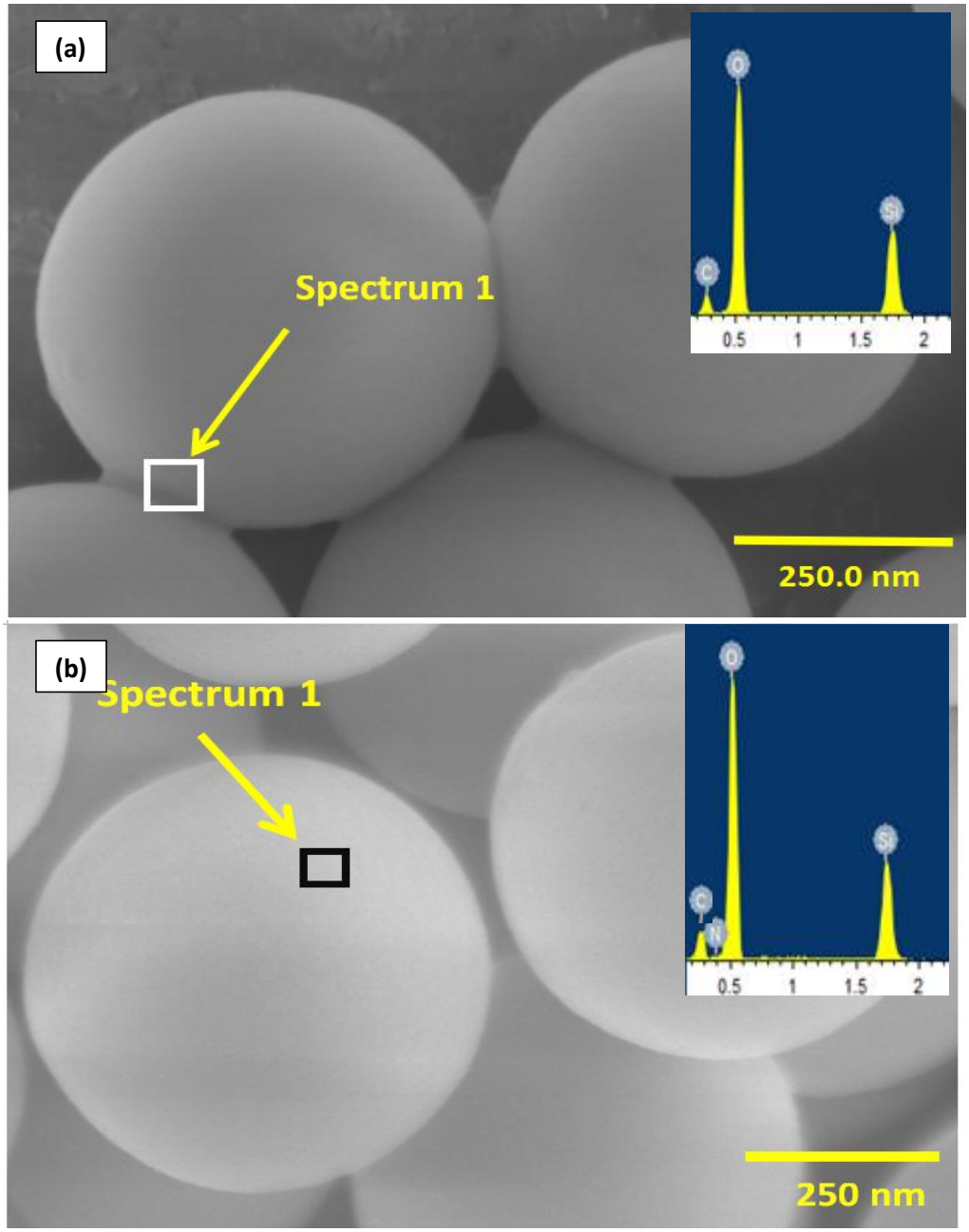


Figure 4.6: EDX analysis of SiO_2 (a) and SiO_2/GOx (b).

4.4.2 Ultraviolet-Visual Spectrophotometer (UV-Vis) Analysis

In this research, the absorbance of MOF-199, SiO_2, MOF-199/GOx were SiO_2/GOx were measured in order to investigate the presence of GOx after immobilization using electrostatic adsorption thus proving the viability of this method.

Figure 4.7 demonstrates that both MOF-199/GOx and SiO_2/GOx exhibited the absorbance peak at 280 nm wavelength. Meanwhile, there was no absorbance peak at 280 nm observed on both bare SiO_2 and MOF-199. The result obtained was similar with chemical property of GOx on which the absorbance of GOx was exhibited at 280 nm wavelength (Wong *et al.,* 2008; Keilin *et al;* 1948).

GOx is an oxidase and react optimally at pH 6.5- 7.0 with a very intense UV fluorescence with 278-280 nm due to the absorbance of tryptophan (Luca *et al.*, 2007; Wong *et al.,* 2008). The fluoresence were excited st around 270 nm before trapped into gelatin and protein membrane of GOx. The absorbance of GOx at around 280 nm resulted in the amine group (N-H) absorbance at 280 nm (Wong *et al.,* 2008; Balashanmugam *et al.,* 2015). This is due to GOx, that is an enzyme which composed of amine group (Luca *et al.*, 2007; Balashanmugam *et al.,* 2015). Therefore absorbance peak exhibited at 280 nm on both MOF-199/GOx and SiO_2/GOx nanocomposites indicated the presence of GOx after immobilization GOx consists of flavoproteins which exhibited the the optimum absorption peak of 280 nm (Luca *et al.,* 2007; Wong *et al.,* 2008).

These results provide another clear evidence of GOx presence on both nanocomposites fter immobilization suggesting successful immobilization method using electrostatic adsorption method.

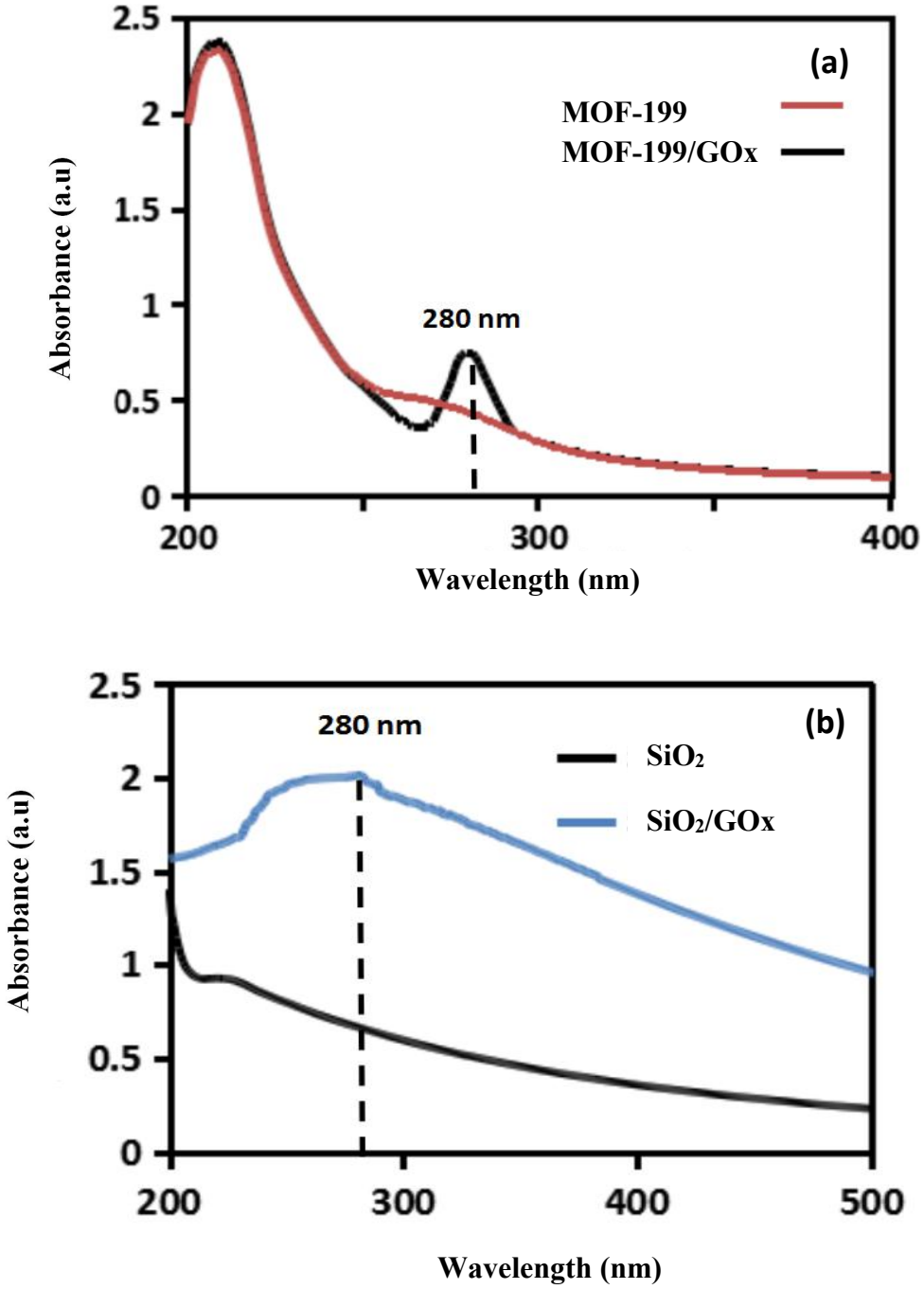


Figure 4.7: UV-Vis of MOF-199 and MOF-199/GOx (a); SiO_2 and SiO_2/G (b).

