ENHANCEMENT OF GRAPHENE BASED ENZYMATIC GLUCOSE BIOFUEL CELL

ARMAN AMANI BABADI

INSTITUTE OF GRADUATE STUDIES
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

ENHANCEMENT OF GRAPHENE BASED ENZYMATIC GLUCOSE BIOFUEL CELL

ARMAN AMANI BABADI

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF GRADUATE STUDIES
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: ARMAN AMANI BABDI

Registration/Matric No: HHC140010

Name of Degree: DOCTORE OF PHILOSOPHY

Title Thesis: ENHANCEMENT OF GRAPHENE BASED ENZYMATIC GLUCOSE BIOFUEL CELL

Field of Study: BIONANOTECHNOLOGY (APPLIED SCIENCE)

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyrighted work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyrighted work;
(5) I hereby assign all and every right in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

ENHANCEMENT OF GRAPHENE BASED ENZYMATIC GLUCOSE BIOFUEL CELL

ABSTRACT

Nowadays, scientists and engineers around the world, attracted by the variety of high applicable implantable devices, supplying their required energy to sense, monitor, pump drugs, vibrate and report the collected data in sensing, wireless transmission devices in military service, homeland security, environmental monitoring and biomedical applications. This power source should be environmentally friendly with long lifetime and even implantable as well as capable of producing energy from the living organisms' fluids. These required properties light up the idea of using enzymatic biofuel cells (EBFCs) to generate the power. Glucose oxidase (GOx) is the most common enzyme which employs widely in the fabrication of glucose biofuel cells (GBFCs). Unfortunately, short lifetime and poor electron transfer of the enzyme to the electrode surface, limit GBFCs applications. Recent studies have indicated that 3-Dimensional graphene is a relatively novel material with unique properties that could make it useful in enzymatic biofuel cells. The graphene offers high electrical conductivity and highly porous structure. According to recent studies, carbon nanostructures can be widely used to immobilize the enzymes and transfer the electron from the enzyme active site to the electrode surface as well. In this project, 3-dimensional graphene is synthesized from the reduced graphene oxide. The synthesized 3D graphene employed to immobilize the glucose oxidase (GOx) on the surface of the glassy carbon (GC) electrode and enhance the electron transfer between them. The power output of the biofuel cell measured by employing the resistance box. The power output of 46.34 μW achieved, which corresponded to the power density of 164 μW cm^{-2} at 0.4 V. Furthermore, the results of testing fabricated EBFC reveal the recognizable enhancement in an enzyme lifetime of 230 days that is the great improvement compared with the literature. In addition, nano-hybrid of the Carbone Nanotubes (CNTs) and 3DG prepared and study as an alternative

nanomaterial to immobilize the enzyme on the surface of the electrode. The CNTs/3DG nano-hybrid improved the lifetime of the enzyme to 230 days and increased the output power density to 253 μWcm^{-2}. Owing to these results, novel 3DG and CNT/3DG nano-hybrid, enhanced the performance of the EBFCs, increase the lifetime of the enzymes, overcome the enzyme leaching challenge and finally improve the electron transfer between the enzyme active site and the electrode surface. The hybridized CNT/3DG nanocomposite is highly porous and conductive, which introduce it as a suitable candidate to immobilize GOx and fabricate EBFCs. The fabricated GBFC could generate the power for implantable devices, drug pumps, and biosensors and replace the usual batteries which commonly use to power the mentioned devices. 3DG and CNT/3DG could be used as an effective immobilization nanomaterial in the field of enzymatic biofuel cells, related research, such as biosensors, bioreactors, and even bring the commercial applications of implantable biofuel cell into the reality.

Keywords: glucose biofuel cell, 3_dimensional graphene, glucose oxidase, bio-anode.

PENINGKATAN GRAFEN BERDASARKAN ENZYMATIC GLUCOSE BIOFUEL CELL

ABSTRAK

Kini, saintis dan jurutera di seluruh dunia, tertarik dengan pelbagai alat implan yang boleh dipakai yang tinggi, membekalkan tenaga yang mereka perlukan untuk merasakan, memantau, mengepam dadah, bergetar dan melaporkan data yang dikumpul dalam penderiaan, peranti penghantaran wayarles dalam perkhidmatan tentera, keselamatan tanah air, pemantauan alam sekitar dan aplikasi bioperubatan. Sumber kuasa ini harus mesra alam dengan jangka hayat yang panjang dan dapat ditanamkan serta dapat menghasilkan tenaga daripada cecair organisma hidup. Ciri-ciri yang diperlukan ini menyala idea menggunakan sel biofuel enzim (EBFCs) untuk menjana kuasa. Glukosa oksidase (GOx) adalah enzim yang paling biasa yang digunakan secara meluas dalam pembuatan sel biofuel glukosa (GBFCs). Malangnya, jangka hayat pendek dan pemindahan elektron miskin enzim ke permukaan elektrod, hadkan aplikasi GBFCs. Kajian terbaru menunjukkan bahawa graphene 3-Dimensi adalah bahan yang agak baru dengan sifat-sifat unik yang dapat menjadikannya berguna dalam sel-sel biofuel enzim. Graphene ini menawarkan kekonduksian elektrik yang tinggi dan struktur berliang. Menurut kajian baru-baru ini, karbon nanostructures boleh digunakan secara meluas untuk melancarkan enzim dan memindahkan elektron dari tapak aktif enzim ke permukaan elektrod juga. Dalam projek ini, graphene 3-dimensi disintesis dari oksida graphene yang dikurangkan. Grafena 3D yang disintesis digunakan untuk melumpuhkan glukosa oksidase (GOx) pada permukaan elektrod karbon berkilau (GC) dan meningkatkan pemindahan elektron di antara mereka. Output kuasa sel biofuel diukur dengan menggunakan kotak penentangan. Output kuasa mencapai 46.34 μW dicapai, yang berkepadatan dengan ketumpatan kuasa 164 μW cm^{-2} pada 0.44 V. Tambahan pula, hasil ujian EBFC direka bentuk mengungkapkan peningkatan yang

dapat dikenali dalam seumur hidup enzim 230 hari yang merupakan peningkatan yang besar berbanding dengan kesusasteraan. Di samping itu, nano-hibrida Karbon Nanotubes (CNTs) dan 3DG disediakan dan belajar sebagai nanomaterial alternatif untuk melancarkan enzim pada permukaan elektrod. CNTs / 3DG nano-hybrid meningkatkan enzim seumur hidup hingga 230 hari dan meningkatkan ketumpatan kuasa output ke 253.36 μWcm^{-2}. Disebabkan keputusan ini, novel 3DG dan CNT/3DG nano-hibrida, meningkatkan prestasi EBFC, meningkatkan hayat enzim, mengatasi cabaran pencairan enzim dan akhirnya meningkatkan pemindahan elektrod antara tapak aktif enzim dan permukaan elektrod. Nanocomposite CNT / 3DG yang hibridisasi sangat berliang dan konduktif, yang memperkenalkannya sebagai calon yang sesuai untuk melancarkan GOx dan membuat EBFC. 3DG dan CNT / 3DG boleh digunakan sebagai nanomaterial immobilization berkesan dalam bidang sel biofuel enzimatik, penyelidikan yang berkaitan, seperti biosensor, bioreaktor, dan bahkan membawa aplikasi komersil sel biofuel yang implan ke realiti.

Katakunci: sel biofuel glukosa, graphene 3_dimensional, glukosa oksidase, bio-anod.

ACKNOWLEDGMENTS

My sincere gratitude goes to my supervisors, Dr. Samira Bagheri and Late Prof Dr. Sharifah Bee Abd Hamid for their constant guidance, valuable advice, and inspiration throughout the progress of this project. Without the direct support and persistent involvement from them in this work, the completion of this research and thesis would not have been an easy task.

I wish to express my special thanks for the financial support from the University of Malaya Grand Challenge Grant (Grant No. GC001A-14AET) and University of Malaya Postgraduate Research Grant (Grant No. PG101-2014B).

Last but not least, I wish to thank my beloved wife, Ms. Reyhaneh Farghadani and my parents, for their love and unconditional support. Also to my friends, I am very grateful for your continuous unflinching support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

2D	:	Two-dimensional
3DG	:	3-Dimensional Graphene
A	:	Ampere
Au	:	Gold
BFC	:	Biofuel Cell
cm	:	Centimeter
CNT	:	Carbon Nano Tube
CV	:	Cyclic Voltammetry
DET	:	Direct Electron Transfer
e^-	:	Electorn
EBFC	:	Enzymatic Biofuel Cell
EIS	:	Electrochemical Impedance Spectra
FAD	:	Flavin Adenine Dinucleotide
FESEM	:	Field Emission Scanning Electron Microscopy
FTIR	:	Fourier Transform Infrared Spectroscopy
g	:	Gram
GBFC	:	Glucose Biofuel Cell
GCE	:	Glassy Carbon Electrode
GDH	:	Glucose Dehydrogenase
Glu	:	Glucose
GOx	:	Glucose Oxidase
h	:	Hour
H_2SO_4	:	Sulfuric Acid
H_3PO_4	:	Phosphoric Acid
HCl	:	Hydrogen Chloride

HRTEM	:	High-Resolution Transmission Electron Microscopy
$KMnO_4$	:	Potassium Permanganate
L	:	Liter
LED	:	Light-Emitting Diode
m	:	Meter
MET	:	Mediated Electron Transfer
MFC	:	Microbial Fuel Cell
min	:	Minute
mL	:	Milliliter
Mm	:	Millimolar
mm	:	Millimeter
NAD	:	Nicotine Adenine Dinucleotide
OCV	:	Open Circuit Voltage
PBS	:	Phosphate-buffer saline
pH	:	Potential of Hydrogen
PQQ	:	Pyrroloquinoline Quinone
sec	:	Second
V	:	Volt

CHAPTER 1: INTRODUCTION

1.1 A Brief Overview of Biofuel Cells

Enzymatic biofuel cells represent an emerging technology that can create electrical energy from biologically renewable catalysts and fuels. Biofuel cells are favorable next generation of green energy sources (Devadas B., Mani V. *et al.*, 2012). Enzymatic biofuel cells (EBFCs) utilize enzymes to generate electricity during an electrochemical oxidation reaction occurring at the electrodes. They are unique compared to the conventional energy systems, cost saving, utilizing renewable substances to generate electricity, the selectivity of the enzyme towards the fuel, ability to work at physiological pH and temperature (Zebda A., Renaud L. *et al.*, 2010) and supplying the small-scale power (Lin J., Trabold T. A. *et al.*, 2013; Topcagic S. & Minteer S. D., 2006). All these benefits make them a suitable choice to power up implantable medical devices such as pacemakers and micro-drug pumps or even applied in wastewater treatment (Barton S. C., Gallaway J. *et al.*, 2004; Gao F., Yan Y. *et al.*, 2007; Sun J., Hu Y. *et al.*, 2009), drug delivery (Zhou M., Zhou N. *et al.*, 2012), biosensors (Zhou M., Kuralay F. *et al.*, 2012), remote sensing, communication devices in bioelectronics (Chen T., Barton S. C. *et al.*, 2001), self-powered sensors (Deng L. & Dong S., 2015a), and bio-electrocatalytic logic gate (Meredith M. T. & Minteer S. D., 2012). Due to the high selectivity of glucose via the glucose oxidase (GOx), this enzyme had been chosen among the various redox enzymes to be applied in biofuel cell, (Unnikrishnan B., Palanisamy S. *et al.*, 2013). Glucose biofuel cell (GBFC), utilizing glucose oxidase (GOx) (EC 1.1.3.4) for glucose oxidation and laccase for dioxygen reduction to generate sufficient power from a mammal's body fluids. The glucose-oxygen reaction catalyze by GOx is as following scheme:

$$\underbrace{C_6H_{12}O_6}_{Glucose} \xrightarrow{GOx} \underbrace{C_6H_{10}O_6}_{D-glucono-1,5-lactone} + 2e^- + 2H^+ \quad \text{Equation 1.1}$$

$$C_6H_{10}O_6 + H_2O \rightarrow \underbrace{C_6H_{12}O_7}_{Gluconic\ Acid} \quad \text{Equation 1.2}$$

$$\frac{1}{2}O_2 + 2e^- + 2H^+ \xrightarrow{laccase} H_2O \quad \text{Equation 1.3}$$

GOx oxidized glucose to produced-gluconolactone and electrons (Bahartan K., Amir L. *et al.*, 2012). A single implanted enzymatic GBFC can power a light-emitting diode (LED), or a digital thermometer (Zebda A., Cosnier S. *et al.*, 2013). According to the above equation, it could be concluded that no toxic byproducts generated by GBFCs and they are totally environmentally friendly. While biofuel cells can utilize the abundant glucose or other carbohydrate sources present in animal fluids, the most challenging parts in a biofuel cell fabrication remain unsolved; efficient immobilization of the enzymes on the electrode surface (Liu C., Alwarappan S. *et al.*, 2010). Unfortunately, there are some limitations in GOx immobilization on the electrode surface; the poor electron transfer between the active site of the enzyme and the electrode, enzyme leaching, and short lifetime of the enzyme (House J. L., Anderson E. M. *et al.*, 2007; Yang X., Hua L. *et al.*, 2003). Based on our knowledge, up to now, several methods have developed to increase the biological function, stability, and efficacy of GOx immobilization onto the electrode surface.

Entrapping or covalently immobilizing GOx in stable matrices, including nano and mesostructured metal oxides (Cao H., Zhu Y. *et al.*, 2008; Fang B., Zhang C. *et al.*, 2011), metal nanoparticles (Baby T. T., Aravind S. S. J. *et al.*, 2010; Bharathi S. & Nogami M., 2001), conducting polymers (Alwarappan S., Liu C. *et al.*, 2010; "Back matter," 1986; Ekiz F., Yuksel M. *et al.*, 2010), mesostructured silica (Blin J. L., Gérardin C. *et al.*, 2005), sol–gel matrix (Chen Q., Kenausis G. L. *et al.*, 1998; Jia W.-

Z., Wang K. *et al.*, 2007), carbon nanotubes (Lin Y., Lu F. *et al.*, 2004; Periasamy A. P., Chang Y.-J. *et al.*, 2011), graphene (Kang X., Wang J. *et al.*, 2009; Shan C., Yang H. *et al.*, 2009; Zhou K., Zhu Y. *et al.*, 2010), etc., leads to the enhanced electron transfer and improved enzyme stability (Joshi R. K., Gomez H. *et al.*, 2010).

Among the immobilization carriers used to date, nano-structured composite and hybrid materials, including nanoparticles, nanofibers, and carbon-based nanomaterials, are the focus of intense fundamental and applied research (Misson M., Zhang H. *et al.*, 2015). Graphene, carbon allotropes, is the key element which can both immobilize the enzyme on the electrode surface and provide an electrical communication between enzyme active site which buried deeply in the protein structure and the electrodes (Putzbach W. & Ronkainen N. J., 2013).

Graphene due to its extraordinary properties, such as high specific surface area (Xie T., Lv W. *et al.*, 2013), easily modified surface area (Sur U. K., 2012), very good mechanical and thermal stability, chemical inertness (Lee W., Lee J. U. *et al.*, 2013), and excellent electronic properties are increasingly used for applications in various fields such as catalysis, sensing, environmental remediation, and energy storage. The large surface area of graphene-based nanomaterials and electrical property creates an ideal immobilization support for various biomolecules including enzymes (Ge J., Yang C. *et al.*, 2012; Gokhale A. A., Lu J. *et al.*, 2013; Jin L., Yang K. *et al.*, 2012; Pavlidis I. V., Vorhaben T. *et al.*, 2012). Numerous studies of DET behavior of GOx on graphene decorated electrodes have been reported (Jiang B., Yang K. *et al.*, 2012; Kane R. S. & Stroock A. D., 2007; Pavlidis I., Vorhaben T. *et al.*, 2012; Shao Q., Wu P. *et al.*, 2012; Zhang Y., Zhang J. *et al.*, 2012). In the recent years, several strategies have been employed for the exploitation of graphene material based modified electrodes towards biofuel cell applications (Zheng W., Zhao H. Y. *et al.*, 2010). In this study, we aim to fabricate the GOx/3DG/GCE bioanode and investigate the GBFCs performance.

1.2 Problem Statement

While the abundant presence of glucose and other carbohydrate sources in animal fluids, and a variety of catalyzing enzymes promote the endless usage of enzymatic biofuel cells, the most challenging parts in their fabrication remain unsolved; efficient immobilization of the enzymes on the electrode surface. Unfortunately, there are some limitations in the immobilization of enzyme on the electrode surface, coupled with the poor electron transfer between the enzyme active site and the electrode, enzyme leaching, and short lifetime of the enzyme. Based on our knowledge, up to now, several methods developed to increase the biological function, stability, and efficacy of enzyme immobilization on the electrode surface.

Extraordinary physical and chemical properties of 3D graphene, such as highly porous structure, easily modified surface area, excellent electrical and mechanical properties; make it a good candidate to employ in the enzyme immobilization and electron transfer in the field of the enzymatic biofuel cell.

1.3 Research Scope

There are four main stages in this work: Synthesis, Fabrication, Characterizations, and Optimization of EBFC which is presented in Table 1.1. The synthesis and characterization of 3DG are investigated in this study. It will then be used to fabricate the EBFCs and enhance the performance of graphene base enzymatic biofuel cell.

Table 1.1: Overview of research scopes

STAGE 1: Synthesis of 3D Graphene	Self-assemble 3D Graphene Synthesis (Hydrothermal method) using the Synthesized Graphene Oxide via chemical exfoliation
	pH Oxidation duration & Acid concentration Reaction temperature Types of reducing agent
STAGE 2: Fabrication of Modified Glassy Carbon Electrode via 3D Graphene	Glassy Carbon Electrode Modified with 3D Graphene
	3D Graphene concentration Enzyme concentration Immobilization time
STAGE 3: Characterizations of Modified Glassy Carbon Electrode	Characterization Tools
	FESEM, HRTEM, (morphological) Raman, FTIR
STAGE 4: Enzymatic biofuel cell performance test	Analytical Tools
	Cyclic Voltammetry Open-circuit Voltage Stability Polarization Curve EIS

1.4 Objectives

The aim of this research is to immobilize the enzyme onto the electrode surface using a 3D graphene to enhance the Glucose BioFuel Cell (GBFC) efficiency to generate the power. The glucose chosen as a stock fuel is firstly due to its high concentration in human blood which might suggest the application of GBFC to implant in a human body. Secondly, glucose is a very dense energy source which has the ability to produce up to 16 kJg^{-1} during the complete oxidation of each molecule and releasing 12 electrons as well.