4.4.3 Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

The FT-IR spectrum of MOF-199 exhibited similar transmittance peaks as compared to previous studies with the presence of C-CH_2, C-O, C-OH, C-H (alkenes) and OH bonds (Table 4.2). (Nguyen *et al.*, 2012; Tranchemontagne *et al.*, 2008). Meanwhile

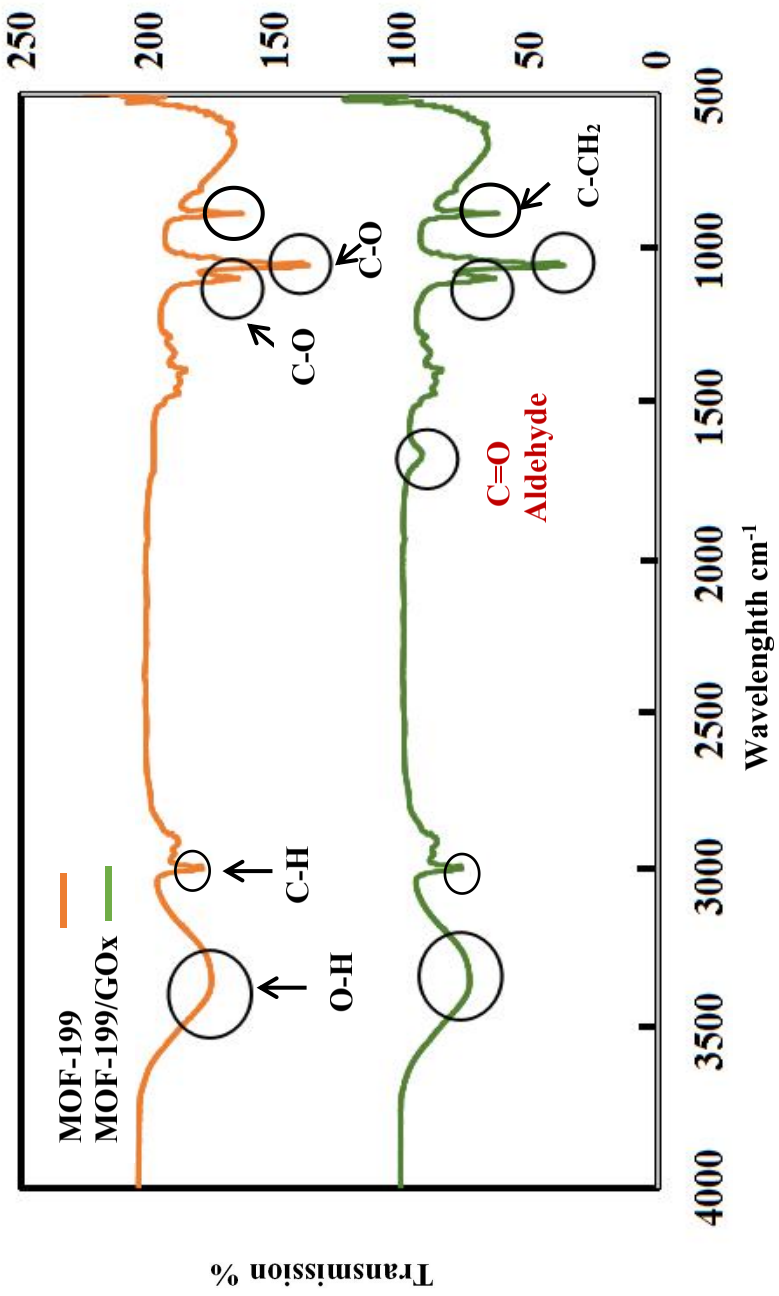
the FT-IR in Figure 4.8, the spectra for MOF-199/GOx exhibited one new bending vibration (Vbend) at ca. 1720 cm^{-1} (Xue *et al.*, 2016). This peak demonstrates C=O aldehyde respectively (Xue *et al.*, 2016). The C=O aldehyde presence due to the glucose oxidation of GOx which produces aldehydes when it was reduce into gluconic acid (Shokri *et al.*, 2014).

Figure 4.9 shows the FT-IR spectra for SiO_2 and SiO_2/GOx. The peaks exhibited for SiO_2 spectrum were Si-O, Si-O-Si, acidic O-H and OH (Table 4.3). Acidic OH spectra were exhibited on both SiO_2 and SiO_2/GOx due to incomplete ionization of water (Wang *et al.,* 2006). These spectra match the FT-IR transmission spectra from previous studies proving that there was no impurity in the sample (Shokri *et al.*, 2014). Similar with MOF-199/GOx, the SiO_2/GOx exhibited a bending peak (Vbend) at ca. 1722 cm^{-1} attributed to the vibration of C=O aldehyde. Hence, from the results obtained, it can be concluded that there were presence of GOx onto both MOF-199/GOx and SiO_2/GOx nanocomposites thus proving the success of immobilization procedure using electrostatic adsorption.

Based on the literature review, majority of conventional immobilizations of enzymes require strong chemical interactions between the enzymes and supports. As result, strong bonds were produced between both of surfaces. Nonetheless, these strong bonds often connect with the active sites of the enzyme which resulted in major loss in enzymatic activities (Alberts *et al.*, 2002). Therefore, in this research, the new method of electrostatic adsorption method did not produce any chemical bond formation between GOx and nanocomposites. However, the electrostatic force between nanocomposite and GOx did retain the GOx attachment strongly thus the presence of aldehyde bond from GOx could be observed using FT-IR analysis. Nevertheless, the strong electrostatic forces between the surfaces of nanomaterials and GOx were formed instead without causing any pivotal loss in catalytic activity of GOx.

Table 4.2: FT-IR spectra of MOF-199 and MOF-199/GOx.

Bond	Wavenumber (cm^{-1}) MOF-199	Wavenumber (cm^{-1}) MOF-199/GOx
$C\text{-}CH_2$	886	895
C-O	1048	1052
C-OH	1105	1125
C=O Aldehyde	-	1720
C-H Stretch	2980	2987
OH (free)	3200-3500	3200-3500



CFigure 4.8: FT-IR analysis of MOF-199 and MOF-199/GOx

Table 4.3: FT-IR spectra of SiO_2 and SiO_2/GOx.

Bond	Wavenumber (cm^{-1}) SiO_2	Wavenumber (cm^{-1}) SiO_2/GOx
Si-O	885	890
Si-O-Si Stretching	1038	1041
C=O Aldehyde	-	1722
Acidic O-H	2982	2960
O-H	3250-3450	3200-3450

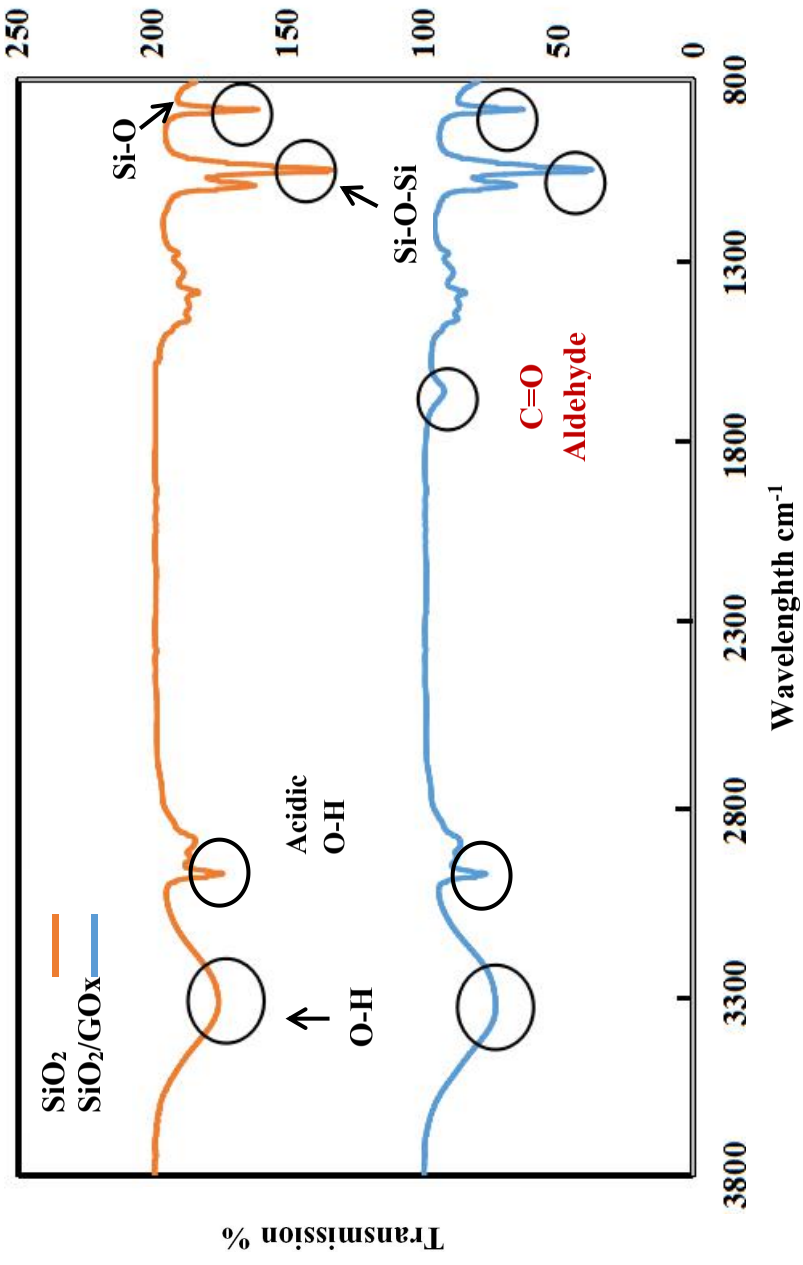


Figure 4.9: FT-IR analysis of SiO_2 and SiO_2/GOx

4.5 Optical Glucose Measurement

Figure 4.10 illustrates the the comparison of absorbance values suggesting corresponding glucose measurement between GOx, MOF-199/GOx and SiO_2/GOx in different glucose concentrations ranging from 1.0- 5.0 mM. The graphs plotted were in accordance to Beer's law (Dahlqvist *et al.*, 1961). Beer's law is an mathematical formula that expresses measurement of concentration of an analyte dissolved is proportional towards to its absorbance value (Kovács *et al.*, 2008; Mark *et al.*, 2002; Poots 1967; Dahlqvist *et al.*, 1961).

The results demonstrate the proportional increment of absorbance with the increase of glucose concentration for GOx, MOF-199/GOx, and SiO_2/GOx respectively. In addition, in order to completely following the Beer's law, the linear range correlation (R^2) of the absorbance values should be within the range of 0.5 to 1.0 on where 1.0 indicated the absolute relationship. This is to ensure that strong evidence of proportional relationship between concentration and the absorbance values.