Hence, the specific objectives of this research are as follows:

i. To synthesize highly porous 3-dimensional graphene-based nanostructure via hydrothermal method

ii. To fabricate the enzymatic bioelectrode by immobilization of enzyme on the synthesized 3D Graphene-based nanostructure

iii. To investigate and optimize the power density of the fabricated biofuel cell

1.5 Organization of Thesis

This dissertation adopts a University Malaya style guide to the presentation, logically aimed and systematically rendered to enhance understanding of the research. The thesis is divided into five chapters as follows:

Chapter one highlights the background of the study, the problems existing in this area which builds motivation for this project, and the objectives of this research.

Chapter two presents the literature review, which covers the history and type of the fuel cells and their structure. This chapter shows that there have been many experimental studies regarding the fabrication of EBFCs. However, no work has been published on comparing the effect of 3DG and 3DG/CNT hybrid in GBFCs as a bioanode.

Chapter three explains the methodology for conducting this research project. Preparation of materials and using them for fabrication of GBFC described schematically and the characterization equipment explained in details by adding some photographs and figures.

Chapter four presents the experimental results which are analyzed by different characterization techniques.

Chapter five demonstrates the comprehensive conclusions along with recommendations for further work.

CHAPTER 2: LITERATURE REVIEW

2.1 Fuel Cell

Fuel cells are the devices capable of extracting the chemical energy from the variety of material which called fuel and converts it into useful electrical energy (Li X., Zhang L. *et al.*, 2009). Fuel cells are similar to batteries, but the major difference is in a nature of fuels which supply the electricity. Batteries contain a certain amount of fuel or chemical energy which consume during the electricity generation process and finally ends whereas the fuel cell is not limited to the amount of reserved fuel and can generate power by continuously supply of fuel (Mano N., Mao F. *et al.*, 2003). All the fuel cells contain two electrodes (anode and cathode) placed in the electrolyte that could be separated by the semipermeable membrane which facilitates the free flow of ions among the electrodes (A. K. Shukla, P. Suresh *et al.*, 2004). The common aspect of all fuel cells is the reduction of Oxygen (or so-called air) in the cathode (Figure 2.1).

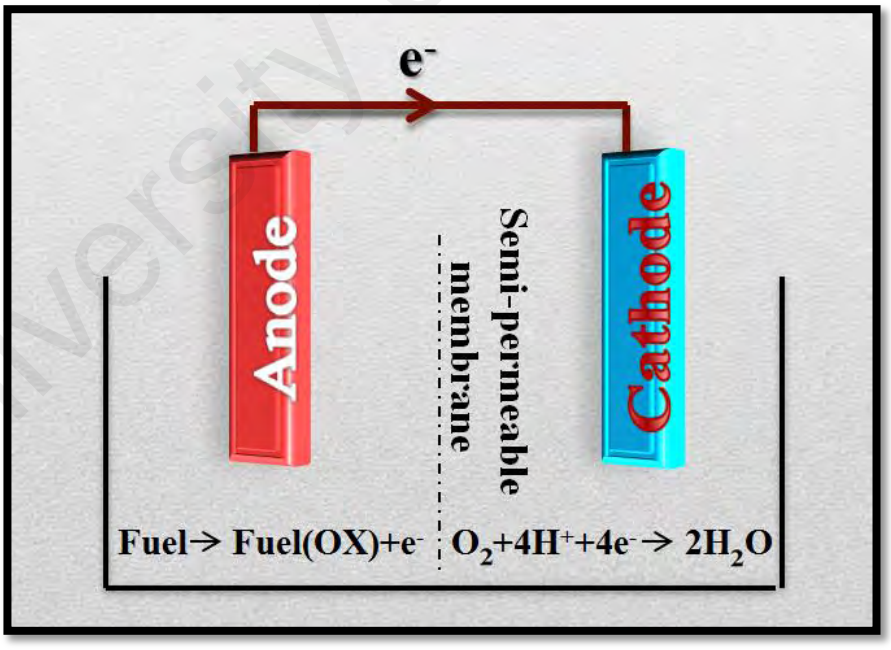

Figure 2.1: General fuel cell schematics

For many years, fuel cells regarded as only of academic interest due to their three main disadvantages; short lifetime, high cost, and the most challenging part electrode

development (Zhao C.-e., Gai P. *et al.*, 2017). Fuel cells have the capability to replace the domestic and military power generation exactly in the place which is required. Currently, two different kinds of fuel cells are under development; organic biofuel and inorganic biofuel (Damiano L., Jambeck J. R. *et al.*, 2014; Suberu M. Y., Mustafa M. W. *et al.*, 2014). The current inorganic fuel cells developed based on the hydrogen-oxygen fuel cell while organic biofuel cells vary based on the catalysts choice (Chandan A., Hattenberger M. *et al.*, 2013).

2.2 Biofuel Cells

A Biofuel cell is the offspring of fuel cell technology and biotechnology (Palmore G. T. R. & Whitesides G. M., 1994). Biofuel cells (BFCs) are the sort of fuel cells employs biocatalysts for their power generation process (Osman M., Shah A. *et al.*, 2011). Furthermore, those fuel cells which drive the energy from biological carbon fixation sources at ambient temperature and pressure also considered as biofuel cells. BFCs are the potential green energy source and an alternative solution for the future energy demand (Wang Z. L. & Wu W., 2012). BFCs can utilize microbes, enzymes and other biological organelles to generate the power from biological fuels originated from plants or animals. Due to the ability of biofuel cells to utilize waste products and other variety of materials, it has been purposed that BFCs can be developed to operate in different variety of situation, from implantable device in human body up to applications in spacecraft (Prasad K. P., Chen Y. *et al.*, 2014; Zhou M. & Dong S., 2011). Similar to fuel cells, BFCs are complex of two electrodes which called bioanode and biocathode. In addition, some biofuel cells might have a semipermeable membrane that separates the bioelectrodes. However enzymatic biofuel cells and enzyme-based biosensors might look similar regarding using the bio-anode there is a recognizable difference between them. The critical aspect of enzyme-based biosensors is to detect and report the

presence of specific material even in small amount while enzyme-based biofuel cells use the substrate to generate the power and need a sufficient amount of it.

Every biofuel cell generates energy by an electrochemical reaction of fuel and oxidant in two separated electrodes (Barton S Calabrese, Gallaway Josh *et al.*, 2004). The released electrons travel through the external circuit from the anode toward the cathode to reduce the terminal electron acceptor which in most of the cases is oxygen. The electrons use the difference in electrochemical potential between cathode and anode to flow through the external circuit (Harnisch F. & Schröder U., 2009; Logan B. E. & Regan J. M., 2006). The biofuel cells power extraction method from the biofuel varies according to the complexity of biofuel. Biofuel cells can be broken down into 3 subclasses; microbial fuel cells (fuel cells employ microorganisms to generate power), mitochondrial fuel cells (fuel cells employ mitochondrial to generate power), and enzymatic biofuel cells (fuel cells employ enzymes to generate power) (Najafpour G., 2015).

2.2.1 Microbial Fuel Cells

In the microbial fuel cell (MFC) the whole organism employs to catalyze the reaction and generate the power(Mohan S. V., Velvizhi G. *et al.*, 2014). The microorganisms contain multiple enzymes which act as a bioreactor. However, the MFCs distinguished from its elder brother, based on the fabricated product. The whole system of MFCs led to direct electricity production while the bioreactors products should undergo more process to generate the power. Since the first successful effort to fabricate the functional MFC in 1991 (Habermann W. & Pommer E., 1991), lots of interests attracted toward this technology. With the beginning of space age in 1960s, the research in this field boosted due to microbe application in fabricating the bioanode. MFCs have the advantage of complete oxidation of biofuel and also long lifetime 3-5 years. However,

they are limited by their low power density due to the slow transport of biofuel from the extracellular matrix through the cellular membrane (Rabaey K. & Verstraete W., 2005).

MFCs basic consist of four main parts; an anode, a cathode, buffer solution and a permeable membrane (Logan B. E. & Elimelech M., 2012). The anode contains microbes (biocatalyst), while the buffer solution provides substrate (such as sugar), maintain the pH and facilitate the ion transfer between the electrodes (proton exchange membrane). The microbes break down substrate to hydrogen ions (H^+) and electrons (e^-) in the anode. In nature, the hydrogen ion and electron combine with the oxygen and produce the H_2O. This procedure which occurs in the aerobic respiration system has no power generation. So, in order to generate a power, the anode chamber should have anodic condition while the cathode electrode surrounded by the oxygen. This condition will force the cell to balance the electric charge between the electrodes by allowing the H^+ ions to travel through a permeable membrane to the cathode while the electrons can only travel to the cathode through the external circuit (Du Z., Li H. *et al.*, 2007; Zeng K. & Zhang D., 2010). The electrons current through the external circuit generate the power and at the cathode, destination will combine with hydrogen ions and oxygen molecules and produce water. Figure 2.2 picturing a diagram of this procedure.

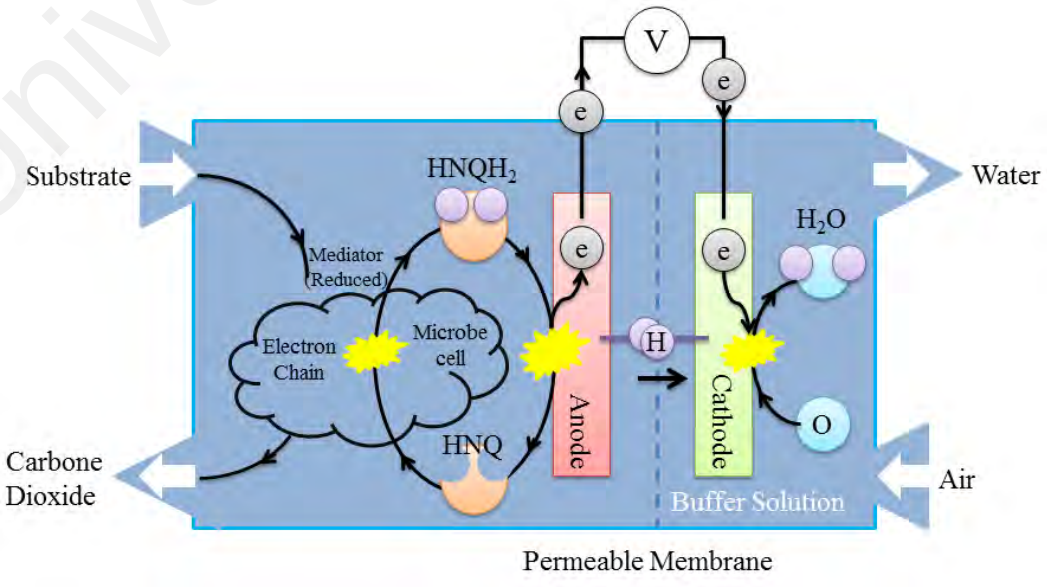

Figure 2.2: Diagram of MFC.

The mediators improve the performance of MFCs intensely by transferring the electron between the internal reducing center and the external electrode.

2.2.2 Mitochondrial Biofuel Cells

Mitochondria are the cell powerhouse containing a complex of enzymes that completely oxidize the biofuel through the Kreb's cycle and run the electron transport chain of metabolism. Mitochondria are the living organelle in charge of power conversion in the cytoplasm of living cells of animals, plants, and fungi (Arechederra R. & Minteer S. D., 2008; Bhatnagar D., Xu S. *et al.*, 2011). These power generation factories perform the complete and fast oxidization of biofuel which provides a high energy density and no toxic byproducts.

Mitochondria biofuel cells are the new class of biofuel cell that employs the whole organelle (Deng L. & Dong S., 2015b). While the complete oxidation of biofuel is the highly desirable attribute of mitochondrial biofuel cell, the lack of reproduction ability, complexity, and necessity of working in highly stable condition, are their weak points (Hubenova Y. & Mitov M., 2015). Figure 2.3 represents the schematic view of mitochondrial base bioanode.

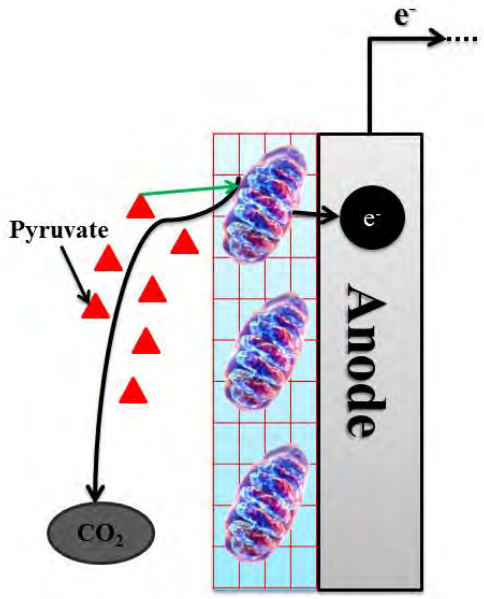

Figure 2.3: Schematic of a mitochondrial bioanode

2.2.3 Enzymatic Biofuel Cells

Enzymes are catalytic proteins which accelerate the reaction without increasing the temperature or pressure. The enzymes contain an active site which adapts conformationally to the substrate as a key-lock principle (Figure 2.4). Another essential component of oxidoreductases enzymes is co-factors. They have a non-protein structure and might have a tight or loose attachment to the enzyme (Bard A. J., Stratmann M. *et al.*, 2003).

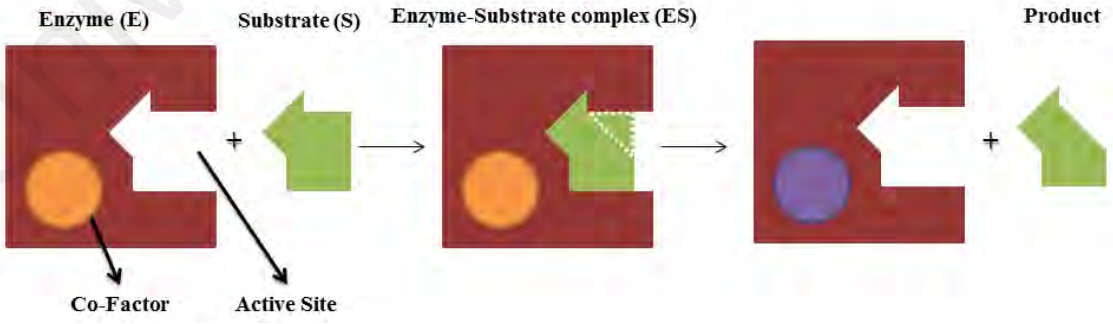

Figure 2.4: Schematic of the enzyme lock and key principle.

As the figure 2.4 shows, the enzymes have the high specificity toward the substrate. It means that among all the molecules and material in the fluid or solution, they will

effectively form an enzyme-substrate complex with their relative substrate and catalyze the reaction to generate the product.

All the currently known enzymes divided into six groups base on the catalyzed reaction (Hult K. & Berglund P., 2007). Among those six groups, the oxidoreductases are the ones which used in the bioelectrochemical field. They transfer the H^+ ions and electrons that result in the reduction or oxidation reactions (Cannon R. D., 2016). By using a bioelectrochemical system which can harvest the produced electrons during the oxidoreductase reaction, electricity could be generated from the enzyme activity. This is the principle of the enzymatic biofuel cells (EBFCs) operation. Like another kind of biofuel cells, the EBFCs also consists of anode, cathode and electrolyte buffer. The EBFCs require no membrane compare to another type of fuel cells (Zhu B., Raza R. *et al.*, 2011). However, a large electrode surface area is required to generate the acceptable range of power. The general scheme of EBFC is demonstrated in figure 2.5.

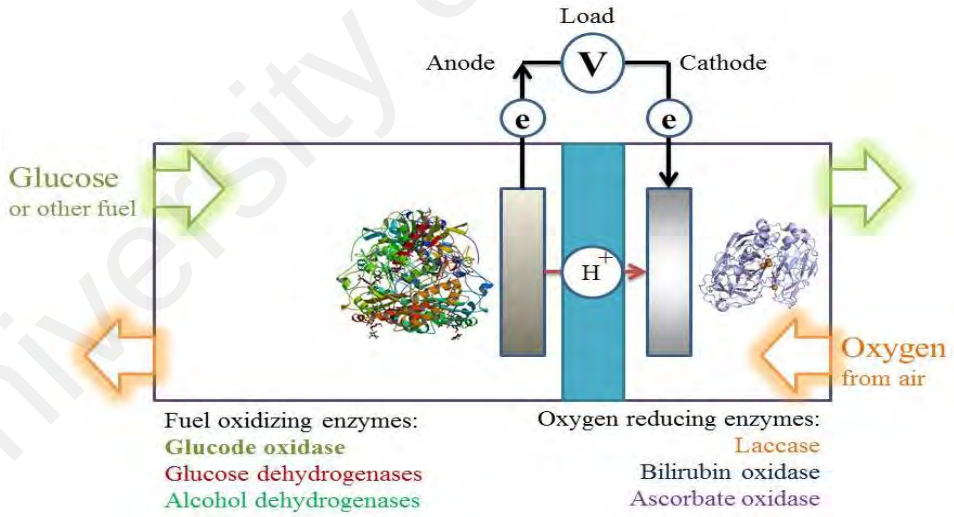

Figure 2.5: General scheme of the enzymatic biofuel cell

The GOx enzyme, oxidases the fuel (glucose as demonstrated in figure 2.5) in the anode. The catalyzed reaction release electrons and protons (hydrogen ions) that travel through external circuit and electrolyte to the cathode respectively. In the cathode, the

oxidant (oxygen according to the figure 2.5) will reduce to the H_2O (water) (Ó Conghaile P., Falk M. *et al.*, 2016). The whole explained cell reaction is as below:

$$C_6H_{12}O_6(GLU) + {}^{1}/_{2}\,O_2 \xrightarrow{GOx} C_6H_{10}O_6(gluconolactone) + H_2O \quad \textbf{Equation 2.1}$$

The EBFCs employ the isolated form of the enzymes depends on the substrate of interests. Though there is a variety of 3000 enzymes to choose for fabricating the electrodes, the key element of developing EBFC is a selection of suitable electrode modification method. The modified electrodes have to preserve the ideal activity of the enzyme and also the electron transfer between the enzyme active site and the electrode surface. Due to the fact that enzyme active site is buried deeply in the prodeeptructure of the enzyme, a wiring system between the enzyme active site and the surface of the electrode seems unavoidable (Karajić A., 2015).

Despite the variety of oxidoreductase enzymes to choose for the biofuel cell, most of the researchers employing glucose oxidase (GOx) and glucose dehydrogenase (GDH) in their study to fabricate the bioanode (Shan C. *et al.*, 2009).

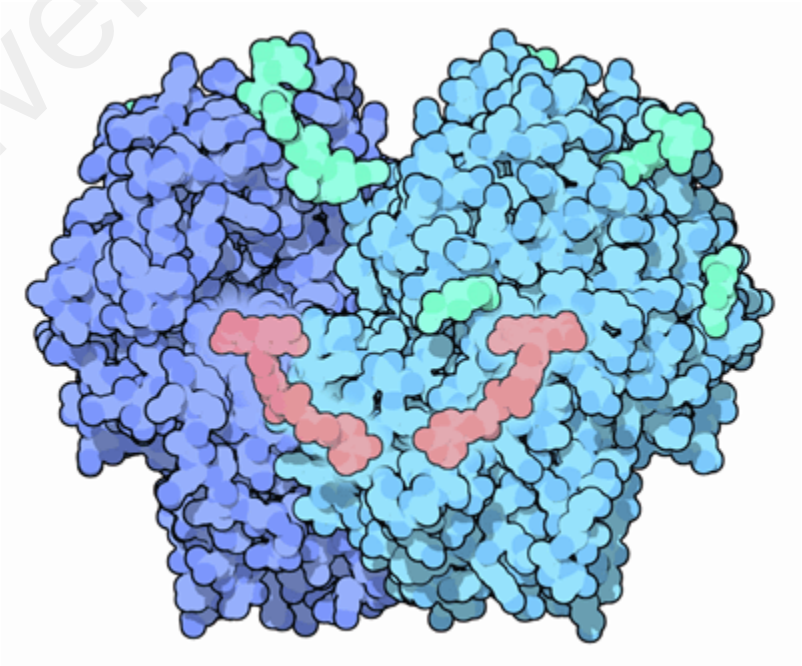

Figure 2.6: Schematic structure of GOx.

2.2.3.1 Bioanode

Enzymatic bioanodes vary based on their design, but most of them have to follow the basic principles such as; large negative OCP, high fuel efficiency and stability to improve the overall performance of enzymatic biofuel cells. In order to achieve these requirements, the natural properties of the enzyme, electron transfer mechanism, and the substrate selectivity of enzyme should be considered in the bioanode designing procedure.

Therefore, there might be a variety of candidate which could be used as a biocatalyst. Among those all, glucose oxidase and glucose dehydrogenase attract the most attention in the design process of glucose biofuel cell. The principal properties of these two enzymes explained in continue.

(a) ***Glucose oxidase***

Glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4) is a nucleic acid containing protein (flavoprotein) that transfer an electron to oxygen and catalyze D-glucono-delta-lactone and hydrogen peroxide biosynthesize by oxidizing the first hydroxyl group of β-D-glucose. The most common source of glucose oxidase is the fungus *Aspergillus niger*, which isolated enzyme utilized for industrial applications such as glucose monitoring. The niger-derived Glucose oxidase (GOx) is highly substrate specific for glucose while other GOxs are capable of oxidizing other sugars, including maltose. Enormous studies have reported higher glucose specificity of GOx and its application to measuring the amount of glucose in the mixture with other sugars (Keilin & Hartree, 1948). GOx is a pH-sensitive enzyme (Kleppe, 1966). Debility of native GOx to handover electrons to the electrode, make this enzyme a valuable candidate to be applied in third-generation glucose sensor structure by connecting the redox center of enzymes to the electrode. In order to approach this connection, several molecular wires,

such as osmium complex-linked polymers (Vijayakumar et al., 1996), artificial cofactor derivatives (Willner & Willner, 2001), thienoviologens (Albers et al., 1997), CNTs (Tsai et al., 2005), or nanofibers (Vamvakaki et al., 2006) employed. Figure 2.7 reveals the cyclic voltammograms for co-immobilization of PPD and GOx at the GC/CNT/PB electrode.

As it has demonstrated in figure 2.6, GOx is composed of two subunits with the molecular weight of 80,000. Each subunit contains Flavin dinucleotide cofactors and also a glycoprotein backbone. Table 2.1; represent the brief properties of GOx.

Table 2.1: Properties of GOx.

source	*Aspergillus niger*	
molecular weight	155,000	
pH optimum	6	
cofactor	FAD	
co-substrate	Oxygen	
Km (Glucose)	30mM	
substrate	b-D Glucose 2-Deoxi-D- Glucose 6-methyl-D-Glucose D-Mannose a-D- Glucose	Relative Rate: 100 Relative Rate: 25 Relative Rate: 2 Relative Rate: 1 Relative Rate: 0.6

One of the alternative enzymes which are capable of replacing the Glucose oxidase is Glucose dehydrogenases that will be explained in details.

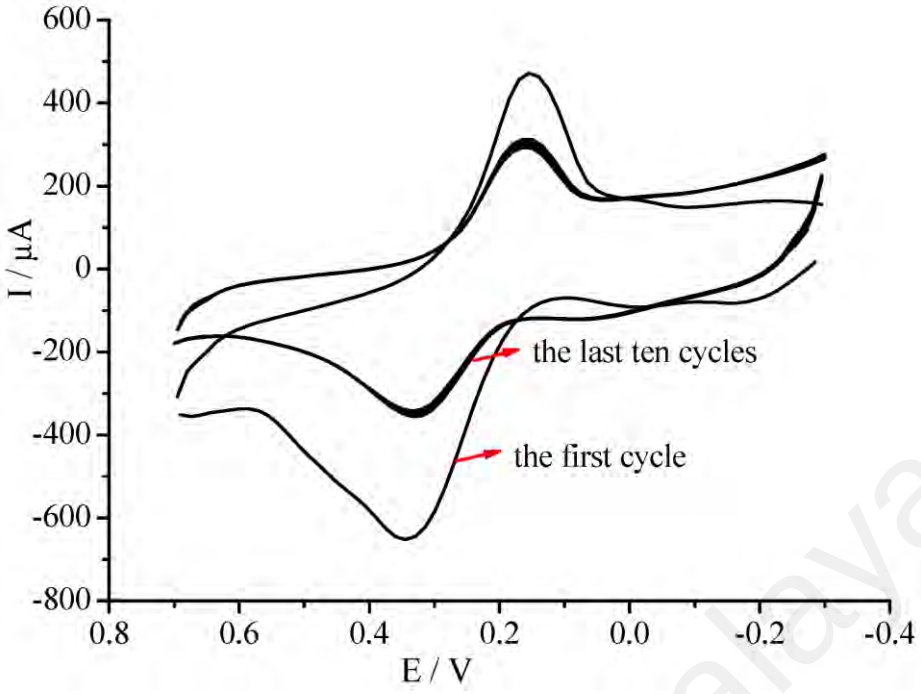

Figure 2.7: Cyclic voltammograms of GC/CNT/PB modified the electrode in phosphate buffer (pH 7.4) containing 5 mM phenylenediamine and 10 mg/mL GOx at a scan rate of 50 mV/s (Yao et al., 2012).

(b) ***Glucose Dehydrogenase***

Glucose dehydrogenases belong to oxidoreductases family that transfer electrons to numerous natural and synthetic electron acceptors instead of oxygen and classified according to employed redox cofactors. This non-protein component and primary electron acceptor cofactors are Nicotine adenine dinucleotide (NAD), Pyrroloquinoline quinone (PQQ) and Flavin adenine dinucleotide (FAD).

Pyrroloquinoline Quinone Glucose dehydrogenase

Pyrroloquinoline quinone (PQQ) is the third common redox cofactor found in enzymes, which the only biosynthesis in the ribosome of prokaryotes during an unknown pathway. Quinoprotein GDH (EC 1.1.5.2) subdivided into at least two separate groups of GDHs: PQQ GDH-A which binds to the membrane and PQQ GDH-B which dissolved in water, both utilize PQQ as the redox cofactor. In bacteria, the

PQQ GDH-A is the terminal oxidation of glucose in the respiratory chain. The PQQ GDH-B utilized in rapid glucose sensing due to its higher catalytic efficiency compare to GOx activity (more than 25 times) while reporting fatal errors compare to GOx.

Nicotine Adenine Dinucleotide (Phosphate)-Dependent Glucose Dehydrogenase

Dehydrogenase Glucose-1-dehydrogenase [NAD(P) GDH, EC 1.1.1.47] found in the massive amount of prokaryotes and eukaryotes. The β-D-glucoseNAD(P)$^{+1}$-oxidoreductase enzyme transfers the electron of the hydroxyl group of glucose to NAD+ or NADP+ (first electron acceptor) and oxidizes the glucose. While most NAD(P) GDHs use specifically glucose as a substrate, some of them are unable to recognize the second hydroxyl group and react with mannose, 2-amino-2-deoxy- D-glucose, and glucosamine instead of glucose. The extracted NAD (P) GDH from *Bacillus megaterium* utilized to monitor the blood sugar. Figure 2.8 shows the obtained cyclic voltammograms.

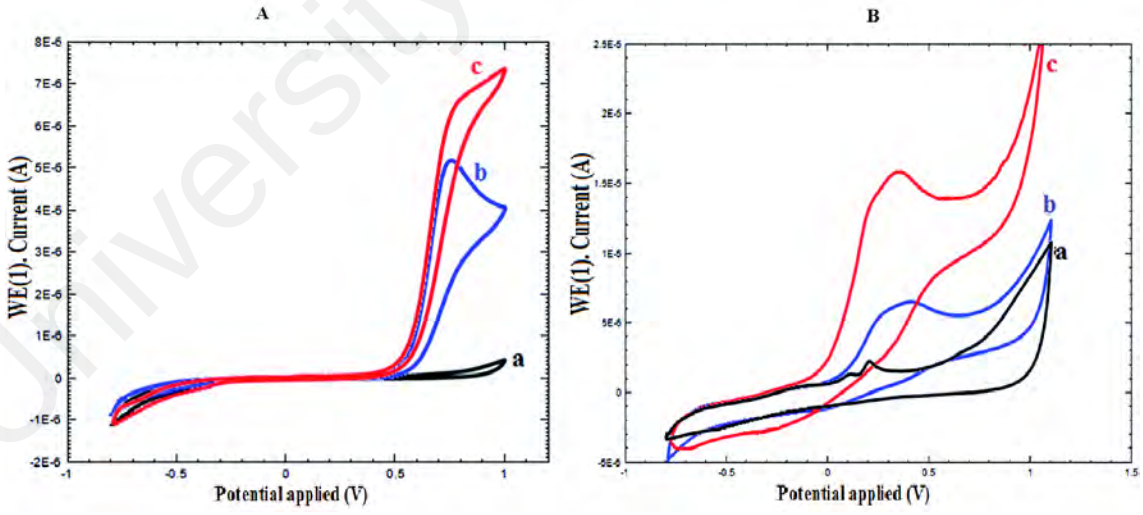

Figure 2.8: Cyclic voltammograms of (A) GDH/PAMAM/GCE and(B) GDH/poly-HT/PAMAM/GCE in the presence of 10 mM NAD+ and (a) in the absence, (b) in the presence of 10 mM glucose without irradiation and (c) with irradiation. Supporting electrolyte: 0.1 M PBS (pH 7.0) (Dilgin D. G. & Gökçel H. İ., 2015)

Flavin Adenine Dinucleotide Dependent Glucose Dehydrogenase

The FAD-dependent GDHs (FAD GDH, EC 1.1.99.10) are well-known due to their use in sensor applications. The cyclic voltammograms of the ferrocene polymeric mediator and FAD-GDH in phosphate buffered saline (PBS) reported in Figure 2.9. The electron transfer from $FADH_2$ to the ferrocene moieties and reduce it to ferrocenium which re-oxidize again by transferring the electrons to the electrode.

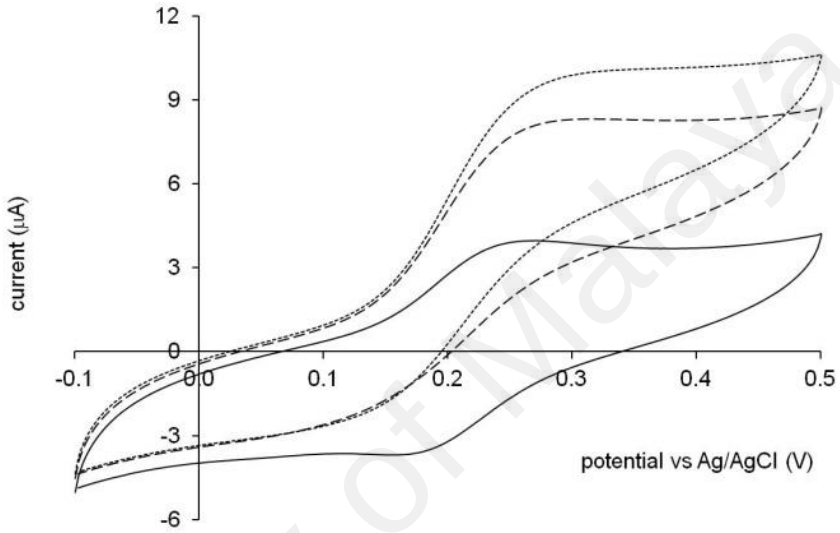

Figure 2.9: Cyclic voltammograms: 1.5mg/mL FAD-GDH, 9 mg/mL ferrocene polymeric mediator in 0.01M pH 7.4 PBS, scan rate 5 mV/s, without (solid line), with 2.5 mM glucose (broken line) & 5.0mM glucose (dotted line) (Babadi A. A., Bagheri S. *et al.*, 2016).

2.2.3.2 Biocathode

The cathode is the reduction site of the biofuel cells. The common chemical reaction occurs in the cathode presented in the equation;

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \qquad \text{Equation 2.2}$$

The electrons that went through the circuit to generate current react with the hydrogen ions which migrated to the cathodic side of the cell and the oxygen to produce water molecules.

In order to prepare biocathode, two different positively charged enzymes could be immobilized on the surface of the electrode; Bilirubin oxidase and Laccase. In addition, a catalytic metal such as platinum could be a suitable choice for the cathode site.

(a) ***Bilirubin Oxidase***

One of the important chemical reactions in energy converting system is oxygen reduction. One of the enzymes which are capable of reducing oxygen to water is Bilirubin Oxidase (BOD). Bilirubin Oxidase (EC 1.3.3.5) is an oxidoreductase enzyme that discovered in 1981 by Tanaka and Murao. This enzyme specifically catalyzes the oxidation reaction of bilirubin to biliverdin and primarily used in the jaundice diagnostic. It is the best enzyme to reduce oxygen due to its lowest required over potential energy to catalyze the reaction (Shan C. *et al.*, 2009). Figure 2.10 represents the cyclic voltammograms of BOD.

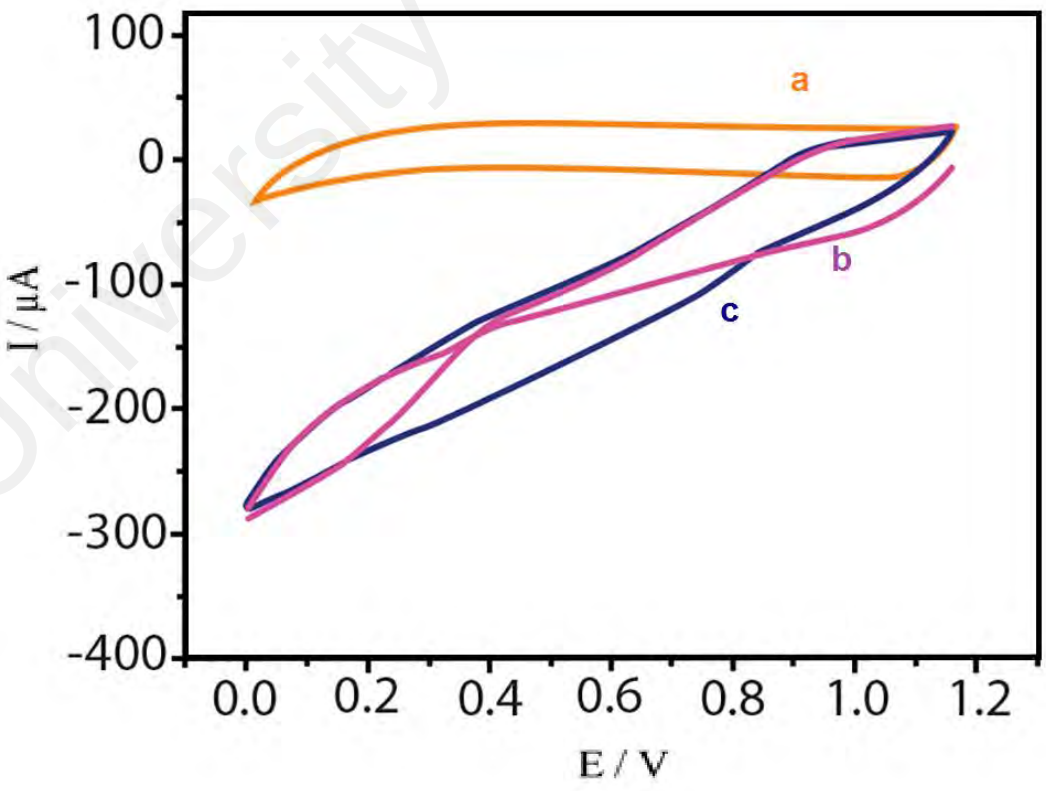

Figure 2.10: CVs of immobilized BOD on SPGE; (a) in absence of O_2, (b) first scan, (c) second scan. Scan rate 5 mV/s (Tasca F., Farias D. *et al.*, 2015).

(b) ***Laccase***

Laccase (EC 1.10.3.2) is a polyphenol oxidase enzyme found in a variety of organisms. Belonging to the multicopper enzymes family same as BOD, laccase also is a copper-containing enzyme. Laccase was firstly defined by Yoshida (1883). Laccases (LAC) play an important role in the degradation of lignin in nature and use oxygen as the second substrate for its enzymatic reaction. LAC is capable of oxidizing quinol using molecular oxygen (Fernandes A. J. T., 2011).