The results obtained showed that not only the absorbance values of MOF/GOx and SiO_2/GOx were proportional with the increment of glucose concentration, both of the MOF-199/GOx and SiO_2/GOx nanocomposites reacted accurately with GOx producing a significantly good R^2 values. From the results, the GOx, MOF-199/GOx and SiO_2/GOx illustrate proportional relationship with the increment of glucose concentrations from 1.0 to 5.0 mM with linear range of 0.994, 0.999 and 0.988 respectively. The result also shows that the absorbance for SiO_2/GOx was lower as compared to MOF-199/GOx. This result was due to the structure of support. The MOF-199 is porous thus could provide higher adsorption and enzyme loading of GOx as compared to SiO_2. Therefore, the amount of GOx reacted with glucose in MOF-199/GOx was higher than SiO_2/GOx (Gupta *et al.,* 2011). Nonetheless, both fot the nanostructured composites were able to produce high significant R values. These results suggested that both SiO_2/GOx and MOF-199/GOx were able to react with glucose with high accuracy suggesting the successful immobilization method using electrostatic adsorption (Palanisamy *et al.*, 2012; Amerov *et al.*, 2004). These results suggested that the electrostatic adsorption method were successfully applied

to produce active glucose sensing nanocomposites for both MOF-199/GOx and SiO_2/GOx.

Current glucose biosensors are applied using blood plasma as medium of glucose reading. However, conventional glucose sensors could only detect as low as 10.0 mg/mL (0.56 mM) with 10^{-2} mM sensitivity (Patel *et al.*, 2015). Meanwhile, the mean glucose concentration for normal condition and diabetic mellitus were 1.23 mM and 4.22 mM respectively (Abikshyeet *et al.*, 2012; Patel *et al.*, 2015) Thus, current glucose biosensors are not possible to be used for salivary glucose measurement (Patel *et al.*, 2015). Hence, the measurement of high LOD sensing materials (MOF-199/GOx and SiO_2/GOx) which match the salivary glucose concentrations were attempted. The optical glucose measurements were conducted using UV-Vis analysis in glucose concentration from 1.0 mM-5.0 mM. The consideration of choosing this range of glucose concentrations were based from previous studies (Arslan *et al.*, 2011; Kong *et al.*, 2009). In addition, the aim of this research is to fabricate high stability with high LOD sensing material possibly for salivary glucose detection. The range of 1.0- 5.0 mM were plausible to initiate salivary glucose measurement simulated for diabetic patients.

Based on the Eq 3.1 from Chapter 3.8.1, the reaction between glucose ($C_6H_{12}O_6$) and GOx led to redox reaction that oxidized glucose to produce gluconic acid ($C_6H_{12}O_7$). The oxygen (O_2) was reduced to produce hydrogen peroxides (H_2O_2) instead (Kong *et al.*, 2009; Arslan *et al.*, 2011). Therefore, from this forward reaction, the concentration of glucose could directly be measured with the production of gluconic acid when reacted to GOx. Hypothetically, the increment of glucose concentration will increase the reaction between glucose and GOx, resulting to the increase of absorbance which yielding more concentration of gluconic acid (Kovács *et al.*, 2008;Kong *et al.*, 2009; Arslan *et al.*, 2011).

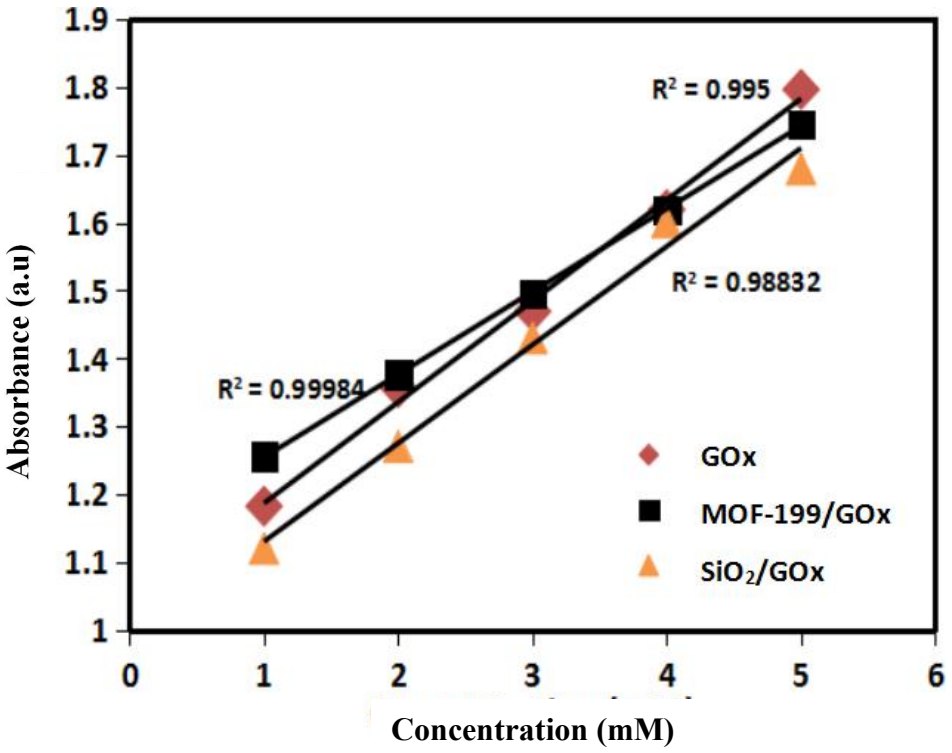


Figure 4.10: UV-Vis analysis on optical measurement of different concentration of glucose using GOx, MOF-199/GOx and SiO_2/GOx.

4.6 Amperometric Glucose Measurement

The results obtained demonstrated the proportional relationship between the current produced and increment of glucose concentration from 1.0 mM to 5.0 mM to free GOx, MOF-199/GOx and SiO_2/GOx suggesting the successful reaction between GOx and glucose. From the Figure 4.11, the linear range obtained for GOx, MOF-199 and SiO_2/GOx when reacted with glucose from 1.0- 5.0 mM were 0.988, 0.996 and 0.999. All of the ranges were approaching to absolute 1 which supported the optical measurement results as well as solid evidences of active GOx after immobilization process. These patterns are similar with the glucose measurement using light absorbance as in Figure 4.10.

Apart of optical glucose measurement, the concentration of glucose could be measured based on the current produced in the reaction (amperometric). In order to fulfill conventional glucose biosensors measurement, glucose solution was also

measured using amperometric measurement. Amperometric measurement is the measurement of current produced by the electron movement which is applied in current glucose biosensor's measurement (Clarke *et al.*, 2006; Amerov *et al.*, 2004; Ang *et al.,* 2015).

Similar to the optical glucose measurement analysis, the production of current is proportional towards the glucose concentration. This is due to the rate of electron transfer that is increased with the increment of glucose concentration, reacting with GOx. Based on the Eq. 3.1, the reaction of glucose with GOx involved the redox reaction on where the glucose was oxidized into gluconic acid and the oxygen (O_2) was reduced into hydrogen peroxides (H_2O_2). These reactions resulted in electron movement which produced current (Kong *et al.*, 2009; Arslan *et al.*, 2011). Based, on these reactions, the current produced were proportional towards the concentration of the analyte suggesting the direct measurement of glucose concentrations based on current reading. Therefore, The higher the concentration of the glucose, the higher the rate of electron movement that would result in higher production of current (mA) (Zdarta *et al.,* 2018).

As compared to the reaction of free GOx with MOF-199/GOx and SiO_2/GOx nanocomposites, both reactions of nanocomposites with glucose provided better linear range than reaction of free GOx and glucose. The reason is because, MOF-199 and SiO_2 act as the 'stabilizer' to provide better support for GOx when reacted with glucose thus providing stability for GOx (Zdarta *et al.,* 2018). The results obtained also demonstrated better linear range as compared to previous studies, The linear range obtained for gel entrapment of gelatin and SiO_2 on platinum electrode (gel-SiO_2/Pt) gave the linear range of R^2=0.833 from 0.0 mM-16.0 mM of glucose concentations (Le *et al.*, 2010). Meanwhile, the MOF growth on platinum nanoparticles did provide a linear range for less than 0.95 which had proved that electrostatic adsorption was capable to produce a good nanocomposite materials for application of glucose biosensor (Le *et al.*, 2010). To conclude, the produced nanocomposites using electrostatic adsorption were capable to be applied for glucose sensing material fabrication.

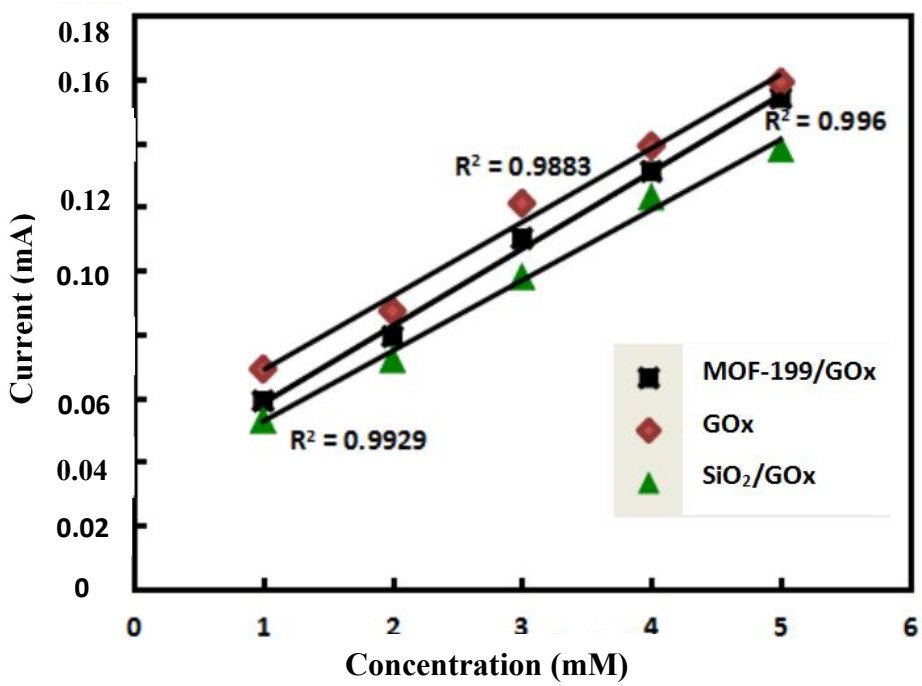


Figure 4.11: Amperometric analysis on GOx, MOF-199/GOx and SiO_2/GOx of different concentration of glucose.