2.3 Electron Transfer Mechanisms

The key point during the biofuel cell operation, regardless of its organic part which could be enzymes or microbes, is an efficient transfer of an electron from the catalyzing site to the surface of the electrode. Research that has been done up to now, suggests two possible mechanisms in the case of electron transfer; Mediated Electron Transfer (MET) and Direct Electron Transfer (DET). Each of these mechanisms has their benefits over the other one. MET employs the redox active materials as electron relays which harvest the electron from catalyze site and carry to the electrode. The DET developed with the view to improve the stability of the immobilized enzyme. Carbon nanotube (CNT) and its composites are the suitable candidates to achieve this aim and in addition directly wiring the enzyme (Chaubey A. & Malhotra B. D., 2002). Electron Transfer Mechanisms is an important point in the EBFCs field, due to the fact that the generated power is dependent both on enzyme activation rate and also enzyme regeneration rate (in the case of DET) or enzyme regeneration rate and mediator regeneration rate (in the case of MET).

2.3.1 Mediated Electron Transfer

The majority of the enzymes used in bioanode or biocathode have an inefficient transfer of an electron from the enzyme active site to the surface of the electrode (Figure 2.11). Therefore, some elements or polymers should be employed which contribute directly to enzymes or indirectly with cofactors (Moehlenbrock M. J. & Minteer S. D., 2008). The preferable mediators are the ones that can work with NAD^+ and FAD-dependent enzymes. The mediator can be solved in the buffer solution, polymerized or immobilized on the surface of the electrode. MET provides the faster electron transfer compare to the DET. The common mediators that used these days in the EBFCs are; conducting salts, altered vitamins, and organic dyes. The generated current is the product of oxidation of reduced mediator on the surface of the electrode (Holzinger M., Le Goff A. *et al.*, 2012).

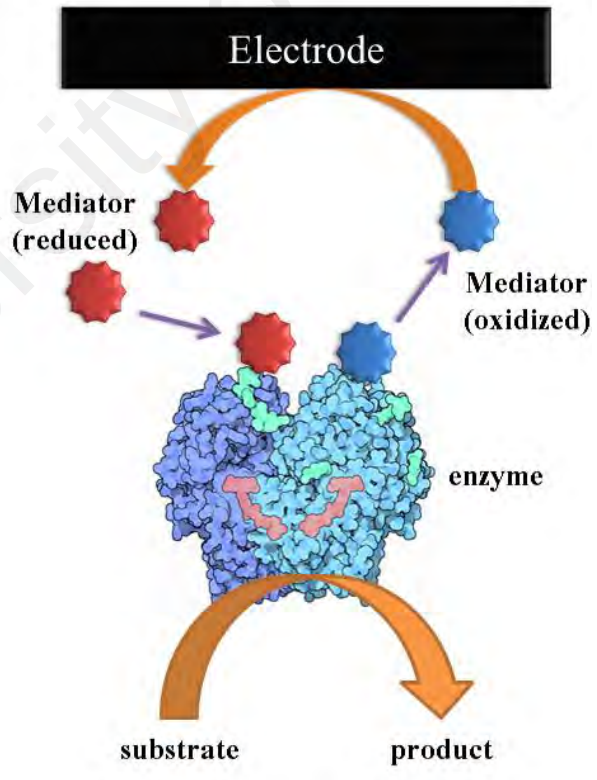

Figure 2.11: MET mechanism utilized in EBFCs.

2.3.2 Direct Electron Transfer

During the DET mechanism, electrons directly travel from enzyme active site to the surface of the electrode without using any mediator. The current generated directly from the re-oxidation of enzyme reduced form on the surface of the electrode. The novel material that brings DET to the reality is CNTs and carbon-based nanomaterials. CNT and graphene outer layer is hydrophobic which strongly attached with the enzyme and provide a wiring matrix which harvests the electron from the enzyme active site (Bullen R. A., Arnot T. *et al.*, 2006). In addition, DET has been observed in the PQQ dependent enzyme due to the availability of the haem group in their construction. In comparison with DET, the obtained power from MET is higher while the reported lifetime using the DET is longer. The less power generation of DET mechanism could be overcome by controlling the orientation of immobilized enzyme in the manner that the enzyme redox site approximately facing the electrode surface. In the figure 2.12, DET mechanism in the biofuel cell is pictured which is much simpler than the MET mechanism (Degani Y. & Heller A., 1987; Mu S. & Xue H., 1996).

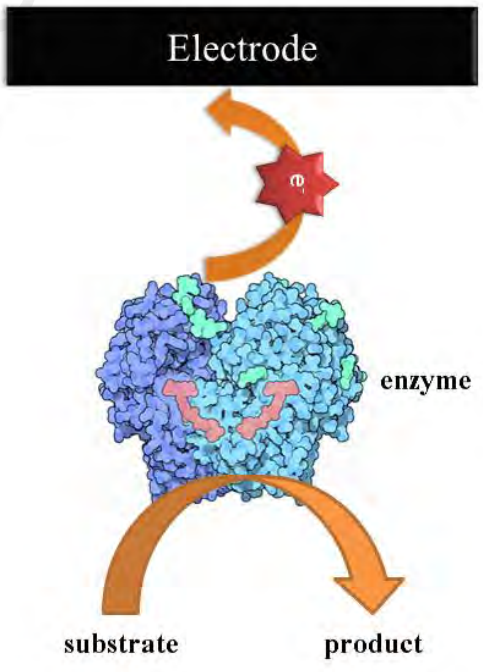

Figure 2.12: Direct Electron Transfer utilize in EBFCs.

Employing MET techniques will increase the electron transfer rate and subsequently the currents. However, it would decrease the biofuel cell generated a voltage. It is because of the influence of mediator potential on the potential of enzymatic. It means that with the purpose of electron transfer occur; in the case of oxidation, mediator potential has to be more positive than the enzyme potential and in the case of reduction, it has to be more negative. Considering all these challenges, the utilization of DET approach is more favorable for EBFCs applications (Ramanavicius A. & Ramanaviciene A., 2009).

2.4 Immobilization of Enzymes

One of the requirements in the EBFCs designing process is the enzyme immobilization. During this process, the enzyme would be attached or localized on the surface of the electrode which both increase the enzyme electron transfer rate and also enzyme lifetime while on the other hand, successful enzyme immobilization decrease the enzyme leakage. According to the literature, there are four main immobilization strategies employ in the biosensors and EBFCs; simple physical adsorption, covalent attachment, cross-linking and entrapment (Lim J., Cirigliano N. *et al.*, 2007). It also should be considered that in some cases, enzyme immobilization is a combination of all mentioned techniques. In continue, all these methods are introduced and discussed.

2.4.1 Physical Adsorption

This is the simplest method of immobilization which developed base on the hydrogen bonds, salt linkages, and Van der Waal's forces (figure 2.13). It is based on the physical interaction of the enzyme and electrode surface. In addition, due to not employing any reagent comparing to chemical methods, it is less disruptive to the

enzyme protein structure. Furthermore, physical adsorption is cheap, easy to carry out and minimum steps of activation required (Kragl U., Greiner L. *et al.*, 1999).

The well-known hydrophobic reaction is categorized in this method. CNTs which have been mentioned in the direct electron transfer section absorb and intertwine to the enzyme as a result of its hydrophobic nature (Sheldon R. A., 2007).

Unfortunately, due to the weak nature of involved bonds, physical adsorption process is unstable. Changes in temperature, pH, and ionic strength can desorb the enzyme from the electrode surface (Guiseppi-Elie A., Lei C. *et al.*, 2002).

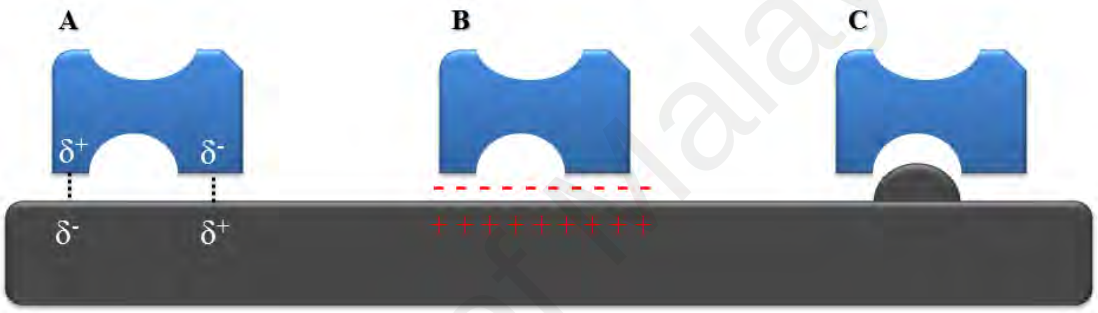

Figure 2.13: Physical adsorption mechanisms; A) Van der Waals forces, B) ionic bonding, C) affinity binding.

2.4.2 Electrostatic Attraction

Electrostatic attraction basic is similar to the physical adsorption but it is an active process. This method, use the benefits of ionic nature of the enzymes. Proteins ionic charge can be controlled by pH changes. In an ambient pH, proteins could be cation, anion or zwitterion. It makes enzymes capable of attaching to the electrode with the opposite charge using the ionic binding. In addition, applying a charge to the electrode would draw enzymes out of the solution and immobilize them on the electrode surface that significantly improves the enzyme loading (Du Toit H. & Di Lorenzo M., 2014).

However, changing the electrode charge could make the enzyme detachment even after the enzyme immobilization. Thus, to have the functional EBFC it is vital to use the negatively charged enzyme for the bioanode and the positively charged one for the

biocathode. The limitation of this method reveals when paying attention to the fact that the pH of the biofuel cell should be fixed. It would limit the choice of the right complimentary charged enzyme in that specific pH.

2.4.3 Covalent Coupling

This is an irreversible method which explains the wide usage of this method in the field of enzyme immobilization. It is also known as a surface functionalization method as a result of using terminal groups to form a covalent bond. The peptide bond (covalent bond) with either the amine or carboxylic acid terminal groups of the enzyme prevents the enzyme detachment from the electrode surface. However, due to the complicated folded structure of the enzyme, these terminal groups are not accessible most of the time. Therefore, the side chains of lysine (amino group), aspartic and glutamic acids (carboxylic group) used to form a peptide bond while the thiol group of cysteine reserved to form a sulfide bond. Nevertheless, it is important to use those amino acids in a covalent coupling method which do not involve in the active site of the enzyme. In addition, modifying the 3D-structure of the enzyme due to covalent bonds might deactivate the enzyme (Mattiasson B. & Kaul R., 1991).

Meanwhile that the enzyme contains the functional group, the electrode surface also must be prepared using the complementary reactive group. In order to achieve a successful reaction between the enzyme and the functionalized electrode, these groups must be destabilized to become active.

2.4.4 Entrapment

Entrapment method developed based on the obstruction of the enzyme in the polymer compound in the manner that substrates and products can freely pass through but keep the enzyme. The three main tactics of entrapment are; gel encapsulation,

sandwich entrapment, and microencapsulation. As it can be understood from the figure 2.14 entrapment methods were restricted by mass transport limitation through the peripheral membrane (O'driscoll K., 1976).

Though the gel encapsulation prevents the enzyme leakage, the employed electrode is one-time use and it is not suitable for fabrication of reusable electrodes. On the other hand, sandwich entrapment overcomes the limitations of gel encapsulation but still, has to struggle with the mass transport limitations due to its different coating layers. Micro-encapsulation is a combination of gel encapsulation and sandwich entrapment. In this method, the enzyme physically entrapped in the matrix of the membrane on the surface of the electrode. Recent studies report the successful assembly of simple CNT and chitosan matrices (Liu Y., Wang M. *et al.*, 2005), CNT, poly-cation polyethyleneimine and Nafion matrices (Ivnitski D., Branch B. *et al.*, 2006), and dithiol-gold nanoparticle-cystamine matrices constructed by sequential self-assembly (Zhang S., Wang N. *et al.*, 2005).

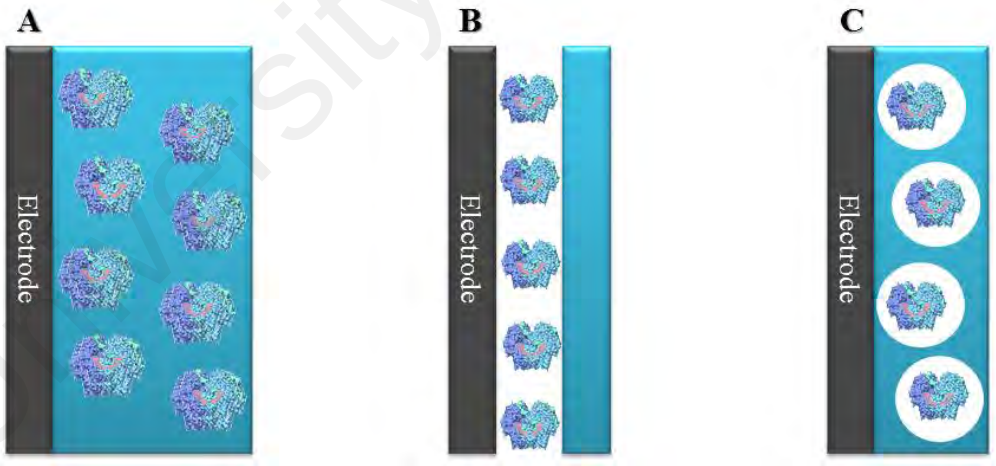

Figure 2.14: Entrapment methods; A) gel encapsulation, B) Sandwich entrapment, C) Micro-encapsulation.

2.4.5 Comparative Analysis of Immobilization Methods

In Table 2.2, the discussed methods to immobilize the enzyme on the surface of the electrode have been compared base on the stability, complexity, preparation time and

the electron transfer mechanism (Bhatia R. B., Brinker C. J. *et al.*, 2000; Katz E. & MacVittie K., 2013; Sahney R., Anand S. *et al.*, 2006; Tsai T.-W., Heckert G. *et al.*, 2009). Looking for the long-lasting irreversible method, covalent coupling would be the best one which does not interfere with enzyme activity whereas the enzyme choices are limited to this method. In terms of time-saving and money-saving, physical adsorption would be the best choice with minimum loss of enzyme activity. The only disadvantage might be enzyme leakage and poor electron transfer, which could be solved using the carbon based nanomaterials, which trapped the enzyme strongly and directly wiring the enzyme redox site to the surface of the electrode.

Table 2.2: Qualitative comparison of different enzyme immobilization techniques

Immobilization Method		Stability	Complexity	Preparation Time	Electron Transfer Mechanism
Physical Adsorption	Van Der Waals Adsorption	Very Low	Low	1 Day	Det/Met
	Ionic Bonding	Low	Low	1 Day	Det/Met
	Affinity Binding	pH Sensitive	Variable	1 Day	Det/Met
Electrostatic Attraction		pH Sensitive	Low	1 Hour	Det/Met
Covalent Coupling		Irreversible	High	1-7 Days	Det/Met
Entrapment	Gel Encapsulation	Irreversible	High	3 Days	Met
	Sandwich Entrapment	Irreversible	Variable	1 Hour	Det/Met
	Micro-Encapsulation	Irreversible	High	1 Day	Det/Met

2.5 Graphene

Graphene, Nano two-dimensional (2D) substructure of carbon with unbelievable surface area of about 2630 $m^2 g^{-1}$ (about the size of a football stadium), possess unique physicochemical properties (high surface area, excellent conductivity, high mechanical strength, and ease of functionalization and mass production) (Shao et al., 2010; Stoller

et al., 2008). Graphene can be called the 'mother of all carbon forms' (Geim & Novoselov, 2007). Some studies reported the conductivity of graphene 64 mS cm^{-1} using a four-point probe method (approximately 60 times more than that of SWCNTs) (Alwarappan et al., 2009; Dai et al., 2007). Unlike CNTs, graphene does not suffer from disadvantages such as; residual metallic impurities that are inherent to the CVD fabrication process and have hindered their exploitation (Banks et al., 2006; Ji et al., 2010). In consequence of Graphene electrical conductivity, large surface area, unique heterogeneous electron transfer and charge carrier rates, widely applicable electrocatalytic activity, and low fabrication costs, it is a suitable applicable for electrochemical implementation (Brownson & Banks, 2010; F Chekin et al., 2012; Chen et al., 2010; Liang & Zhi, 2009; Pumera, 2009). Consequently, graphene was introduced as a favorable electrode material alternative in various applications in order to enhance specific technological fields and particularly the energy storage and generation fields. All these unique properties promising the applications of graphene energy-related issues. Utilizing graphene to develop the existing knowledge and current techniques, groundbreaking performance is expected to surpass that already achieved whilst at the same time proving to be a greener and more energy efficient alternative (Brownson et al., 2011).

2.6 Implantable Enzymatic Biofuel Cell

Implantable biofuel cells produce electricity from the electrochemical glucose and oxygen reaction. In theory, the constant supply of glucose and oxygen together in provide an unlimited source of power generation (Kerzenmacher et al., 2008; Kloke et al., 2012) which can power implants like cardiac pacemakers. The suitable glucose fuel cells for implantation is classified based on their electrocatalyst which facilitate the electrode reactions and divided into three main types; isolated enzymes such as glucose

oxidase (Mano et al., 2003; A Termehyousefi et al., 2015; Zebda et al., 2013a) or abiotic electrocatalysts such as platinum (Kerzenmacher et al., 2008; Kloke et al., 2012) and microbial catalysts have a minor role for the application in implantable glucose fuel cells due to high risk of infection (Inmann & Spensley, 2013). Up to now, many types of research are carried out to generate the power from thermos-electric effect, vibrations or body movements which most of them are not applicable in the real world. Thus, applying glucose fuel cells which generate power, using two common substrates in physiological fluids (glucose and dissolved oxygen) seems more promising. In this case, the enzyme wired electrically to the redox mediators. Recent studies have reported few enzyme-based biofuel cells which are functional *in-vivo*. The challenging issues are implanting the biocatalytic electrodes and extracting the electrical power from small living (Halamkova et al., 2012).

Glucose fuel cells were considered as an interesting potential power source for implanted devices since they utilize the available glucose and dioxygen to generate power. First studies by Drake and colleagues (1970) suggest that an abiotic catalyst could efficiently power an implanted medical device (Drake et al., 1970). GBFCs employ catalysts enzymes which electrically wired by redox mediators (Cracknell et al., 2008; Deng et al., 2008; Habrioux et al., 2008; Togo et al., 2008). Although the MFCs can apply to generate the static power in large scales (Ishii et al., 2008), the enzymatic biofuel cell may employ in vivo for powering small implantable devices like biosensors due to their low power requirements (Heller, 2004; Osman et al., 2011).

With the aim of investigating the sufficient power supply of GBFCs to support electronic devices, especially implantable medical devices, enzyme-based biofuel cells implanted into living lobsters were assessed (Zebda, Gondran, et al., 2011). Series connecting the two implanted biofuel cells in lobsters "living battery" were capable of generating up to 1.2 V open circuit voltage (V_{oc}). In the same way, a set of five series-

connected cells, filled with human serum solution might generate V_{oc} of 3 V which can easily supply the required power of a pacemaker. Normal human physiological glucose concentration and condition during resting or performing physical exercises can guarantee the sustainable operation of the pacemaker. These two system models provide a landscape of future application of GBFCs in sensing, information processing and wireless transmission devices in military service, homeland security, environmental monitoring and biomedical applications. The first prototype of the pacemaker activated by the electrical energy transformed from physiological fluids opens the path for future implantable medical devices (MacVittie et al., 2013). While the dissolved enzymes are only stable for days, immobilizing them on the electrode surface increase their stability by months (Christine M. Moore et al., 2004).

Hereby, we reviewed the applicable enzymes in glucose/O2 biofuel cells, structures of carbon nanomaterials such as CNTs and graphene and focused on the application of functionalized and hybrid carbon-based nanomaterials on biofuel cells.

2.6.1 Current Limitations of Glucose-Oxygen Enzymatic Biofuel Cells

The typical enzymatic fuel cell demonstrates power in the microwatt range and low long-term stability (Ivanov I., Vidakovic-Koch T. *et al.*, 2010). Biofuel cell tests are often performed under quite different conditions (concentration, temperature, pH, mass transport conditions, etc.), which complicates or hampers the comparison between different configurations and the identification of the electrodes and fuel cells with the best performance (Bandodkar A. J. & Wang J., 2016; Krikštolaitytė V., 2014). It is obvious that for straightforward characterization some standardization is needed, and the logical way is the adoption of methods from conventional fuel cells research. Regarding the electrochemical experiments, the importance of steady-state measurements should be underlined. Once an unambiguous characterization of the

biofuel cell performance under steady-state conditions has been done, dynamic experiments for simulation of real applications can be performed. Essential for the future application of enzymatic fuel cells is their long-term stability (Guarnera S., Abate A. *et al.*, 2015). The utilization of a batch container as the conventional electrochemical cell or a beaker raises the problems of substrate depletion and product accumulation. Most of the reported configurations are based on a simple batch type system with a focus on the chemistry and processes occurring at the bioelectrode interface. Flow-through systems offer a possible solution for the long-term stability investigation of biofuel cells. Quite often the stability of biofuel cells is not evaluated at all, or in an inappropriate manner. Numerous studies evaluate the long-term performance by recording the respective polarization curves at different intervals of time, meanwhile storing the fuel cell in the respective solution. These conditions do not represent the actual working conditions of such power-generating devices. More realistic information about the long-term stability can be obtained by constant polarization at potentiostatic or galvanostatic conditions. Amongst the variety of enzymatic fuel cells based on different fuels and oxidants, glucose/oxygen fuel cells are the most renowned type and subject of most extensive research. Their major intended application is associated with implantation and use as power sources for other implantable devices. However, the problem with the low power density is especially pronounced in this case since glucose and oxygen are present in very low concentrations in the human body, which still restrains any real practical applications. Therefore, in the development of glucose-oxygen biofuel cells, apart from the fine-tuning and increase of the voltage, a major focus is the establishment of efficient electron transfer and increase of the currents. On the other side, the low stability is affected by the intrinsic nature of enzymes as biocatalysts as well as some issues associated with the electrode architecture (leaching of the enzyme, leaching of mediator, pH change, etc.). The desire for electron transfer

efficiency and stability constitute the electrode design as the essential step in the development of efficient biofuel cells. Consequently, most of the research until now has been focused on this issue and less attention has been drawn to the overall system design.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

In this work, a glucose-oxygen biofuel cell was studied. During this methodical development of EBFC, 4 stages were highlighted. At the first stage, 3D graphene was synthesized. In order to achieve this aim, graphite oxide was synthesized using the modified Hummer's method and in continue treated with the hydrothermal method to produce 3D graphene. In the next stage, the prepared sample was analyzed using the Raman, Fourier Transform Infrared Analysis (FTIR), High-Resolution Transmission Electron Microscopy (HRTEM) and Field Emission Scanning Electron Microscopy (FESEM). In the third stage, the bio-electrode was synthesized and the enzyme was immobilized. Electrochemical techniques get to work to characterize the fabricated bio-electrode and optimize it in the final stage.

3.2 Synthesizing 3-Dimensional Graphene

The initial step of preparing the 3D graphene is to synthesize graphite oxide from graphite using the modified Hummer's method (Guo H.-L., Wang X.-F. *et al.*, 2009; Hummers Jr W. S. & Offeman R. E., 1958). Graphene oxide was obtained by oxidation of 1 g of graphite flakes with 120 ml of H_2SO_4 (95%), 13 ml of H_3PO_4 (90%) and 6 g of $KMnO_4$ (9:1 mixture of concentrated H2SO4/H3PO4). The mixture was stirred for three days to complete oxidation of the graphite. During oxidation, the color of the mixture changed from dark purple-green to dark brown. To stop the oxidation process, about 150 g of ice cubes were added to the reaction mixture and then 7 ml of H_2O_2 solution was added, the color of the solution mixture was changed to bright yellow indicating the high oxidation level of graphite. The formed graphite oxide was washed three times with 1 M HCl by using centrifugation and repeatedly washed with deionized water until the pH was around 4 to 5. During the washing process with deionized water, the

graphite oxide underwent exfoliation which results in thickening of the graphene oxide solution and forming graphene oxide gel.

A mixture of 1 ml GO aqueous dispersion and 40 ml of deionized water was stirred for 20 min to provide a homogeneous solution. An amount of 10µl hydrazine was added to the mixture while stirring and pH adjusted to 9. The mixed solution sealed in a 50 ml stainless steel autoclave and maintained at 80 °C for 24 hours. The prepared 3D graphene was taken out with tweezers and kept in a freeze drier for 48 h to remove adsorbed water. Dried 3D graphene stored into the sealed Schott bottle for the next step.

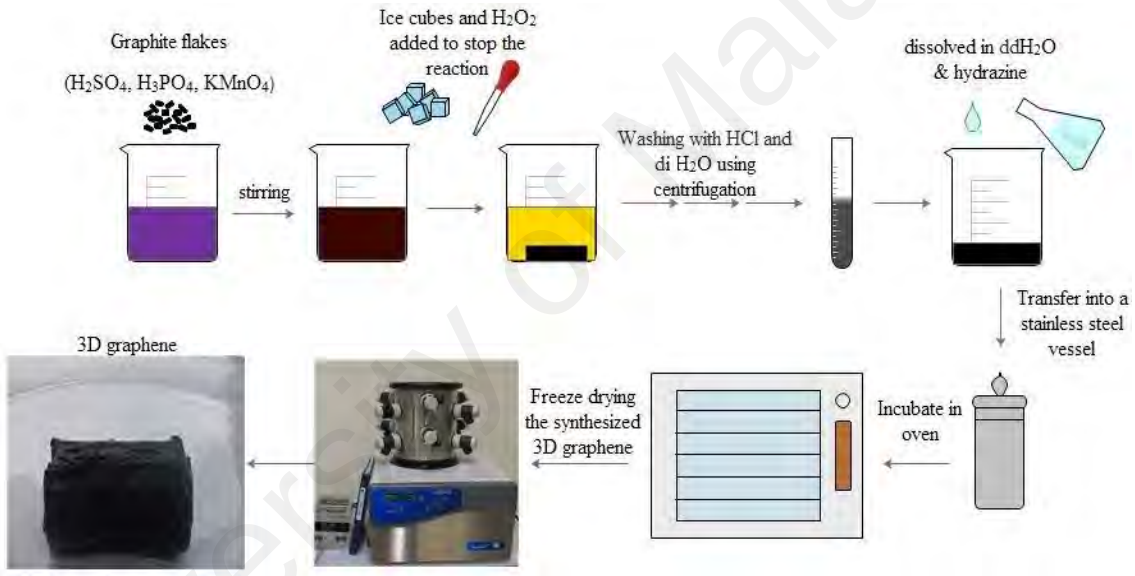

Figure 3.1: Synthesis of 3DG

In addition, to provide a CNT/3DG hybrid, the same procedure as carried out, but the amount of CNT added to synthesized GO before it undergoes the hydrothermal step.

3.3 Characterization

The characteristics of the synthesized 3DG and CNT/3DG hybrid were analyzed using Raman, FTIR, HRTEM, and FESEM.

3.3.1 Raman Spectroscopy

The Raman spectra of all 3D-graphene composite and CNT/3DG hybrid were obtained using a Renishaw inVia™ confocal Raman microscope employing a 532 nm excitation wavelength to confirm the reduction of graphene. Excitation was provided by a HeNe laser (Melles Griot). The exciting laser radiation was coupled into a Zeiss microscope through a wavelength-specific single mode optical fiber. The incident laser beam was collimated via an achromatic lens and passes a holographic band-pass filter before it was focused on the sample through the microscope objective. The sample is located on a piezo-electrically driven microscope scanning stage with an x, y resolution of ca. 3 nm and a repeatability of 5 nm, and z resolution of ca. 0.3 nm and 2nm repeatability. The Raman backscattered radiation was detected by a back-illuminated deep depletion, 1024*128 pixels charge-coupled device camera operating at -82.

3.3.2 Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) is a technique to determine the functional group in the samples. 200 mg of potassium bromide and 1% of the samples were mixed and made the pellets by pressing at 10 tons for preparing KBr disc. The FTIR spectra of samples were characterized by a Bruker IFS 66/S FTIR (Germany) with 4 cm^{-1} resolution. The resolution of the FTIR was set to 4 cm^{-1}, with 16 scans in the wavelength range in between 400 cm^{-1} to 4000 cm^{-1}. The FTIR spectrum was collected, with its peaks labeled. The FTIR spectrum was obtained using a Perkin-Elmer 100 spectrophotometer.

3.3.3 N_2 Sorption Isotherms

Brunauer Emmett Teller (BET) analysis provides precise specific surface area evaluation of materials by nitrogen multilayer adsorption measured as a function of

relative pressure using a fully automated analyzer. The technique encompasses external area and pore area evaluations to determine the total specific surface area. BET provides important information for studying the effects of surface porosity and particle size in many applications. The specific surface area determined by BET is related to the total surface area (reactive surface) since all porous structures adsorb the small gas molecules. The samples could dry out either with nitrogen purging or in a vacuum applying elevated temperatures. The amount of adsorbed gas is correlated to the total surface area of the particles including pores in the surface. The calculation is based on the BET theory. Traditionally nitrogen is used as adsorbate gas. Gas adsorption also enables the determination of size and volume distribution of micropores (0.35 – 2.0 nm).

3.3.4 High-Resolution Transmission Electron Microscopy

Transmission electron microscopy images of as-received compounds were captured using Philips PW 6061 TEM system (Model CM 200, Eindhoven, Netherlands) to analyze the structure of nanomaterials. FEIG-4020, 500 kV high-resolution transmission electron microscopes (HRTEM) was used to study the 3D-graphene composite and CNT/3DG hybrid structure.

Lattice level resolution of HRTEM was used to understand the nature of 3D-graphene composite and CNT/3DG hybrid. Forward and inverse Fourier transform (FFT & Inverse-FFT) analysis, using Gatan, Inc. The samples are ultrasonicated in 95% ethanol before the HRTEM characterization in order to provide a homogeneous solution.

3.3.5 Field Emission Scanning Electron Microscopy

The morphology of 3D-graphene composite and CNT/3DG hybrid were studied by Field Emission Scanning Electron Microscopy (FESEM) that scans the sample with a focused beam of electrons and as a result of bombardment different type of electrons is coming out from the specimen.

The schematic diagram of FESEM can be seen in Figure 3.2. The secondary electrons are collected or detected by a detector. The surface image of the sample is built up by comparing the intensity of these secondary electrons with the scanning primary electron beam. The FESEM images of samples were captured by a scanning electron microscopy of Model: Nova Nanosem 230.

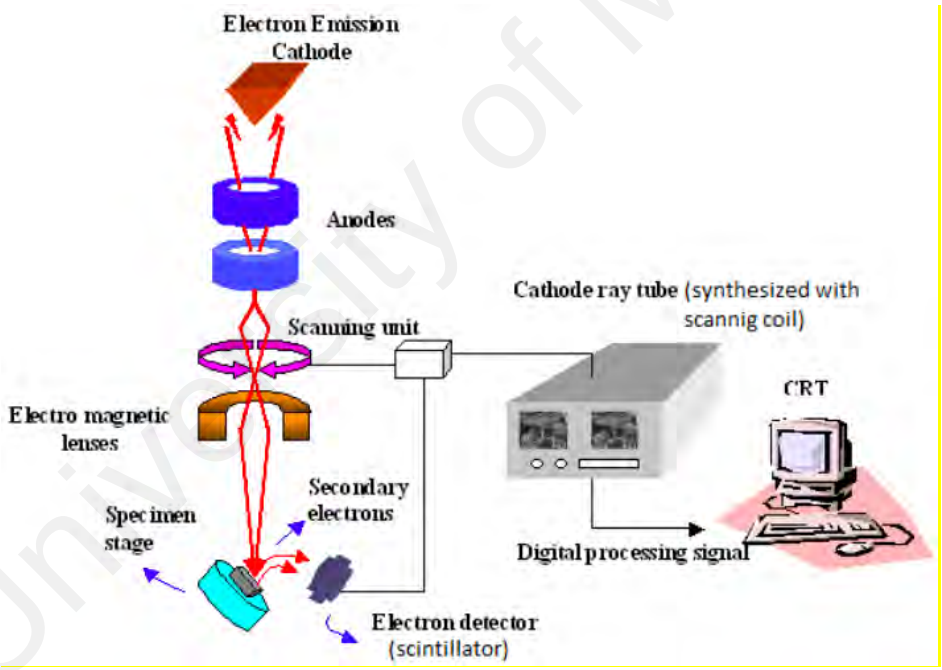

Figure 3.2: Schematic of the working principle of FESEM (Jusman Y., Ng S. C. *et al.*, 2014).

3.4 Bio-electrode Fabrication

A 10 mg of synthesized 3D graphene and CNT/3DG hybrid placed separately in two test tube containing 5 ml ethanol and ultrasonicated for 30 minutes to provide a homogeneous solution and left for 10 minutes at the room temperature to cool down. In the following, step 5 mg of glucose oxidase enzyme added to each solution and mixed gently and stored in the fridge.

In continue, two 3 mm diameter GC electrode was polished with 0.05 mm alumina slurry using a velvet pad. Prior to modification, the electrode placed in a beaker containing 1:1 V/V double distilled water and ethanol and ultrasonicated for 5 min and washed with deionized water and left to dry at room temperature (25±3ºC). After drying of the electrode, a 5 µL of prepared 3D graphene-GOx and 5 µL of prepared CNT\3DG/GOx dispersion was drop cast on each GC electrode and are allowed to dry at a room temperature (25±3ºC) for 15 min.

3.4.1 Electroanalytical Techniques

Electroanalytical methods are a class of techniques in analytical chemistry which study and analyzed by measuring the potential (volts) and/or current (amperes) in an electrochemical cell containing the analyze

3.4.1.1 Cyclic Voltammetry (CV)

Cyclic voltammetry (CV) is a type of potentiodynamic electrochemical measurement and is a sensitive technique for the investigation of chemical kinetics, the current contains quantitative mechanistic information. In cyclic voltammetry, the electrode potential ramps linearly versus time in cyclical phases. The rate of voltage change over time during each of these phases is known as the experiment's scan rate (V/s). The potential is measured between the working electrode (WE) and the reference electrode

(RE), while the current is measured between the working electrode and the counter electrode (CE). These data are plotted as current (i) versus applied potential (E), often referred to as just potential. At some point after the reduction potential of the analyte is reached, the cathodic current will decrease as the concentration of the reducible analyte is depleted. If the redox couple is reversible then during the reverse scan (from t1 to t2) the reduced analyte will start to be re-oxidized, giving rise to a current of reverse polarity (anodic current) to before. The more reversible the redox couple is, the more similar the oxidation peak will be in shape to the reduction peak. Hence, CV data can provide information about redox potentials and electrochemical reaction rates. Depending on the shape of the curves produced and the number of peaks observed CV scans can determine whether a reaction is reversible or irreversible, whether the reaction results in a change of the electrode surface or the electrolyte, how many reaction stages there are and even how many electrons are involved. All electrochemical measurements were performed using a computer controlled Potentiostat/Galvanostat (302N Autolab) that presented in figure 3.3.

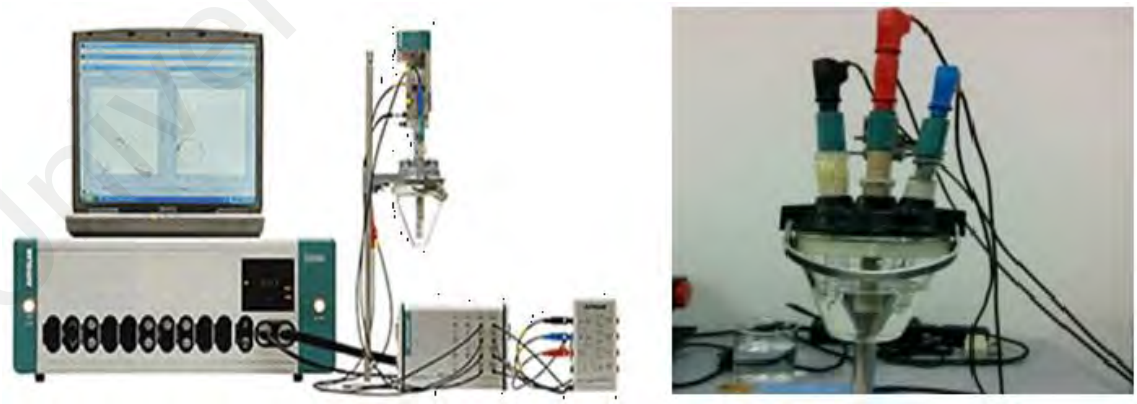

Figure 3.3: Potentiostat/Galvanostat (302N Autolab) and the working cell.