4.7 Limit of Detection

One of the arguments stressed in the problem statement was to produce a high sensitivity glucose sensing material without disrupting both of the GOx activities as well as the support. Therefore, the limit of detection (LOD) analyses were conducted in order to measure the highest limit of both MOF-199/GOx and SiO_2/GOx nanocomposites to measure low concentration of glucose. The LOD measurement of the nanocomposites was intended in order to innovate the high sensitivity glucose sensing nanocomposites for non invasive approach of of glucose measurement particularly in saliva.

Figure 4.12 demonstrates the linear range of LOD for both MOF-199/GOx and SiO_2/GOx were 0.3- 1.0 mM and 0.4-1.0 mM respectively. The results exhibited were also comparable to actual glucose with linear LOD of 0.2 mM-1.0 mM. The results demonstrated satisfactory LOD for both MOF-199/GOx and SiO_2/GOx which provide high LOD for glucose measurement.

Current established glucose biosensors are able to detect as low as 0.6 mM of glucose concentration with average ranging of 10 mg/dL (0.56 mM) to 600 mg/dL (33.3 mM) dominated by four major companies including Bayer, Roche, Abbot, Johnson & Johnson Lifescan (Turner *et. al.,* 2013). Nonetheless, a noninvasive through salivary attempt glucose sensing materials should be able to detect as low as 0.11 mM (normal, fasting person) and 0.43 mM for FSG(Gupta *et al.,* 2017; Abikshyeet *et al.*, 2012; Patel *et al.*, 2015; Arslan *et al.*, 2011). Therefore, through continuous studies and researches, several attempts to produce high sensitivity glucose biosensors were made.

Based on the recent studies on functionalized nanotube titanium (Ti) array, the results demonstrated amperometric linear range of glucose measurement from 0.1 mM to 70 mM with detection limit of 0.032 mM (Gao *et al.*, 2014). In addition, the rhodium nanoparticles with glucose oxidase immobilized on gold nanopartices reported the linear range of 0.05- 0.15 mM glucose measurement with 0.03 mM of LOD (Gao *et al.*, 2014). However as compared to this work, the materials used from previous studies were higher in electrochemical properties thus very expensive. In addition, the immobilization procedures were more complex as compared to this study.

This result supported the successful of high sensitivity glucose sensing nanocomposites using electrostatic adsorption. These results were parallel with current results using the facile method. Therefore the electrostatic adsorption was an ideal immobilization method in producing high sensitivity MOF-199/GOx and SiO_2/GOx nanocomposition.

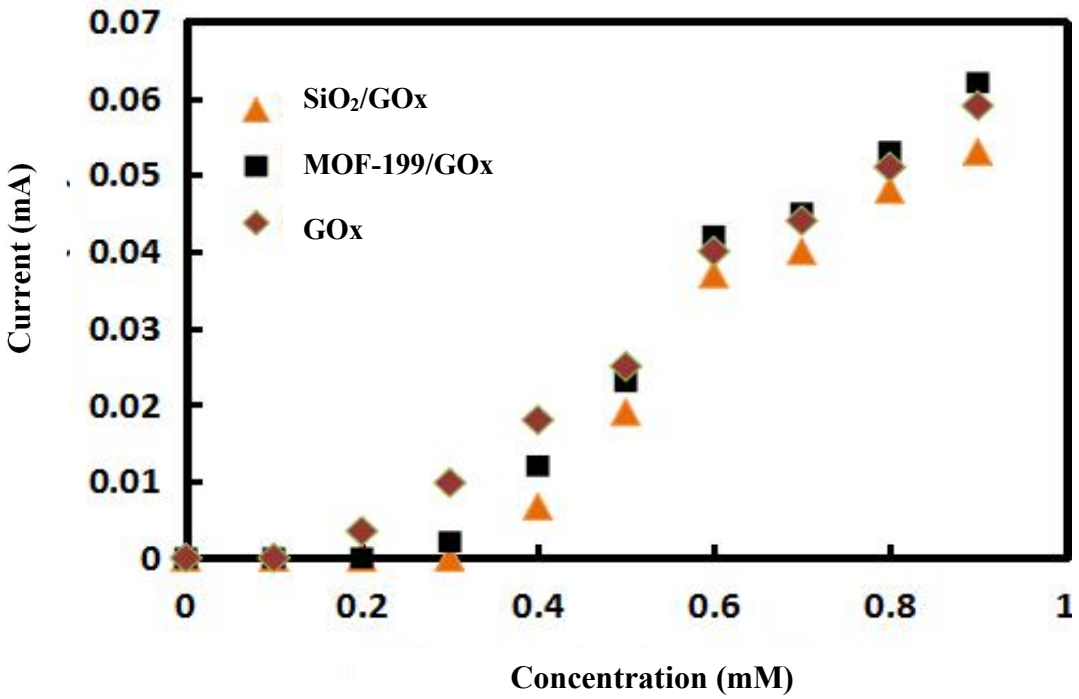


Figure 4.12: Amperometric analysis on LOD of different concentration of glucose using GOx, MOF-199/GOx and SiO_2/GOx.

4.8 Glucose Oxidase (GOx) Stability

Biological enzymes are not stable in extreme ambiances such as pH and temperature of the ambiances (Setlow *et al.,* 2013; Burgess *et al.,* 2010). This is because, most of biological enzymes react within the organism which require sensitive ambiances in order to sustain the continuation of specific cell function. (Fenchel *et al.,* 2012; Powers *et al.,* 1983). The extreme ambiances would inhibit the reaction of enzymes that may harm the specific cell function or corresponding organisms (Fenchel *et al.,* 2012; Powers *et al.,* 1983). Nevertheless, it could not be denied that biological enzymes serve provide great functionality especially in biosensor applications (Xu *et al.*, 2014; Sheldon *et al.*, 2013; Krajewska *et al.*, 2004). This is due to its very high sensitivity which is able to react to specific chemicals (Sheldon *et al.*, 2013; Krajewska *et al.*, 2004). Nonetheless, enzymes are expensive due to its complicated extraction process as well as proper storage (Sahu *et al.*, 2012). Therefore, the least risky immobilization procedures was prominent in order to lower the cost of fabrication of biosensor. In addition to that, a good immobilization procedure could

be measured on how the reusability of immobilized enzymes could be used. This is to ensure the immobilized enzyme could prolong its half life as well as providing better reusability for biosensing application.

In this study, the stability of GOx, MOF-199/GOx and SiO_2/GOx was conducted. GOx, MOF-199/GOx and SiO_2/GOx were reacted in 3.0 mM glucose concentration and the glucose measurement was taken amperometically for 30 days to in order to check its stability as well as their reusability.

The result in Figure 4.13 shows that the glucose measurement was more consistent for MOF-199/GOx and SiO_2/GOx as compared to actual GOx. From the result, all bare GOx, MOF-199/GOx and SiO_2/GOx reacted stably with glucose without massive GOx catalytic loss for 10 days. However, bare GOx experienced massive loss in catalytic activity and partially denatured (half- life) at day 11. Nonetheless, MOF-199/GOx and SiO_2/GOx could still withstand the stability of GOx activity when reacted with glucose for 22 and 20 days respectively. Both MOF-199/GOx and SiO_2/GOx had experienced the half life of GOx activities at day 23 and 21 days respectively. The results also demonstrated that MOF-199/GOx and SiO_2/GOx were more stable and had longer reusability before reaching its half life as compared to bare GOx. Both of MOF-199/GOx and SiO_2/GOx reacted stably in 3.0 mM of glucose before experiencing half life at day 23 and 21 respectively. In addition, the actual GOx could react with glucose stably for 11 days before experiencing its half life.

These results were due to GOx's active sites. GOx was more stable when immobilized with MOF-199 and SiO_2 as compared to bare GOx was because the GOx do not possess actual or rigid shape. Therefore, its tendency to lose the shape is higher compared to the immobilized GOx on a solid support. This would lead towards the alteration of GOx's shape after continuous reaction with substrate thus, GOx would lose its ability to react with glucose (Ang *et al.,* 2015; Moehlenbrock *et al.*, 2011) Thus, maintaining the shape of enzyme's active site throughout the biochemical reaction is very important as a key to unlock specific substrate in order to produce desired product (Moehlenbrock *et al.*, 2011). Therefore, as observed in

the result, both MOF-199/GOx and SiO_2/GOx have higher stability when reacted with glucose compared to bare GOx (Ang *et al.,* 2015). The immobilization on supports have maintained the active site of GOx and could react longer with glucose as compared to bare GOx Ang *et al.,* 2015; Moehlenbrock *et al.*, 2011). The results suggested that the immobilized GOx using electrostatic adsorption is more stable as compared to bare GOx.

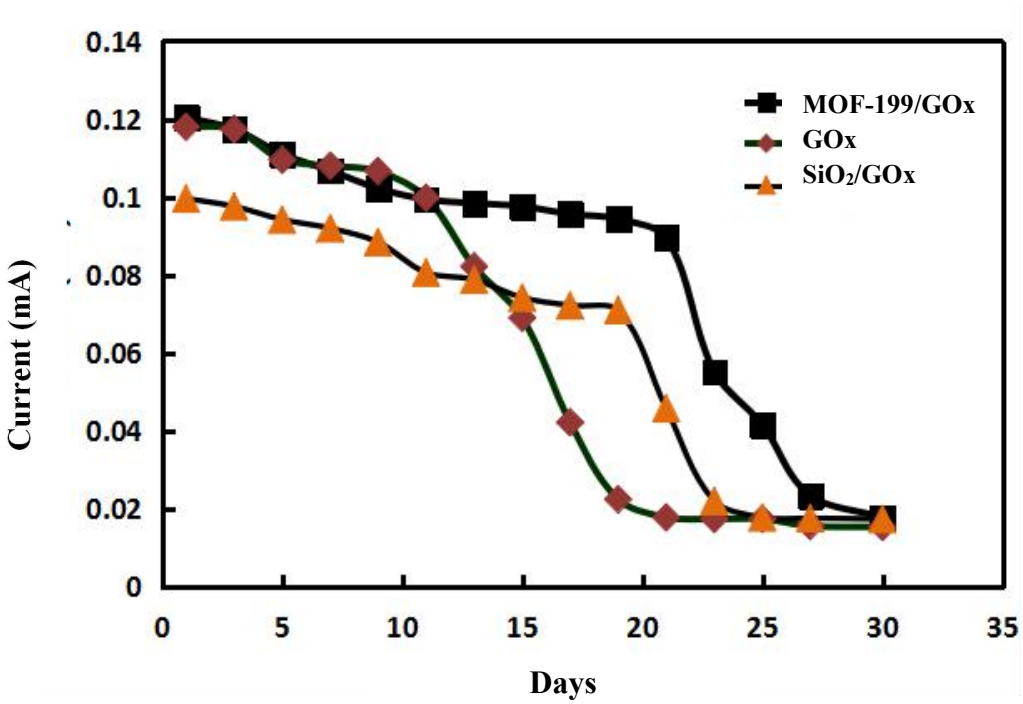


Fig. 4.13: Amperometric analysis on GOx stability (half life) using GOx, MOF-199/GOx and SiO_2/GOx in 3.0 mM of glucose.

4.9 Summary

The characterizations of MOF-199, SiO_2, MOF-199/GOx and SiO_2/GOx were discussed in this chapter. Basically the main purpose of the characterizations is to compare the results between before and after the immobilization of GOx onto MOF-199, SiO_2 nanocomposites in order to validify the new electrostatic adsorption method for GOx immobilization. The characterizations were divided into three main categories which include morphology, physical properties and chemical properties. From the FESEM analyses, there results observed possible GOx particles coating

both MOF-199/GOx and SiO_2/GOx. Furthermore, the optimum temperature and otimum pH for both MOF-199/GOx and SiO_2/GOx were 50°C and 7 respectively. These result supported the actual physical properties of GOx proving the presence of GOx after immobilization. The EDX results exhibited the N element of both MOF-199/GOx and SiO_2/GOx which attributed to the amino group from GOx. In addition, UV-Vis analysis demonstrated the absorabnce peak at ca. 280 nm for both MOF-199/GOx and SiO_2/GOx which attributed to the protein absorbance of GOx. Finally, the FT-IR analysis exhibited the peaks of C=O aldehyde bonds from reaction of GOx and glucose in MOF-199/GOx and SiO_2/GOx which have proved the presence of GOx after immobilization method. These results have provided positive evidences on successful immobilization of GOx onto MOF-199 and SiO_2 nanomaterials using new electrostatic adsorption method.

The application of MOF-199/GOx and SiO_2/GOx were discussed in this chapter. The application of the nanocomposites were focusing on the measurement of GOx activities as well as the stability and LOD of glucose sensing nanocomposites. The method of glucose activity measurement was divided into two which includes optical measurement (UV-Vis) and amperometric measurement (current production). Based on the results discussed, the UV-Vis analysis suggested that both MOF-199/GOx and SiO_2/GOx nanocomposites reacted proportionally with the increment of glucose concentration. Furthermore, the pattern of reaction and LOD of glucose measurement were parallel with actual GOx when reacted to the same glucose solutions thus, proving that the ability in glucose sensing. The amperometric glucose measurement demonstrated similar results as optical measurement on which the production of current is proportional towards the increment glucose concentration. Both MOF-199/GOx and SiO_2/GOx reacted successfully when reacted in different glucose concentrations. The limit of detection (LOD) for MOF-199/GOx and SiO_2/GOx nanocomposites was also measured in order to measure the stability for glucose sensing ability. The LOD for both MOF-199/GOx and SiO_2/GOx nanocomposites were 0.3 mM and 0.4 mM respectively. Both of the LODs were higher than current conventional glucose sensors (0.6 mM) on which provided better LOD to measure lower concentration of glucose. The results suit the objective of the research in which to attempt the fabrication of high stability and high LOD of glucose sensing materials

particularly salivary glucose detection. The stability of GOx from both MOF-199/GOx and SiO_2/GOx nanocomposites were also investigated. Based on the results, both of MOF-199/GOx and SiO_2/GOx reacted more stably in 3 mM of glucose before experiencing half life at day 23 and 21 respectively as compared to actual GOx which could only react stably for 11 days before experiencing half life. Therefore, the results proved that both of MOF-199/GOx and SiO_2/GOx nanocomposites were able to measure low glucose concentration in which in range with salivary glucose concentration. Furthermore, both of the immobilized composites have better reusability as compared to actual GOx which fit the high stability glucose sensing material. This could be the stepping stone in order to fabricate the non invasive glucose sensing materials particularly in salivary detection.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This research was basically focusing on fabrication of highly stability glucose sensing material using facile method of enzyme immobilization. As discussed in chapter 1 and chapter 2, diabetes is a serious illness which has caused major inability for body immune system as well as nerve system. However, diabetes can be controlled with constant glucose measurement and sufficient medicine. The current measurement of glucose however could be very invasive as it involves blood withdrawal (finger pricking). This method could draw serious outcomes if the glucose measurement is done constantly. Therefore, the fabrication for non invasive glucose sensing material was attempted in this study. In order to reach the objective of this research, the limit of detection (LOD) for glucose biosensor needed to be increased. Thus, the MOF-199 and SiO_2 nanomaterials were selected as the support for glucose oxidase (GOx) immobilization. MOF-199 has high porosity in order to maximize the enzyme loading into it. Meanwhile SiO_2 is a non metal nanomaterial which could stabilize the attachment of GOx onto it. This research was also focusing on facile method for enzyme immobilization as the process require high cost and time consuming. Therefore, glucose oxidase (GOx) were immobilized onto MOF-199 and SiO_2 using facile, electrostatic adsorption. The presence of GOx onto nanomaterials were studied by looking into morphological evidences, physical properties, chemical properties and elemental compositions between before and after immobilization of GOx onto the nanomaterials. These are the conclusions made by this research:

1. The evidences of GOx presented on these materials after electrostatic adsorption supported that the immobilization method was successful.

2. The presence of GOx in morphological evidence, optimization characteristics, chemical properties as well as elemental properties on MOF-199/GOx and SiO_2/GOx indicated successful immobilization method of GOx.

- The presence of GOx were observed on MOF-199/GOx and SiO_2/GOx morphologically after immobilization.

- The EDX analysis, the N from amino group suggested the GOx presence on the surface of MOF-199/GOx and SiO_2/GOx by 22% and 1.% respectively.

- The optimum temperature and pH of immobilized GOx were 50ºC and 7 respectively.

- The absorbance peak for MOF-199/GOx and SiO2/GOx was at 280 nm suggested the GOx absorbance.

3. The presence of GOx activities for optical and amperometric measurements, LOD and half life measurement had also proven the active GOx on MOF-199 and SiO_2 after electrostatic adsorption.

- MOF-199/GOx and SiO_2/GOx could measure glucose (optical and amperometric) in 1.0- 5.0 mM of glucose

- The limit of detection (LOD) for both MOF-199/GOx and SiO_2/GOx to 0.3 mM and 0.4 mM.

- Half life for both MOF-199/GOx and SiO_2/GOx were 23 days and 21 days respectively.

These results were prominent in attempt to produce high stability and high LOD glucose biosensing material using facile method which could be attempted in further non-invasive glucose biosensor.

5.2 Recommendations

The GOx nanocomposites could be tested in actual clinical condition, if needed salivary glucose. This study could provide better data for fabrication of actual biosensing material.Various types of nanomaterials could be attempted in producing the GOx nanocomposites using electrostatic adsorption. Furthermore, the characterization methods could be attempted in order to provide better sensitivity of current glucose sensing materials.

REFERENCES

Abikshyeet, P., Ramesh, V., & Oza, N. (2012). Glucose estimation in the salivary secretion of diabetes mellitus patients. *Diabetes, Metabolic Syndrome an Obesity: Targets and Therapy*, *5*, 149.

Agrawal, R. P., Sharma, N., Rathore, M. S., Gupta, V. B., Jain, S., Agarwal, V., & Goyal, S. (2013). Noninvasive method for glucose level estimation by saliva. *Journal of Diabetes and Metabolism*, 4(5), 2-5.

Ahmad, R., & Sardar, M. (2015). Enzyme immobilization: an overview on nanoparticles as immobilization matrix. *Biochemistry and Analytical Biochemistry*, 4(2), 1.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). The shape and structure of proteins. *Molecular Biology of the Cell,* 4,54-89.

Alvarez-Lorenzo, C., & Concheiro, A. (2014). Smart drug delivery systems: from fundamentals to the clinic. *Chemical Communications*, 50(58), 7743-7765.

Altun, S., Çakıroğlu, B., Özacar, M., & Özacar, M. (2015). A facile and effective immobilization of glucose oxidase on tannic acid modified CoFe2O4 magnetic nanoparticles. *Colloids and Surfaces B: Biointerfaces*, 136, 963-970.

American Diabetes Association. (2010). Diagnosis and classification of diabetes mellitus. *Diabetes Care*, 33(Suppl 1), S62.

Amerov, A., Fuller, M., Orendorff, J., & Howse, J. (2014). Real Time Measurements of Acetylene in Ethylene Manufacturing Process. *Field Trials of TDLAS Based Analyzer,* 6(2), 12.

Ang, L. F., Por, L. Y., & Yam, M. F. (2015). Development of an amperometric-based glucose biosensor to measure the glucose content of fruit. *PloS One*, 10(3), e0111859.

Arnold, M. A., & Rechnitz, G. A. (1987). Biosensors based on plant and animal tissue, 30, *Oxford University Press*, New York.

Arslan, F., Ustabaş, S., & Arslan, H. (2011). An Amperometric Biosensor for Glucose Determination Prepared from Glucose Oxidase Immobilized I Polyaniline-Polyvinylsulfonate Film. *Sensors*, 11(8), 8152-8163.

Asif, M. (2014). The prevention and control the type-2 diabetes by changing lifestyle and dietary pattern. *Journal of Education and Health Promotion*, 3 ,1-7.

Assal, J. P., Aoki, T. T., Manzano, F. M., & Kozak, G. P. (1974). Metabolic effects of sodium bicarbonate in management of diabetic ketoacidosis. *Diabetes*, 23(5), 405-411.

Avenas, P. (2012). Etymology of main polysaccharide names. In T*he European Polysaccharide Network of Excellence (EPNOE),* 13-21. Springer, Vienna.

Anuar, N. F. S., Misran, H., Manap, A., & Othman, S. Z. (2015). Review on immobilization of nanoparticles for fabrication of glucose biosensor. *Applied Mechanics and Materials,* 773, 720-724.

Balashanmugam, P., & Kalaichelvan, P. T. (2015). Biosynthesis characterization of silver nanoparticles using Cassia roxburghii DC. aqueous extract, and coated on cotton cloth for effective antibacterial activity. *International Journal of Nanomedicine*, 10(Suppl 1), 87.