3.4.1.2 Chronoamperometry

Amperometry is the term indicating electrochemical techniques in which a current is measured as a function of an independent variable that is, typically, time or electrode potential. Chronoamperometry is the technique in which the current is measured, at a fixed potential, at different times since the start of polarization. Chronoamperometry is typically carried out in unstirred solution and at the fixed electrode, i.e., under experimental conditions avoiding convection as the mass transfer to the electrode.

3.4.1.3 Electrochemical Impedance Spectra

Electrochemical impedance spectroscopy is an advanced tool in corrosion and solid state laboratories that are slowly making its way into the service environment as units are decreased in size and become portable. Impedance Spectroscopy is also called AC Impedance or just Impedance Spectroscopy.

The usefulness of impedance spectroscopy lies in the ability to distinguish the dielectric and electric properties of individual contributions of components under investigation. In order to gain deeper insight into the interfacial charge transfer process within the fabricated bio-electrode, the electrochemical impedance spectra (EIS) were recorded in a frequency range between 0.01 Hz and 100 kHz. All electrochemical measurements were performed using a computer controlled Potentiostat/Galvanostat (302N Autolab).

3.4.1.4 Polarization

In an ideal scenario, the potential difference of a fuel cell would only depend on the difference between the potential at which the fuel can be oxidized and the potential at which the oxidant can be reduced (ie the OCP). In reality, however, the operating potential difference of a fuel cell is always less than the OCP due to polarization (the

reduction of potential difference when current is flowing) of the fuel cell (Baker R. & Zhang J., 2011). Polarization curves (a plot of cell potential against current or current density) are thus used to assess fuel cell performance and to better quantify and characterize the different sources of polarization. Though polarization curves are useful for determining the overall cell performance, it does not give any information about the individual components in the cell. Instead, it gives the indications on the sum total inefficiencies of the system. Polarization curves also give very useful information on the parameters required in order to maximize a fuel cell's performance. Since the same data can be used to determine power density, polarization curves are often coupled with power density curves which show at which potential and cell resistance the power is maximized.

3.4.1.5 Power density

The fabricated bioanode placed inside a glass beaker containing 0.1% or 0.42% glucose solution dissolved in phosphate-buffered saline solution (pH 7.4). Possible air trapped inside the EBFCs was removed by nitrogen purging for 10 mins. To measure the load characteristics of the assembled biofuel cell, the terminals were connected to a variable external resistance (0-12 kΩ), as shown in Figure 3.4. Dividing the calculated power output to the area of the electrode surface would be power density.

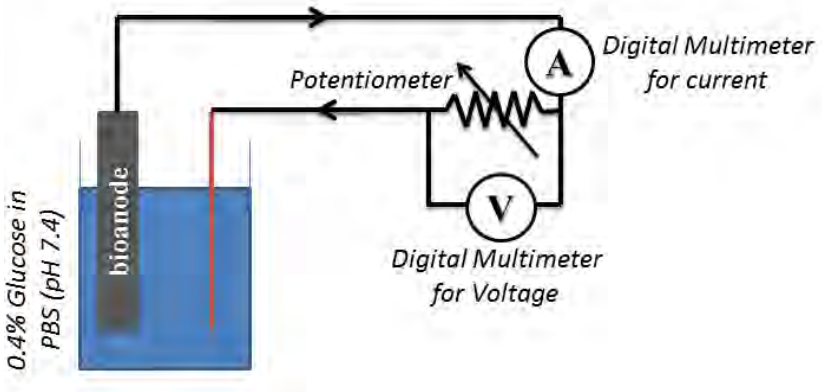

Figure 3.4: Experimental Setup for Biofuel Cell power density characterizations

CHAPTER 4: RESULTS AND DISCUSSION

The aim of this research is to immobilize the enzyme on the surface of the electrode using the highly porous 3D-graphene and also a CNT/3DG hybrid. This chapter is the base of this dissertation, which presents a detailed description of the results obtained through experimentations and scientific analysis of the outcomes. In the first part, the characterization results of synthesized 3D-graphene and CNT/3DG hybrid were presented and the next part showed the electroanalytical properties of fabricated bio-electrode.

4.1 Characterization

The synthesized 3DG and CNT/3DG hybrid were required for further immobilization of GOx and electrode modification. Thus the synthesized 3DG and CNT/3DG hybrid were characterized using Raman, FTIR, BET, HRTEM, and FESEM.

4.1.1 Raman Spectroscopy

RAMAN spectra are a powerful tool to characterize carbon base material. Figure 4.1 reveals the RAMAN spectra comparison between graphene oxide (GO) and 3D graphene. As it can be extracted from figure 4.1, the spectra of all the samples have two vibrations at around 1584 cm^{-1} and 1351 cm^{-1}, which related to the G and D bands of the RGO, respectively. The G band is assigned to the first-order scattering of the E_{2g} phonon and the D band is corresponded to decrease of the size in plane sp^2 domains due to extensive oxidation presented in GO nanosheets. The ratio between the intensities of the D and G bands (I_D/I_G) is a measure of the disorder, as expressed by the sp^2/sp^3 carbon ratio which increases (from 1.184 to 1.386) after the reduction procedure. Compared with graphene oxide (0.93), the 3D graphene (1.01) has slightly higher I_D/I_G

values, which are attributed to a decrease in the size of the sp^2 domains upon the reduction procedure.

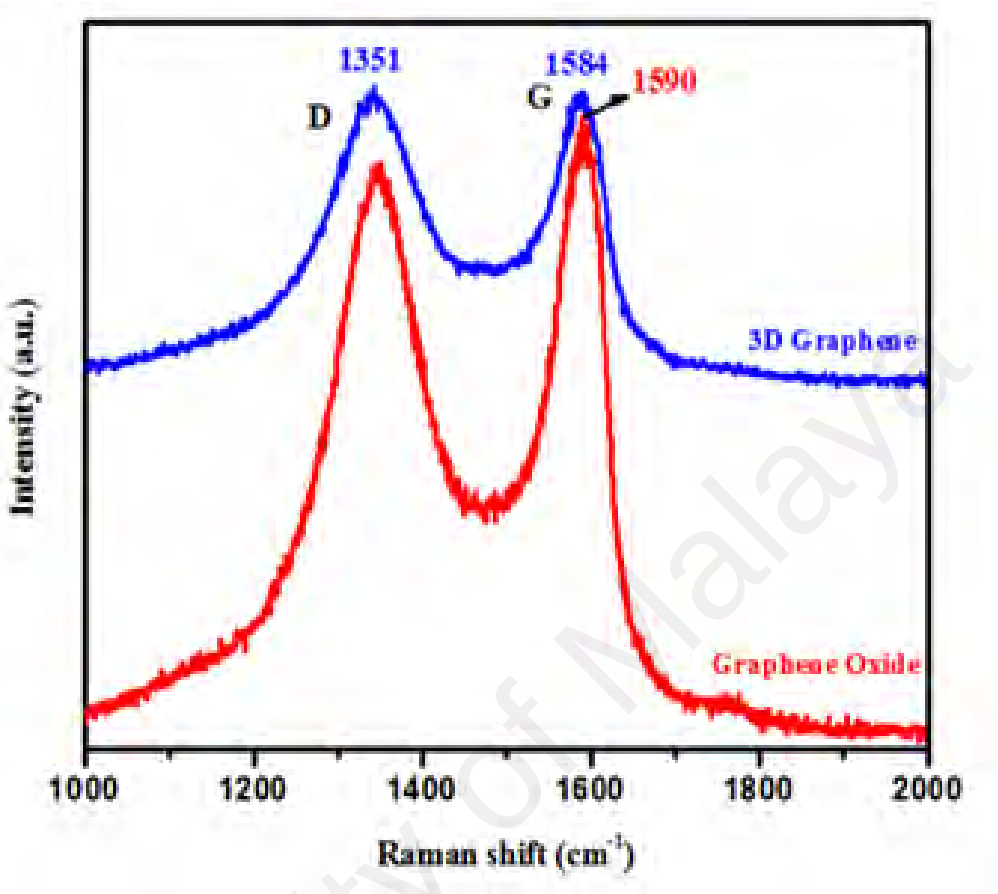

Figure 4.1: RAMAN spectra of graphene oxide (GO) and 3D graphene.

The summary of comparing Raman spectra of graphene oxide (GO) and 3D graphene are presented in Table 4.1.

Table 4.1: RAMAN spectra of graphene oxide (GO) and 3D graphene.

Raman Shift (cm^{-1})		Assignment
GO	1590	in-plane vibrations of sp^2-bonded carbon
	1351	disordered structures of GO due to the extensive oxidation
3DG	1584	indicating the recovery of sp^2 domains

4.1.2 Fourier Transform Infrared Spectroscopy

The result of Fourier transforms infrared spectroscopy (FTIR) comparison between graphene oxide (GO) and 3D graphene presented in figure 4.2. The characteristic FTIR peaks of GO at 1158 cm^{-1} is attributed to C-O stretching vibrations of O-C-O, 1741 cm^{-1} assigned to aldehyde C=O/COOH group stretch- and a broad peak between 2500 cm^{-1} and 3500 cm^{-1} in the IR spectrum of GO is due to the carboxyl O-H stretching and presence of moisture in graphene oxide (Xian T., Yang H. *et al.*, 2014). Deletion of corresponding to the C=O/–COOH, and C-O in the FTIR result of 3D graphene represents a successful synthesis of 3D graphene and deoxidation of GO.

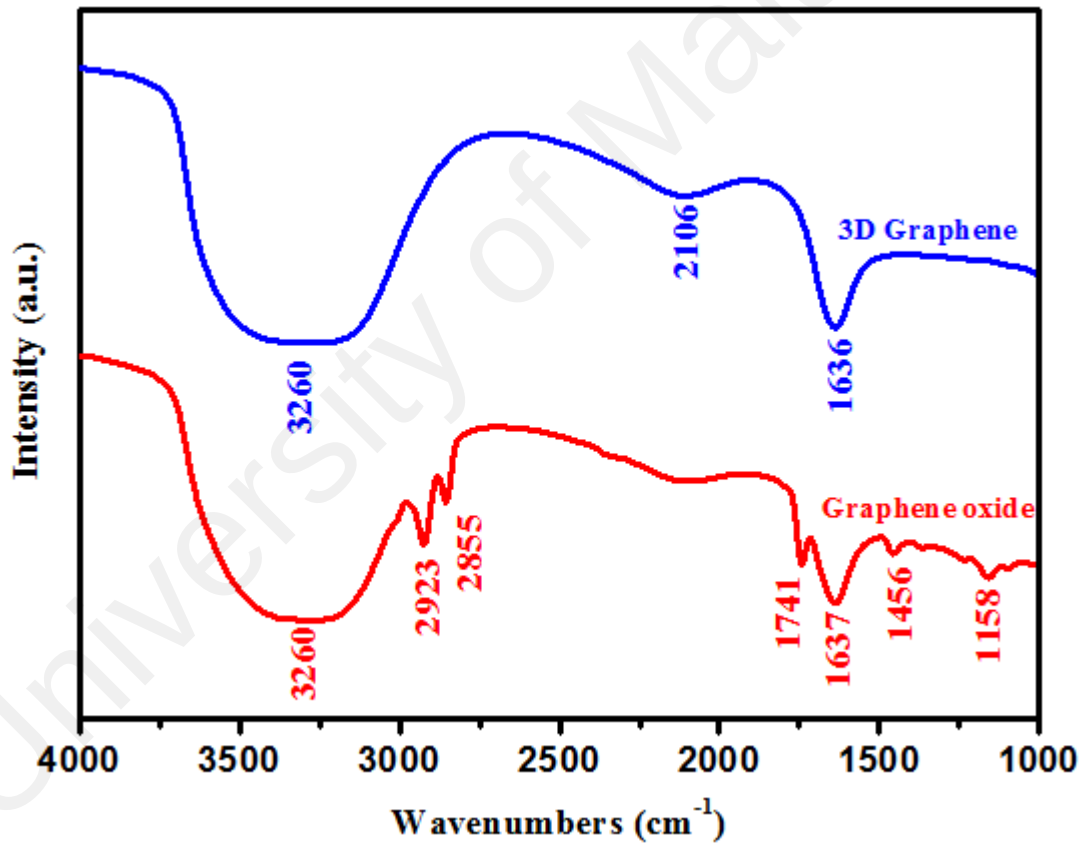

Figure 4.2: FTIR spectra of graphene oxide (GO) and 3D graphene.

The summary comparison of FTIR spectra of graphene oxide versus and 3D graphene was accessible in Table 4.2.

Table 4.2: Comparison of FTIR spectra of graphene oxide versus and 3D graphene

Wavenumber (cm^{-1})		Assignment
GO	1158	O-C-C
	1741	C=O/COOH
	2855-2923	–OH
3DG	1639	C=C
	2106	C-H
	3260	–OH

4.1.3 N_2 Sorption Isotherms

Brunauer Emmett Teller (BET) surface areas and pore volumes were measured on a Micromeritics ASAP 2020 sorptometer using nitrogen adsorption at 77 K which the results for the 3DG are presented in figure 4.3. The specific surface area of N-doped graphene aerogel is 1200 m^2g^{-1}, which was larger than that of graphene oxide (206 m^2g^{-1}). The N2 adsorption-desorption curve and calculating the pore size distribution of synthesizing 3DG from desorption data using the Barrett–Joyner–Halenda (BJH) presents a typical type-IV isotherm and H2 hysteresis loop, which confirms the mesoporous structure of synthesized 3DG. BJH inset shows that abundant mesoporous exist in the 3DG in the range 2–9 nm

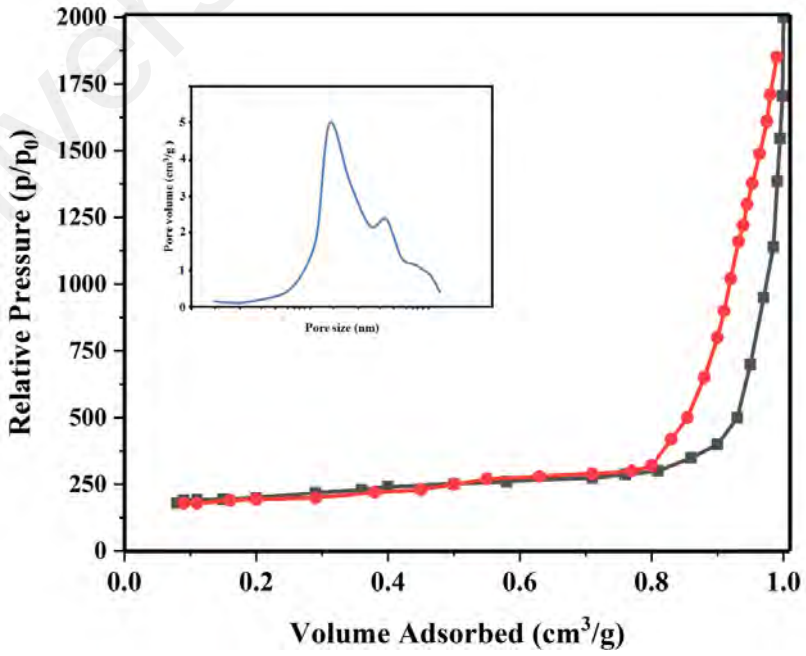

Figure 4.3: Isotherm plot and BJH pore distribution (inset) of N-doped 3DG.

The surface area and pore structures properties of graphene, graphene oxide, and 3DG are elucidated by BET methods in Table 4.3. Based on the BET analysis results, it was found that the surface area of the synthesized 3DG noticeably enhanced compared with the graphene, graphene oxide and graphite. 3DG has higher surface area compared to graphene and graphene oxide because of both its three-dimensional structure and high porosity which these factors increase the surface area. The high surface area of 3DG provides a perfect area in order to immobilize the enzyme. Furthermore, the GOx enzyme also could trap in the porous structure of the 3DG which it also facilitates the enzyme immobilization.

Table 4.3: The texture property of graphite, graphene oxide, graphene and 3-Dimensional graphene

Sample	BET surface area m^2/g
Graphite	1.32
Graphene oxide	23
Graphene	442
3DG	1200

4.1.4 HRTEM and FESEM

Two factors that control the power generation density and long-term activity of the biofuel cells are the enzyme stability and the substrate diffusion towards the electrode surface. The microporous structure of the 3DG can prevent the denaturation of the immobilized enzyme structure, leaching out the problem and also providing sufficient concentration of both glucose and oxygen to the enzymes. Surface characterization of bioanode has been characterized by FESEM and HRTEM analysis. Figure 4.4A shows the HRTEM image of synthesized 3D graphene (3DG), while the FESEM image (Figure 4.4B) reveals the high porous structure of 3DG which increase the biological function, stability and efficacy of GOx immobilization. The Figure 4.4C and 4.4D

confirm the successful hybridization of GOx and 3DG. For further comparison, the HRTEM and FE-SEM of the graphene oxide can be found in Appendix A.