Bajaj, S., Jawad, F., Islam, N., Mahtab, H., Bhattarai, J., Shrestha, D., & Aung, M. W. (2013). South Asian women with diabetes: psychosocial challenges and

management: consensus statement. *Indian Journal of Endocrinology and Metabolism*, 17(4), 548.

Bankar, S. B., Bule, M. V., Singhal, R. S., & Ananthanarayan, L. (2009). Glucose oxidase—an overview. *Biotechnology Advances*, 27(4), 489-501.

Behera, S., Ghanty, S., Ahmad, F., Santra, S., & Banerjee, S. (2012). UV-visible spectrophotometric method development and validation of assay of paracetamol tablet formulation. *Journal of Analytical and Bioanalytical Techniques*, 3(6), 151-7.

Bollella, P., Schulz, C., Favero, G., Mazzei, F., Ludwig, R., Gorton, L., & Antiochia, R. (2017). Green synthesis and characterization of gold and silver nanoparticles and their application for development of a third generation lactose biosensor. *Electroanalysis,* 29(1), 77-86.

Brena, B., González-Pombo, P., & Batista-Viera, F. (2013). Immobilization of enzymes: a literature survey. *Immobilization of Enzymes and Cells,*15-3, Humana Press, Totowa, NJ.

Bruen, D., Delaney, C., Florea, L., & Diamond, D. (2017). Glucose sensing for diabetes monitoring: recent developments. *Sensors*, 17(8), 1866.

Buchanan, T. A., Xiang, A. H., & Page, K. A. (2012). Gestational diabetes mellitus: risks and management during and after pregnancy. *Nature Reviews Endocrinology*, 8(11), 639.

Burgess, S. A., Lindsay, D., & Flint, S. H. (2010). Thermophilic bacilli and their importance in dairy processing. *International Journal of Food Microbiology*, 144(2), 215-225.

Carda, C., Mosquera-Lloreda, N., Salom, L., Gomez de Ferraris, M. E., & Peydró, A. (2006). Structural and functional salivary disorders in type 2 diabetic patients. *Medicina Oral Patologia Oral y Cirugia Bucal*, 11(4), 209.

Cendrowski, K., Chen, X., Zielinska, B., Kalenczuk, R. J., Rümmeli, M. H., Büchner, B., & Borowiak-Palen, E. (2011). Synthesis, characterization, and photocatalytic properties of core/shell mesoporous silica nanospheres supporting nanocrystalline titania. *Journal of Nanoparticle Research*, 13(11), 5899-5908.

Chase, H. P. (2000). Understanding insulin-dependent diabetes. Denver, CO: *Barbara Davis Center for Childhood Diabetes*. University of Colorado Health Sciences Center.

Chiang, J. L., Kirkman, M. S., Laffel, L. M., & Peters, A. L. (2014). Type 1 diabetes through the life span: a position statement of the American Diabetes Association. *Diabetes Care,* 37(7), 2034-2054.

Cho, N. H., Shaw, J. E., Karuranga, S., Huang, Y., da Rocha Fernandes, J. D., Ohlrogge, A. W., & Malanda, B. (2018). IDF Diabetes Atlas: global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Research and Clinical Practice*, 138, 271-281.

Dahlqvist, A. (1961). Determination of maltase and isomaltase activities with a glucose-oxidase reagent. *Biochemical Journal*, 80(3), 547.

Damborsky, P., Vitel, J., & Katrlik, J. (2016). Optical biosensors. *Essays In Biochemistry*, 60(1), 91–100.

Datta, S., Christena, L. R., & Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials. *3 Biotech*, 3(1), 1-9.

Dhotel, A., Chen, Z., Delbreilh, L., Youssef, B., Saiter, J. M., & Tan, L. (2013). Molecular motions in functional self-assembled nanostructures. *International Journal of Molecular Sciences*, 14(2), 2303-2333.

Diabetes Control and Complications Trial Research Group. (1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *New England Journal of Medicine*, 329(14), 977-986.

Drummond, T. G., Hill, M. G., & Barton, J. K. (2003). Electrochemical DNA sensors. *Nature biotechnology*, *21*(10), 1192.

Du, Y., & Dong, S. (2016). Nucleic Acid Biosensors: Recent Advances and Perspectives. *Analytical Chemistry*, 89(1), 189–215.

Duarte, A. I., Moreira, P. I., & Oliveira, C. R. (2012). Insulin in central nervous system: more than just a peripheral hormone. *Journal of Aging Research*, 2012, 1-21

Ekanayake, E. M. I., Preethichandra, D. M. G., & Kaneto, K. (2008). Effect of Glucose Oxidase Immobilizing Techniques on Performances of Nano Scale Polypyrrole Glucose Biosensors. *Japanese Journal of Applied Physics*, 47, 1321.

Fan, M., Dai, D., & Huang, B. (2012). Fourier transform infrared spectroscopy for natural fibres. In *Fourier Transform-Materials Analysis,* 3, 45-64.

Fenchel, T., Blackburn, T. H., & King, G. M. (2012). Bacterial biogeochemistry: the ecophysiology of mineral cycling. *Academic Press*. 3, 27-52.

Ferri, S., Kojima, K., & Sode, K. (2011). Glucose Electrochemistry: Review of Glucose Oxidases and Glucose Dehydrogenases: A Bird's Eye View of Glucose

Sensing Enzymes. *Journal of Diabetes Science and Technology*, 5(5), 1068-1076.

Flitsch, S. L., & Ulijn, R. V. (2003). Sugars tied to the spot. *Nature*, 421(6920), 219.

Forouhi, N. G. ,& Wareham, N. J.(2010). Epidemiology of diabetes. *Medicine*, 38(11), 602-606.

Guariguata, L., Whiting, D. R., Hambleton, I., Beagley, J., Linnenkamp, U., & Shaw, J. E. (2014). Global estimates of diabetes prevalence for 2013 and projections for 2035. *Diabetes Research and Clinical Practice*, 103(2), 137-149.

Gopal, K., Saleme, B., Al Batran, R., Aburasayn, H., Eshreif, A., Ho, K. L., & Park, E. A. (2017). FoxO1 regulates myocardial glucose oxidation rates via transcriptional control of pyruvate dehydrogenase kinase 4 expression. *American Journal of Physiology-Heart and Circulatory Physiology*, 313(3), H479-H490.

Guo, X., Liang, B., Jian, J., Zhang, Y., & Ye, X. (2014). Glucose biosensor based on a platinum electrode modified with rhodium nanoparticles and with glucose oxidase immobilized on gold nanoparticles. *Microchimica Acta*, 5(181), 519-525.

Gupta, M. N., Kaloti, M., Kapoor, M., & Solanki, K. (2011). Nanomaterials as matrices for enzyme immobilization. *Artificial Cells, Blood Substitutes, and Biotechnology*, *39*(2), 98-109.

Gupta, S., Nayak, M. T., Sunitha, J. D., Dawar, G., Sinha, N., & Rallan, N. S. (2017). Correlation of salivary glucose level with blood glucose level in diabetes mellitus. *Journal of Oral and Maxillofacial Pathology*, 21(3), 334.

Guzik, U., Hupert-Kocurek, K., & Wojcieszyńska, D. (2014). Immobilization as a strategy for improving enzyme properties-application to oxidoreductases. *Molecules*, 19(7), 8995-9018.

Hahn, Y. B., Ahmad, R., & Tripathy, N. (2012). Chemical and biological sensors based on metal oxide nanostructures. *Chemical Communications*, 48(84), 10369-10385.

Hameed, I., Masoodi, S. R., Mir, S. A., Nabi, M., Ghazanfar, K., & Ganai, B. A. (2015). Type 2 diabetes mellitus: from a metabolic disorder to an inflammatory condition. *World Journal of Diabetes*, 6(4), 598.

Harper, A., & Anderson, M. R. (2010). Electrochemical glucose sensors—developments using electrostatic assembly and carbon nanotubes for biosensorconstruction. *Sensors*, 10(9), 8248-8274.

Hoarau, M., Badieyan, S., & Marsh, E. N. G. (2017). Immobilized enzymes: understanding enzyme–surface interactions at the molecular level. *Organic & Biomolecular Chemistry*, 15(45), 9539-9551.

House, J. L., Anderson, E. M., & Ward, W. K. (2007). Immobilization techniques to avoid enzyme loss from oxidase-based biosensors: a one-year study. *Journal of Diabetes Science and Technology*, 1(1), 18-27.

Hu, F. B. (2011). Globalization of diabetes: the role of diet, lifestyle, and genes. *Diabetes Care*, 34(6), 1249-1257.

Husain Q (2017). Nanomaterial as novel supports of the immobilization of amylolytic enzymes and their applications: A review. *Biocatalysis,* 3, 37–53

Husain, Q., (2010). Peroxidase mediated decolorization and remediation of waste water containing industrial dyes: A review. *Reviews in Environmental Science and Bio/Technology*, 9(2),117-140.

Jin, Z., Zhou, Q., Chen, Y., Mao, P., Li, H., Liu, H., ... & Li, Y. (2016). Graphdiyne: ZnO Nanocomposites for High-Performance UV Photodetectors. *Advanced Materials*, *28*(19), 3697-3702.

Johnson, K. A. (2008). Role of induced fit in enzyme specificity: a molecular forward/reverse switch. *Journal of Biological Chemistry*, 283(39), 26297-26301.

Kalogeris, T., Bao, Y., & Korthuis, R. J. (2014). Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning. *Redox Biology*, 2, 702-714.

Keilin, D., & Hartree, E. F. (1948). Properties of glucose oxidase (notatin): Addendum. Sedimentation and diffusion of glucose oxidase (notatin). *Biochemical Journal*, 42(2), 221.

Kim, N. Y., Adhikari K. K., Dhakal, R., Chuluunbaatar, Z., Wang C., & Kim, E. S. (2015). Rapid, sensitive and reusabledetection of glucose by a robust radiofrequency integrated passive device biosensor chip, Scientific Reports, 5, 7807.

Kong, T., Chen, Y., Ye, Y., Zhang, K., Wang, Z., & Wang, X. (2009). An amperometric glucose biosensor based on the immobilization of glucose oxidase on the ZnO nanotubes. *Sensors and Actuators B*: *Chemical*, 138(1), 344-350.