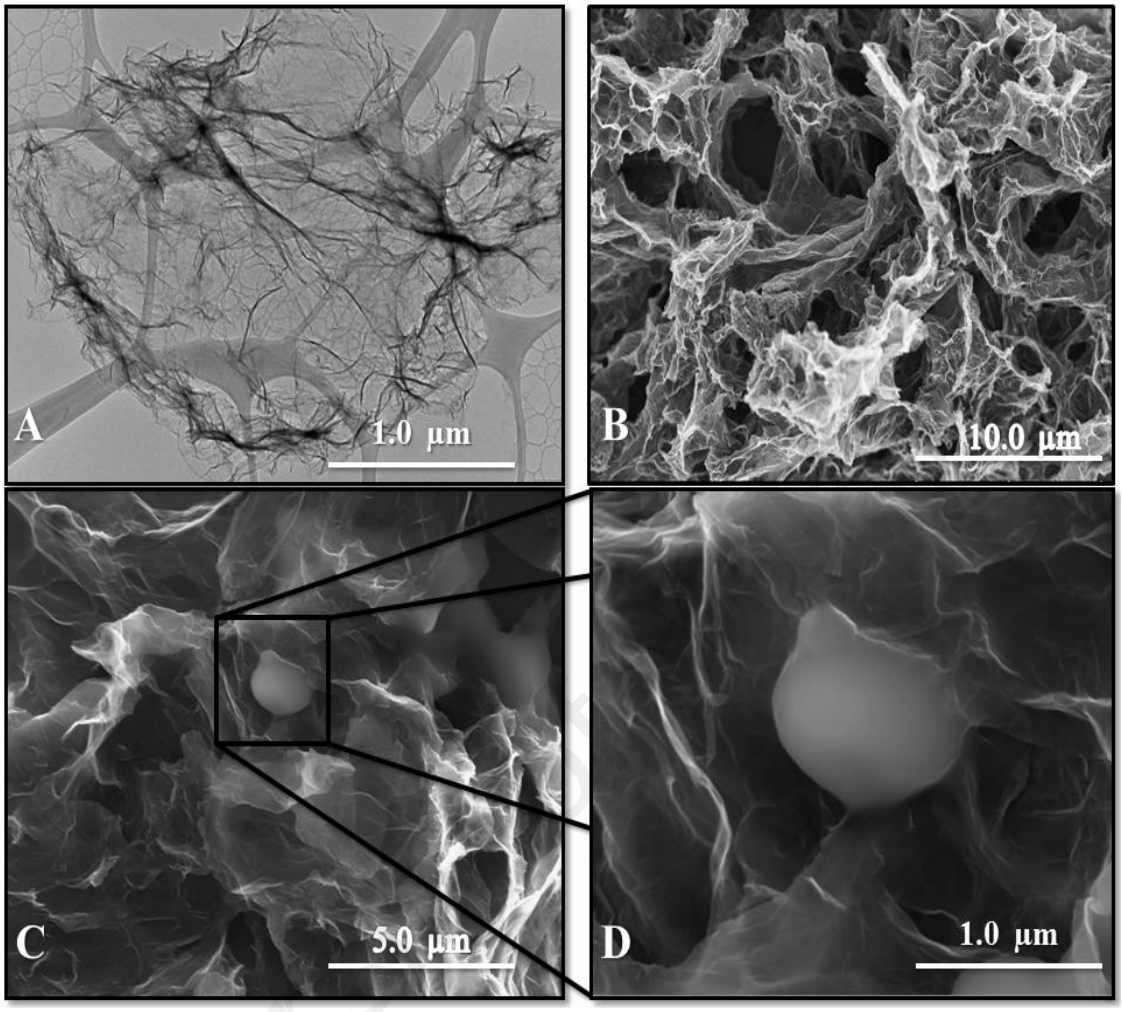

Figure 4.4: (A) HRTEM image of synthesized 3DG, (B) FESEM image of synthesized 3DG, (C) The hybrid of GOx and 3DG and (D) immobilized GOx via 3DG.

The multiporous structure of 3DG and wire like nature of CNTs that enfold the GOx prevent the immobilized enzyme from denaturation and leaching while facilitating the electron transfer from enzyme to the electrode and improve the enzyme stability. Surface characterization of CNT/3DG/GOx has been investigated by FESEM and HRTEM analysis. Figure 4.5A shows the FESEM image of 3DG and its highly porous structure. The FESEM image of hybridized CNT/3DG is presented in figure 4.5B. Figure 4.5C reveal the hybridized CNT/3DG while figure 4.5D shows the successful immobilization of GOx on the CNT/3DG biocomposite support. Finally, the figure 4.5E

represents the clear HRTEM image of hybridized CNT/3DG which the linear shape CNTs are distributed among the 3DG and hybridized together.

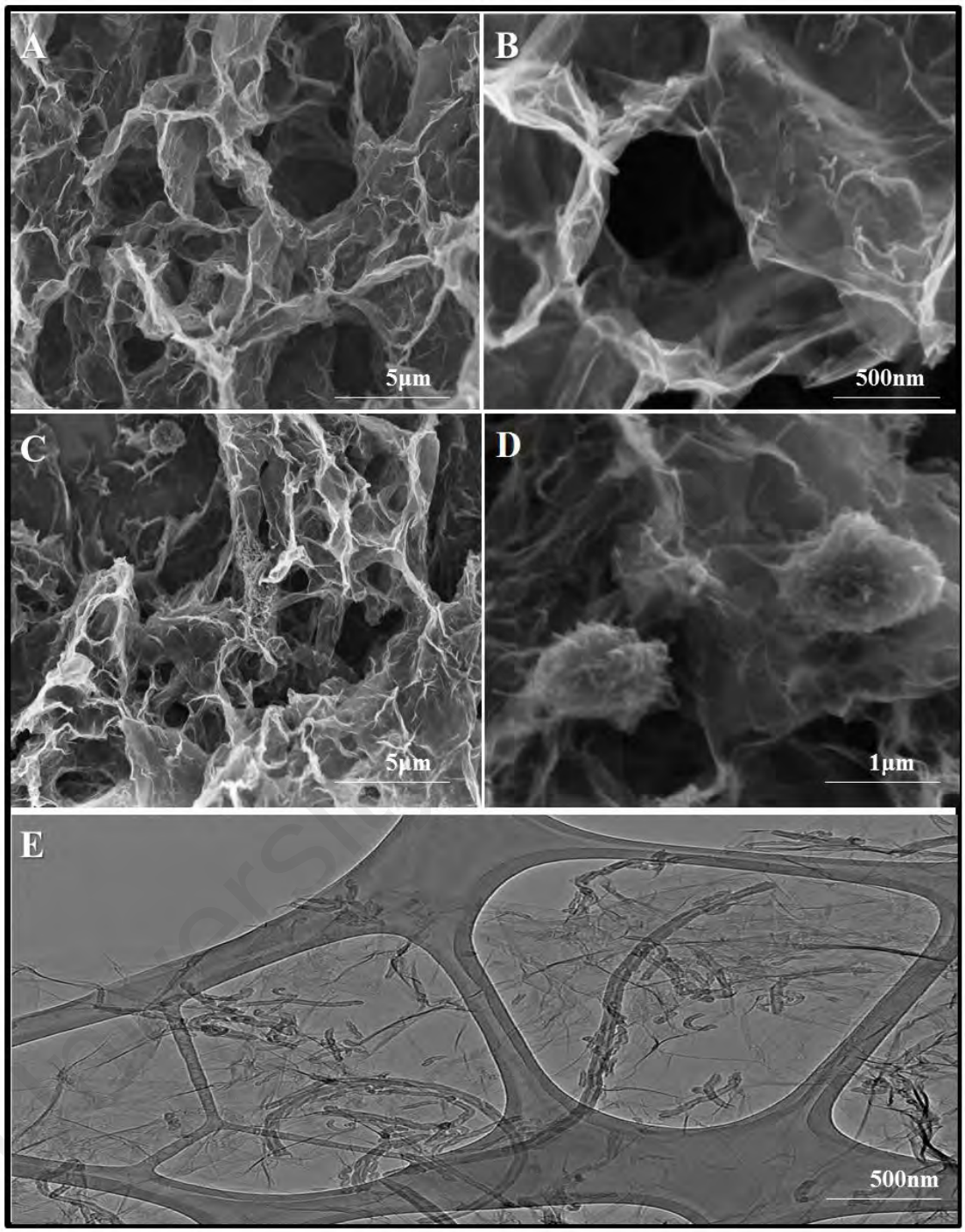

Figure 4.5: (A) FESEM image of 3DG, (B) magnified FESEM image of hybridized CNT/3DG, (C) FESEM image of hybridized CNT/3DG, (D) successfully immobilized GOx on hybridized CNT/3DG, (E) HRTEM image of hybridized CNT/3DG.

4.2 Enzymatic Biofuel Cell Performance

4.2.1 Cyclic Voltammetry (CV)

In order to confirm the successful immobilization of GOx at GCE/3D graphene-GOx cyclic voltammetry technique employed in this study. Figure 4.6 presents cyclic voltammetry result of the GCE/3D graphene-GOx at pH 7 which reveals a pair of well-defined redox peaks at the -0.43 V and -0.39 V. However, GCE/GOx graphene does not show any electrochemical peaks during the cyclic voltammetry studies. These results suggest that electrochemically prepared 3D graphene-GOx biocomposite has the ability for efficient electron transfer to the electrode surface.

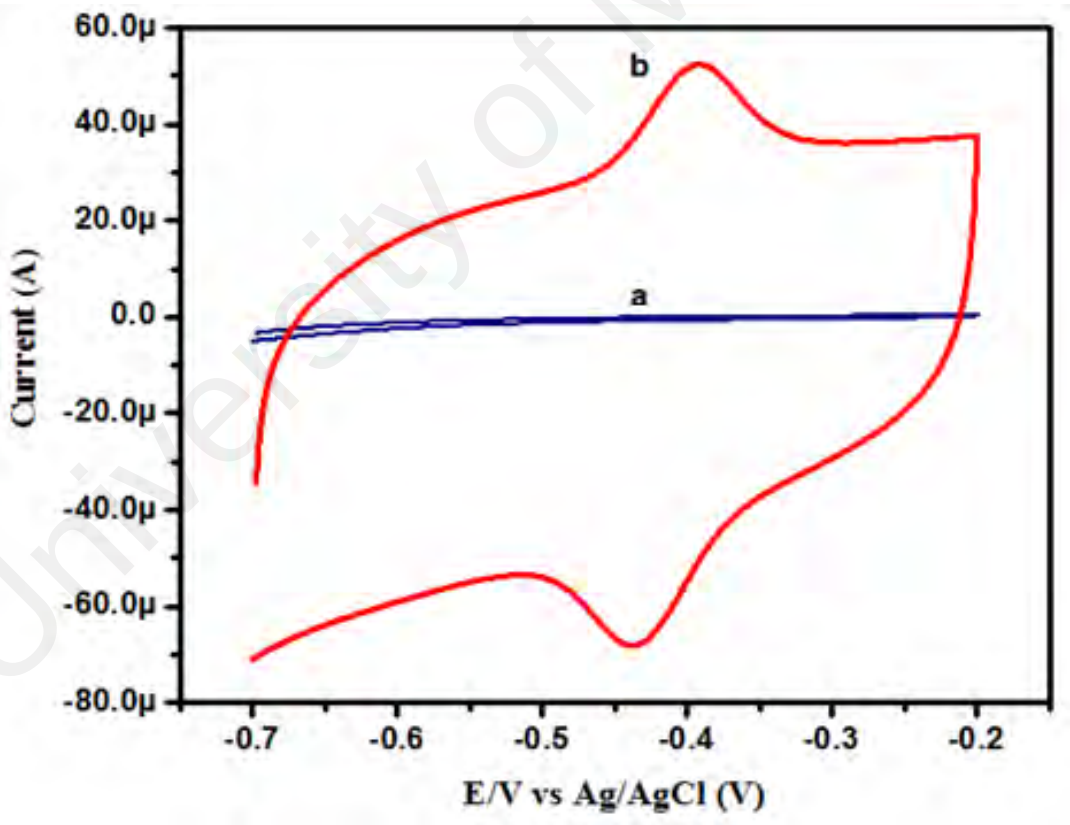

Figure 4.6: Cyclic voltammetry results of the GCE/3D graphene-GOx at pH 7; (a) Bare GCE, (b) Cyclic voltammetry result of GCE/3D graphene-GOx.

The effect of different scan rate on the GCE/3D graphene-GOx also was studied by varying the scan rates from 0.01 V to 0.2 V in N_2 saturated solution at pH 7. According to Figure 4.7, the effect of scan rate is linearly dependent on the peak current and also the peak potential was shifted towards the negative direction by increasing the scan rates from 10 to 200 mV s^{-1}. From the results, we establish that the electron transfer of GOx at the electrode surface as a surface-controlled quasi-reversible process, and as well GOx retain its bioactivity even at higher scan rates and maintained its electrochemical properties.

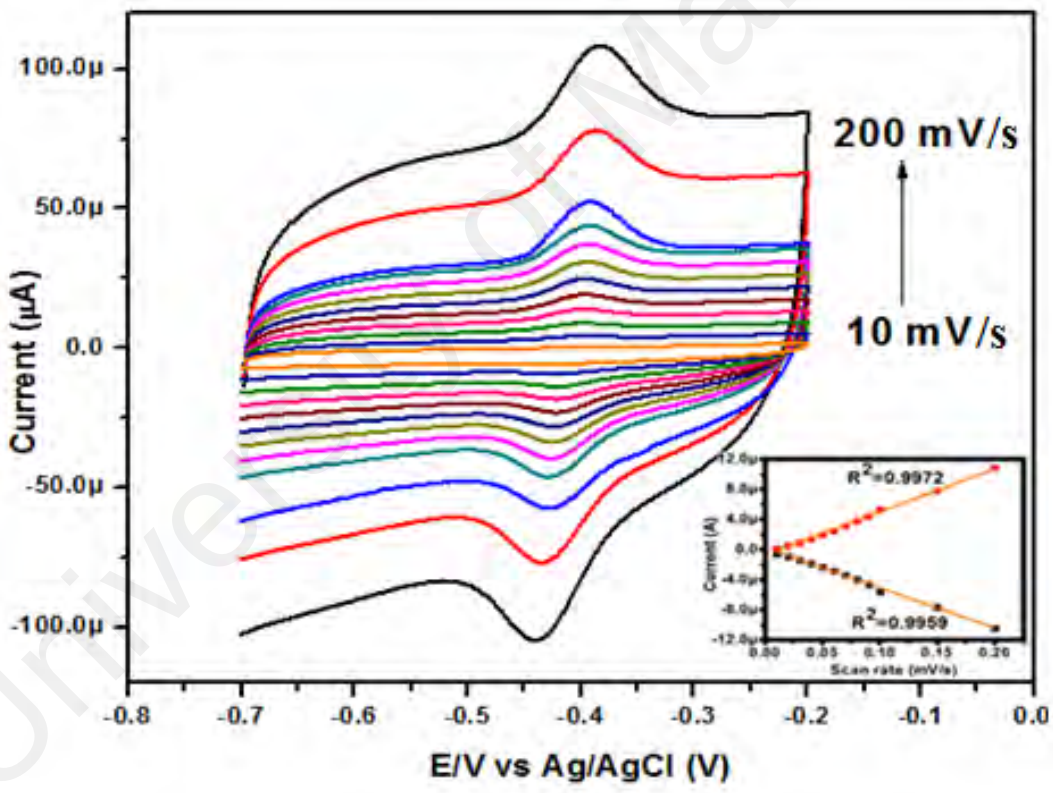

Figure 4.7: CVs obtained at GCE/3D graphene-GOx at different scan rates in N_2 a saturated solution at pH 7. The scan rates from inner to outer are: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200 mV s^{-1}. The inset shows the linear dependence of Ipa (■) and Ipc (■) on scan rate (10 to 200 mV s^{-1}).

In figure 4.8, the effect of pH on cyclic voltammetry was presented. It shows the cyclic voltammograms result of GCE/3D graphene-GOx in buffer solutions with different pH values at a scan rate of 100 mV s^{-1}. The cyclic voltammograms show the effect of different pH range on the negative shift of the redox peaks with increasing pH values from 4 to 10 at the scan rate of 100 mV s^{-1}. The fabricated BFC shows the best efficiency at the pH 7 due to the fact that GOx structure conserves in this pH and the three-dimensional structure of the enzyme in the higher or lower pH would change which result in of the enzyme dysfunction or inefficiency.

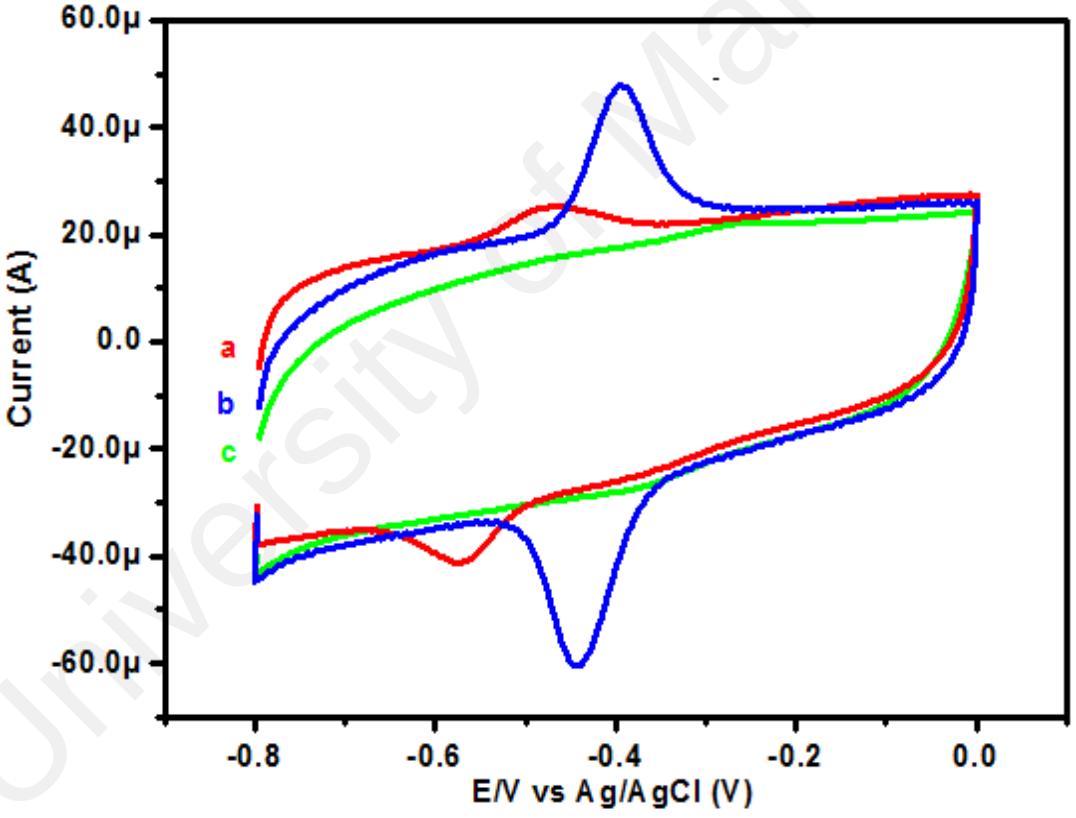

Figure 4.8: The cyclic voltammograms of GCE/3D graphene-GOx at a scan rate of 100 mV s^{-1} in phosphate buffer saline solutions at different pH values; (a) pH 10, (b) pH 7 and (c) pH 4.