Kovács, I., Hermann, J., O'Neill, H. S. C., Gerald, J. F., Sambridge, M., & Horváth, G. (2008). Quantitative absorbance spectroscopy with unpolarized light: Part II. Experimental evaluation and development of a protocol for quantitative analysis of mineral IR spectra. *American Mineralogist*, 93(5-6), 765-778.

Koyun, A., Ahlatcolu, E., Koca, Y., & Kara, S. (2012). *A Roadmap of Biomedical Engineers and Milestones*, Rijeka: Intechopen, 115-124.

Krajewska, B. (2004). Application of chitin-and chitosan-based materials for enzyme immobilizations: a review. *Enzyme and Microbial Technology*, 35(2), 126-139.

Lakhtakia, A. (1990). *Selected Papers on Natural Optical Activity*. Bellingham, Washington: SPIE Optical Engineering Press, 24-56.

Le, T. T. T., Tran, P. D., Pham, X. T., Tong, D. H., & Dang, M. C. (2010). Glucose oxidase immobilization on different modified surfaces of platinum nanowire for application in glucose detection. *Advances in Natural Sciences: Nanoscience and Nanotechnology*, 1(3), 035004.

Leskovac, V., Trivić, S., Wohlfahrt, G., Kandrač, J., & Peričin, D. (2005). Glucose oxidase from Aspergillus niger: the mechanism of action with molecular oxygen, quinones, and one-electron acceptors. *The International Journal of Biochemistry & Cell Biology*, 37(4), 731-750.

Liu, Y., Dong, X., & Chen, P. (2012). Biological and chemical sensors based on graphene materials. *Chemical Society Reviews*, 41(6), 2283-2307.

Lorenz, R. A., Malone, J. I., Nathan, D. M., & Peterson, C. M. (2003). Tests of Glycemia in Diabetes. *Diabetes Care*, 26, 1-12.

Lubert, K. H., & Kalcher, K. (2010). History of electroanalytical methods. *Electroanalysis*, 22(17-18), 1937-1946.

Luca, P., Lepore, M., Portaccio, M., Esposito, R., Rossi, S., Bencivenga, U., & Mita, D. G. (2007). Glucose determination by means of steady-state and time-course UV fluorescence in free or immobilized glucose oxidase. *Sensors*, 7(11), 2612-2625.

Magon, N., & Seshiah, V. (2011). Gestational diabetes mellitus: Non-insulin management. *Indian Journal of Endocrinology and Metabolism*, 15(4), 284.

Mahadi, N., Misran, H., & Othman, S. Z. (2016). Synthesis and Characterizations of MOF-199 using PODFA as Porogen for CO2 Adsorption Applications. *Key Engineering Materials*, 694, 44-49.

Makaram, P., Owens, D., & Aceros, J. (2014). Trends in nanomaterial-based non-invasive diabetes sensing technologies. *Diagnostics*, 4(2), 27-46.

Marchetti, P. (2016). Islet inflammation in type 2 diabetes. *Diabetologia*, 59(4), 668-672.

Mark, H. L., & Griffiths, P. R. (2002). Analysis of noise in Fourier transform infrared spectra. *Applied Spectroscopy*, 56(5), 633-639.

Misran, H., Yarmo, M. A., & Ramesh, S. (2013). Synthesis and characterization of silica nanospheres using nonsurfactant template. *Ceramics International*, 39(2), 931-940.

Misson, M., Zhang, H., & Jin, B. (2015). Nanobiocatalyst advancements and bioprocessing applications. *Journal of the Royal Society Interface*, 12(102), 2 0140891.

Moehlenbrock, M. J., Toby, T. K., Pelster, L. N., & Minteer, S. D. (2011). Metabolon catalysts: an efficient model for multi-enzyme cascades at electrode surfaces. *ChemCatChem*, 3(3), 561-570.

Mohamad, N. R., Marzuki, N. H. C., Buang, N. A., Huyop, F., & Wahab, R. A. (2015). An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnology & Biotechnological Equipment*, 29(2), 205-220.

Monnier, L., & Colette, C. (2009). Target for glycemic control concentrating on glucose. *Diabetes Care*, 32(suppl 2), S199-S204.

N. F. Saiful Anuar, H. Misran, M. Ahmad Salim, S.Z. Othman.(2016). Facile Method of Enzyme Immobilization on Silica Quasi-Nanospheres (SiO_2) Synthesized Using PODFA for Biosensing Applications. *Key Engineering Materials*. 694,67-71,

Nguyen, L. T., Nguyen, T. T., Nguyen, K. D., & Phan, N. T. (2012). Metal–organic framework MOF-199 as an efficient heterogeneous catalyst for the aza-Michael reaction. *Applied Catalysis A: General*, 425, 44-52.

Ozougwu, J. C., Obimba, K. C., Belonwu, C. D., & Unakalamba, C. B. (2013). The pathogenesis and pathophysiology of type 1 and type 2 diabetes mellitus. *Journal of Physiology and Pathophysiology*, 4(4), 46-57.

Palanisamy, S., Devasenathipathy, R., Chen, S. M., Ajmal Ali, M., Karuppiah, C., Balakumar, V.,& Al-Hemaid, F. M. (2015). Direct Electrochemistry of Glucose Oxidase at Reduced Graphene Oxide and β-Cyclodextrin Composite Modified Electrode and Application for Glucose Biosensing. *Electroanalysis*, 27(10), 2412-2420.

Panchbhai, A. S., Degwekar, S. S., & Bhowte, R. R. (2010). Estimation of salivary glucose, salivary amylase, salivary total protein and salivary flow rate in diabetics in India. *Journal of Oral Science*, 52(3), 359-368.

Patel, B. J., Dave, B., Dave, D., Karmakar, P., Shah, M., & Sarvaiya, B. (2015). Comparison and correlation of glucose levels in serum and saliva of both diabetic and non-diabetic patients. *Journal of International Oral Health*, 7(8), 70.

Peng, G., Hou, X., Liu, B., Chen, H., & Luo, R. (2016). Stabilized enzyme immobilization on micron-size PSt–GMA microspheres: different methods to

improve the carriers' surface biocompatibility. *RSC Advances*, 6(94), 91431-91439.

Peyser, T., Dassau, E., Breton, M., & Skyler, J. S. (2014). The artificial pancreas: current status and future prospects in the management of diabetes. *Annalytical of the New York Academy of Science*s, 1311(1), 102-123.

Pfleger B.F., Pitera D.J., Newman J.D., Martin V.J.J., Keasling J.D. (2007). Microbial sensors for small molecules: Development of a mevalonate biosensor. *Metabolic Engineering*, 9(30),38.

Potts, W. J., & Smith, A. L. (1967). Optimizing the operating parameters of infrared spectrometers. *Applied Optics*, 6(2), 257-265.

Powers, E. L. (1983). Physical determinants of radiation sensitivity in bacterial spores. *Advances in Space Research*, 3(8), 73-78.

Rewers, M. J., Pillay, K., De Beaufort, C., Craig, M. E., Hanas, R., Acerini, C. L., & Maahs, D. M. (2014). Assessment and monitoring of glycemic control in children and adolescents with diabetes. *Pediatric Diabetes*, 15, 102-114.

Roglic, G. (2016). WHO Global report on diabetes: A summary. *International Journal of Noncommunicable Diseases*, 1(1), 3.

Röder, P. V., Wu, B., Liu, Y., & Han, W. (2016). Pancreatic regulation of glucose homeostasis. *Experimental & Molecular Medicine*, 48(3), e219.

Sahu, S. K., Thangaraj, M., & Kathiresan, K. (2012). DNA extraction protocol for plants with high levels of secondary metabolites and polysaccharides without using liquid nitrogen and phenol. *ISRN Molecular Biology*, 2012, 1-6.

Salim, Musdalilah Ahmad (2014),. Effect of NH_3 on Structural and Optical Properties of SiO2-CuO Core-Shell Nanostructure. *Applied Mechanics and Materials,* 465, 813-818.

Sergey M. B., Otto S. W. (2008). Optical Biosensors.*Chemical Reviews*,108, 2.

Sharma, S. K., Sehgal, N., & Kumar, A. (2003). Biomolecules for development of biosensors and their applications. *Current Applied Physics*, 3(2-3), 307–316.

Sassolas, A., Blum, L. J., & Leca-Bouvier, B. D. (2012). Immobilization strategies to develop enzymatic biosensors. *Biotechnology Advances*, 30(3), 489-511.

Senkovska, I., & Kaskel, S. (2014). Ultrahigh porosity in mesoporous MOFs: promises and limitations. *Chemical Communications*, 50(54), 7089-7098.

Setford, S. J., & Newman, J. D. (2005). *Enzyme biosensors. In Microbial Enzymes and Biotransformations.* Totowa, New Jersey;Humana Press, 29-60.

Setlow, P., & Johnson, E. A. (2013). Spores and their significance. *Food Microbiology*, 4, 45-79..

Shao, Y., Wang, J., Wu, H., Liu, J., Aksay, I. A., & Lin, Y. (2010). Graphene based electrochemical sensors and biosensors: a review. *Electroanalysis*, 22(10), 1027-1036.

Sheldon, R. A. (2011). Characteristic features and biotechnological applications of cross-linked enzyme aggregates (CLEAs). *Applied Microbiology and Biotechnology*, 92(3), 467-477.

Sheldon, R. A., & van Pelt, S. (2013). Enzyme immobilisation in biocatalysis: why, what and how. *Chemical Society Reviews*, 42(15), 6223-6235.

Shih, C. J., & Smith, E. A. (2009). Determination of glucose and ethanol after enzymatic hydrolysis and fermentation of biomass using Raman spectroscopy. *Analytica Chimica Acta*, 653(2), 200-206.

Shokri, B., Firouzjah, M. A., & Hosseini, S. I. (2009). FTIR analysis of silicon dioxide thin film deposited by metal organic-based PECVD. *Proceedings of 19th International Symposium on Plasma Chemistry Society,* 2631, 26-31.

Singh, J., & Verma, N. (2013). Glucose oxidase from Aspergillus niger: production, characterization and immobilization for glucose oxidation. *Advances in Applied Science Research*, 4, 250-257.

Soares, M. S., Batista Filho, M. M., Pimentel, M. J., Passos, I. A., & Chimenos Küstner, E. (2009). Determination of salivary glucose in healthy adults. *Medicina Oral, Patología Oral y Cirugia Bucal*, 14, (10), 510-513.

South Asian women with diabetes: Psychosocial challenges and management: Consensus statement. *Indian Journal of Endocrinology and Metabolism*, 17(4), 548.

Su, L., Jia, W., Hou, C., & Lei, Y. (2011). Microbial biosensors: a review. *Biosensors and Bioelectronics*, 26(5), 1788-1799.

Thi, T. V. N., Luu, C. L., Hoang, T. C., Nguyen, T., Bui, T. H., Nguyen, P. H. D., & Thi, T. P. P. (2013). Synthesis of MOF-199 and application to CO_2 adsorption. Advances in Natural Sciences. *Nanoscience and Nanotechnology*, 4(3), 035016.

Theint, H. T., Walsh, J. E., Wong, S. T., Voon, K., & Shitan, M. (2019). Development of an optical biosensor for the detection of Trypanosoma evansi and Plasmodium berghei. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 218, 348-358.

Tranchemontagne, D. J., Hunt, J. R., & Yaghi, O. M. (2008). Room temperature synthesis of metal-organic frameworks: MOF-5, MOF-74, MOF-177, MOF-199, and IRMOF-0. *Tetrahedron*, 64(36), 8553-8557.

Turner, A. P. (2013). Biosensors: sense and sensibility. *Chemical Society Review*s, 42(8), 3184-3196.

Tyagi, M., Tomar, M., & Gupta, V. (2013). NiO nanoparticle-based urea biosensor. *Biosensors and Bioelectronics*, 41, 110-115.

Vambergue, A., & Fajardy, I. (2011). Consequences of gestational and pregestational diabetes on placental function and birth weight. *World Journal of Diabetes*, 2(11), 196.

Vashist, S. K., Zheng, D., Al-Rubeaan, K., Luong, J. H., & Sheu, F. S. (2011). Technology behind commercial devices for blood glucose monitoring in diabetes management: A review. *Analytica Chimica Acta*, 703(2), 124-136.

Viswanath, B., Choi, C. S., Lee, K., & Kim, S. (2017). Recent trends in the development of diagnostic tools for diabetes mellitus using patient saliva. *Trends in Analytical Chemistry*, 89, 60-67.

Walcarius, A., Minteer, S. D., Wang, J., Lin, Y., & Merkoçi, A. (2013). Nanomaterials for bio-functionalized electrodes: recent trends. *Journal of Materials Chemistry B*, 1(38), 4878-4908.

Walton, P. W., O'Flaherty, M. R., Butler, M. E., & Compton, P. (1993). Gravimetric biosensors based on acoustic waves in thin polymer films. *Biosensors and Bioelectronics*, 8(9-10), 401-407.

Wang, B., Du, J., Zhu, Z., Ma, Z., Wang, S., & Shan, Z. (2017). Evaluation of Parotid Salivary Glucose Level for Clinical Diagnosis and Monitoring Type 2 Diabetes Mellitus Patients. *BioMed Research International*, 2017, 1-5.

Wang, J. (2001). Glucose biosensors: 40 years of advances and challenges. *Electroanalysis*, 13(12), 983.

Wang, X., & Andrews, L. (2006). Infrared spectra of M (OH) 1, 2, 3 (M= Mn, Fe, Co, Ni) molecules in solid argon and the character of first row transition metal hydroxide bonding. *The Journal of Physical Chemistry A*, 110(33), 10035-10045.

Wilson, R., & Turner, A. P. F. (1992). Glucose oxidase: an ideal enzyme. *Biosensors and Bioelectronics*, 7(3), 165-185.

Wohlfahrt, G., Witt, S., Hendle, J., Schomburg, D., Kalisz, H. M., & Hecht, H. J. (1999). 1.8 and 1.9 Å resolution structures of the Penicillium amagasakiense and Aspergillus niger glucose oxidases as a basis for modelling substrate complexes. *Acta Crystallographica Section D: Biological Crystallography*, 55(5), 969-977.

Wong, C. M., Wong, K. H., & Chen, X. D. (2008). Glucose oxidase: natural occurrence, function, properties and industrial applications. *Applied Microbiology and Biotechnology*, 78(6), 927-938.

Xu, K., Li, W., Liu, Q., Li, B., Liu, X., An, L., & Hu, J. (2014). Hierarchical mesoporous NiCo 2 O 4@ MnO 2 core–shell nanowire arrays on nickel foam for aqueous asymmetric supercapacitors. *Journal of Materials Chemistry A*, 2(13), 4795-4802.

Xue, L., Yang, Y., Zhang, Z. G., Dong, X., Gao, L., Bin, H., ... & Li, Y. (2016). Indacenodithienothiophene–naphthalene diimide copolymer as an acceptor for all-polymer solar cells. *Journal of Materials Chemistry A*, 4(16), 5810-5816.

Yoo, E. H., & Lee, S. Y. (2010). Glucose biosensors: an overview of use in clinical practice. *Sensors*, 10(5), 4558-4576.

Zdarta, J., Meyer, A., Jesionowski, T., & Pinelo, M. (2018). A general overview of support materials for enzyme immobilization: Characteristics, properties, practical utility. *Catalysts*, 8(2), 92.

Zhang, M., Liao, C., Yao, Y., Liu, Z., Gong, F., & Yan, F. (2014). High-Performance Dopamine Sensors Based on Whole-Graphene Solution-Gated Transistors. *Advanced Functional Materials*, 24(7), 978-985.

Zhang, W., Du, Y., & Wang, M. L. (2015). Noninvasive glucose monitoring using saliva nano-biosensor. *Sensing and Bio-Sensing Research*, 4, 23-29.

Zhao, Z., Lei, W., Zhang, X., Wang, B., & Jiang, H. (2010). ZnO-based amperometric enzyme biosensors. *Sensors*, 10(2), 1216-1231.

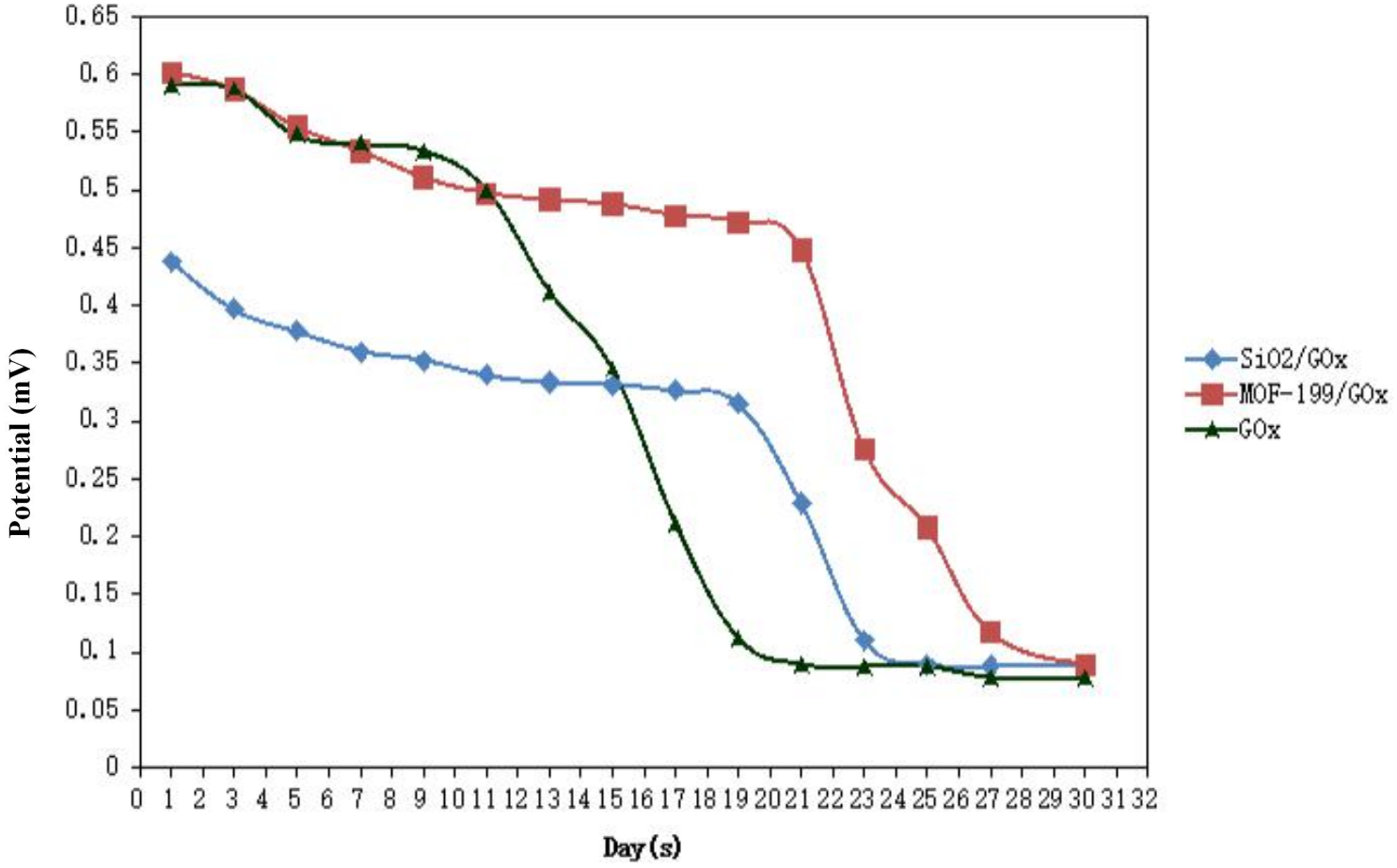
Zhou, L., Jiang, Y., Gao, J., Zhao, X., Ma, L., & Zhou, Q. (2012). Oriented immobilization of glucose oxidase on graphene oxide. *Biochemical Engineering Journal*, 69, 28-31.

Zhu, Y. T., Ren, X. Y., Liu, Y. M., Wei, Y., Qing, L. S., & Liao, X. (2014). Covalent immobilization of porcine pancreatic lipase on carboxyl-activated magnetic nanoparticles: characterization and application for enzymatic inhibition assays. *Materials Science and Engineering: C*, 38, 278-285.

Zucca, P., & Sanjust, E. (2014). Inorganic materials as supports for covalent enzyme immobilization: methods and mechanisms. *Molecules*, 19(9), 14139-14194.

APPENDICES

Appendix I



Potential (mV) against days for GOx activities