Cyclic voltammetry employed to investigate the immobilization of GOx at CNT/3DG/GOx/GCE. According to the Figure 4.9 which demonstrated at pH7, a pair of well-defined redox peaks at -0.45 V and -0.37 V obtained. However, the bare GC electrodes and CNT/3DG modified GCE; do not show any electrochemical peaks during the cyclic voltammetry studies. These results suggest that electrochemically prepared CNT/3DG/GOx biocomposite has the ability for efficient electron transfer to the electrode surface.

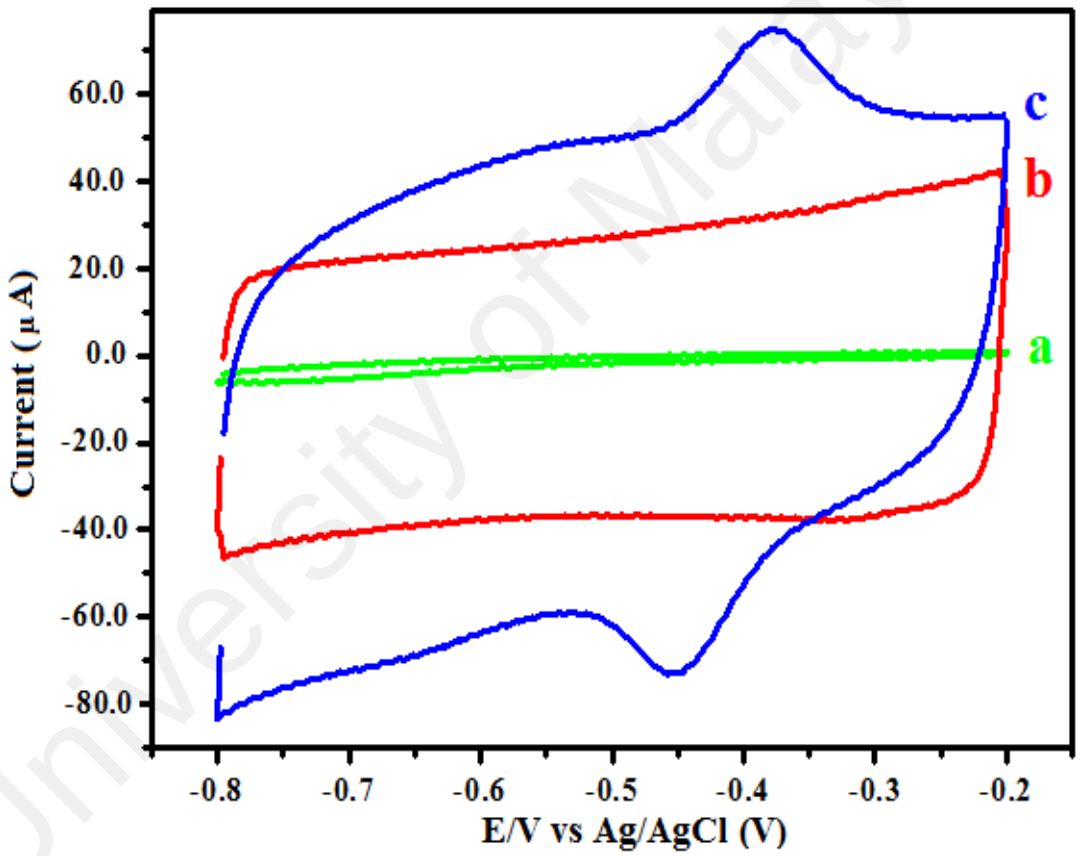

Figure 4.9: Cyclic voltammetry results of the (a) Bare GCE, (b) 3DG/GCE, (c) CNT/3DG/GOx/GCE at pH 7, Scan rate 100 mV s^{-1}

In figure 4.10, the effect of different scan rate at CNT/3DG/GOx/GCE was investigated by varying the scan rates from 0.01 Vs^{-1} to 0.3 Vs^{-1} in N_2 saturated solution at pH 7. As stated by Figure 4.9, the effect of scan rate is linearly dependent on the peak current and also the peak potential was shifted towards the positive direction by increasing the scan rates from 0.01 Vs^{-1} to 0.3 Vs^{-1}. From the results, we concluded that the electron transfer of GOx at the electrode surface as a surface-controlled quasi-reversible process, and as well GOx retain its bioactivity even at higher scan rates.

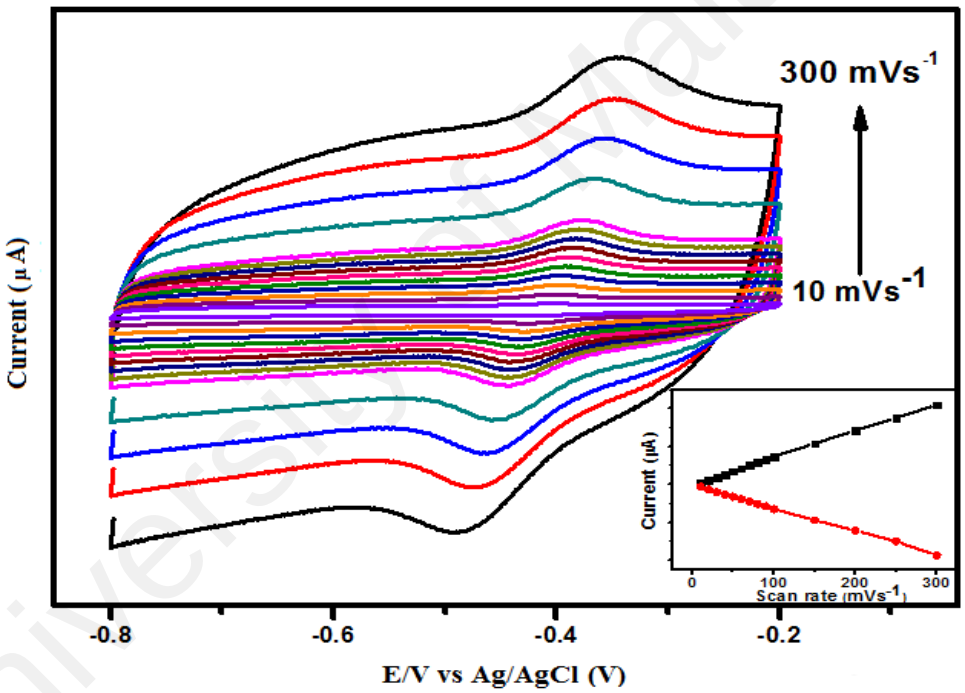

Figure 4.10: Cyclic voltammetry obtained at CNT/3DG/GOx/GCE at different scan rates in N_2 saturated solution at pH 7. The scan rates from inner to outer are: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 and 300 mV s^{-1}. The inset shows the linear dependence of Ipa (■) and Ipc (■) on scan rate (10 to 300 mV s^{-1}).

The effect of the pH variety on the fabricated CNT/3DG/GOx/GCE is presented in figure 4.11. The cyclic voltammograms results are at a scan rate of 100 mVs^{-1} in buffer solutions at pH values of 4, 7 and 10. The cyclic voltammetry shows the effect of different pH range to the negative shift of the redox peaks with increasing pH values from 4 to 10 at the scan rate of 100 mVs^{-1}. According to this figure, the optimum pH for the operation of fabricated BFC is pH 7 which is near to human blood pH and suggests the implantable application of the BFC.

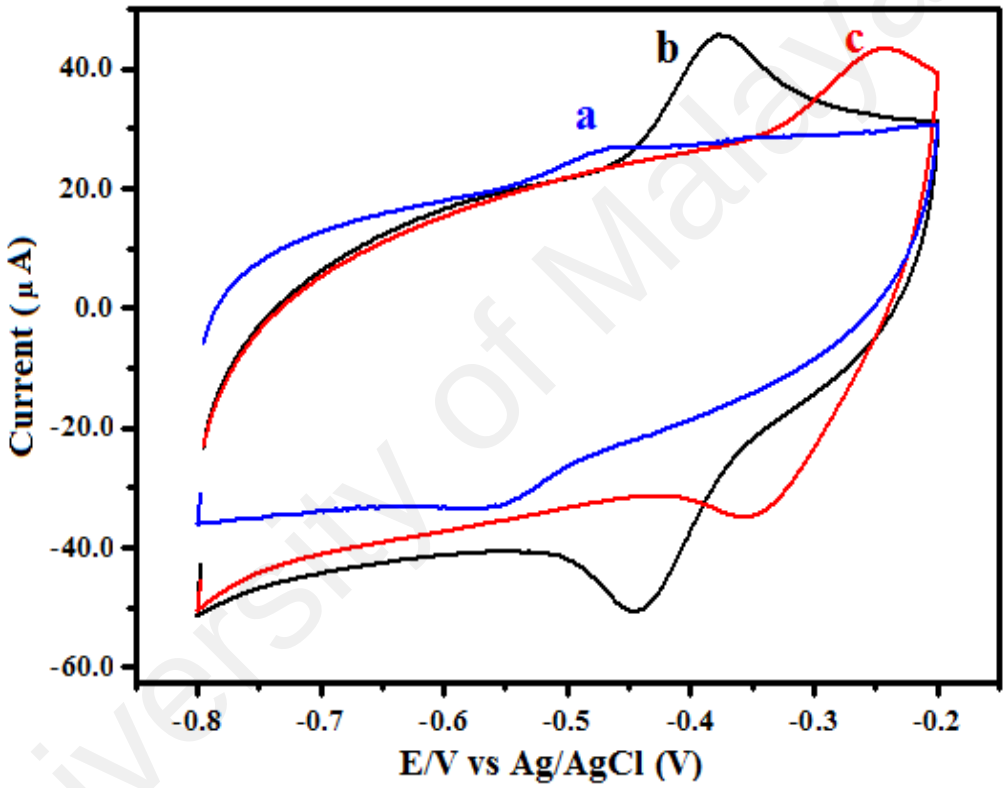

Figure 4.11: The cyclic voltammograms of CNT/3DG/GOx/GCE at different pH values; (a) pH 10, (b) pH 7 and (c) pH 4, at the scan the rate of 100 mV s−1 in buffer solutions

4.2.2 Chronoamperometry

Figure 4.12 displays the GOx/GC electrode Chronoamperometric response based on the current-time response for successive additions of glucose at the applied potential of -0.40 V and O_2-saturated 0.1 M PBS (pH 7) condition. According to the figure 4, the current increased in response to the injection of glucose and reached a steady state within 5 seconds. Increasing the concentration of injected glucose increased the amperometric currents simultaneously and the calibration curve corresponding to amperometric response is linear against the glucose concentration range from 0.005 mM to 3mM. Considering the normal blood glucose level which is between 4 mM and 6 mM, the biofuel cell would be supplied with enough and a suitable amount of glucose in the case of implants applications.

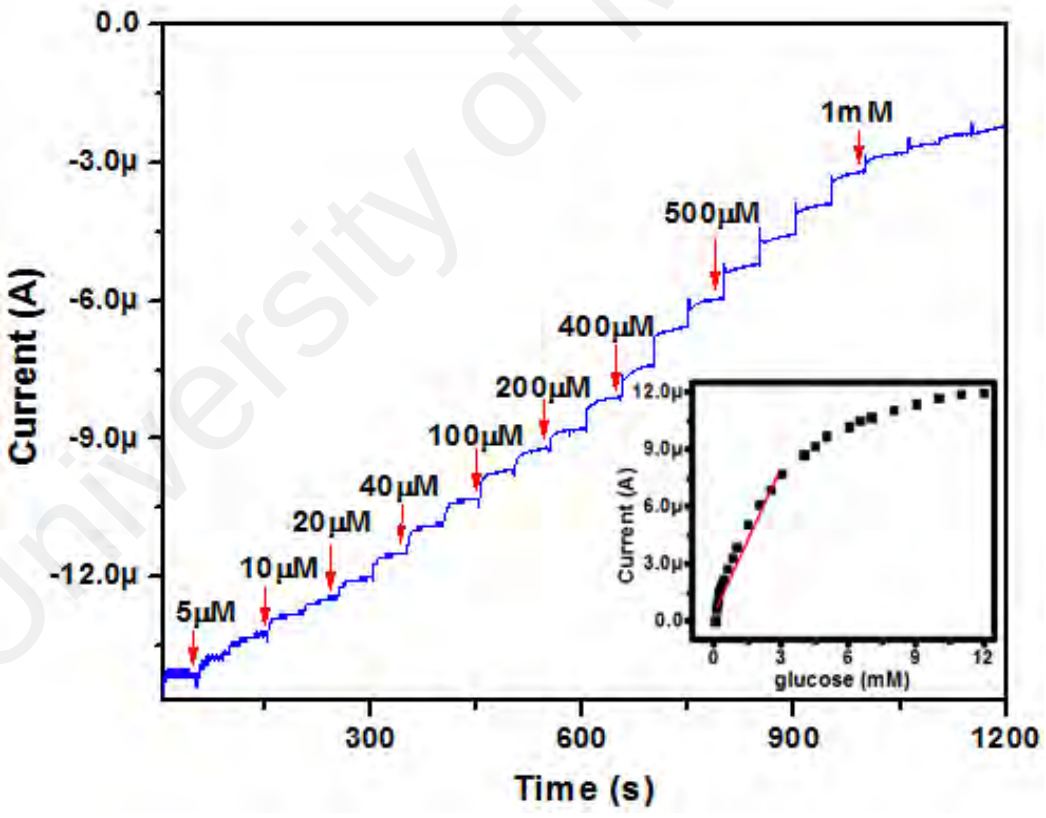

Figure 4.12: Chronoamperometric response of the GCE/3D graphene-GOx in response to successive injections of glucose into N_2 saturated 0.1 M PBS (pH 7.0) at the applied potential of -0.40 V.

Figure 4.13 displays the CNT/3DG/GOx/GCE Chronoamperometric response based on the current-time response for sequential additions of glucose at the -0.40 V potential and 0.1 M PBS (pH 7). This figure shows that the injection of glucose will increase the current and reached a steady state within 5 seconds. Increasing the concentration of injected glucose increased the amperometric currents simultaneously and the calibration curve corresponding to amperometric response is linear against the glucose concentration range from 0.005mM to 6mM. Considering the normal blood glucose level which is between 4 and 6mM, the biofuel cell would be supplied with enough and a suitable amount of glucose in the case of implants applications.

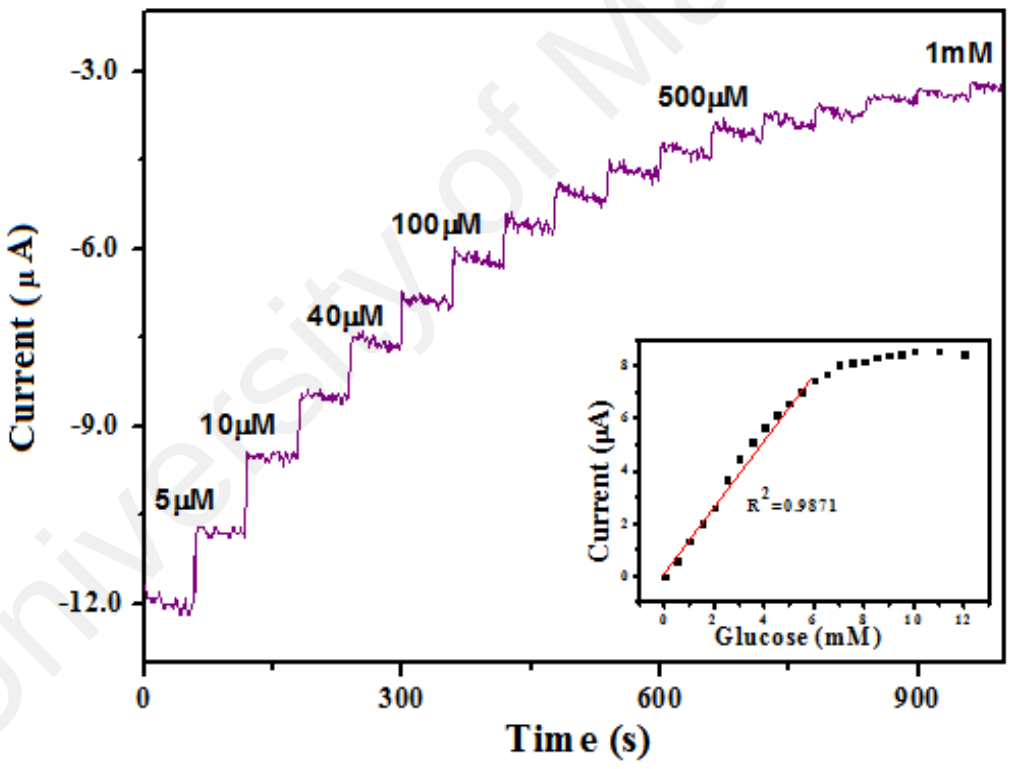

Figure 4.13: Chronoamperometric response of the CNT/3DG/GOx/GCE in response to successive injections of glucose into N_2 saturated 0.1 M PBS (pH 7.0) at the applied potential of -0.40 V.

4.2.3 Electrochemical impedance spectra

In order to investigate the effect of enzyme catalysts on charge transfer of EBC, charge transfer resistances (Rcts) of the EBCs electrochemical impedance spectra were studied. Figure 4.14 represents Nyquist plots (Z' vs. Z'') with the enzyme immobilized CNTs, the enzyme immobilized 3DG and enzyme immobilized CNTs/3DG. The Voigt R-C model circuit (inset of Figure 4.14) is chosen to fit the obtained impedance data.

According to the Nyquist plots, when enzyme catalysts were included, R_{ct} was highest when CNT/3DG/GOx was employed in EBC, while the value was lowest when CNT/GOx was employed. On the other hand, the bare GCE has the highest R_{ct} of the EBC. R_{ct} is proportional to the loading amount of enzyme molecules (GOx molecules) because the GOx molecule is assembled of proteins that have non-conductive property. It deserves to note that x-axis intercepts of the Nyquist plots are similar together. It demonstrates that solution resistances (R_s) of EBCs are not affected by catalyst types.

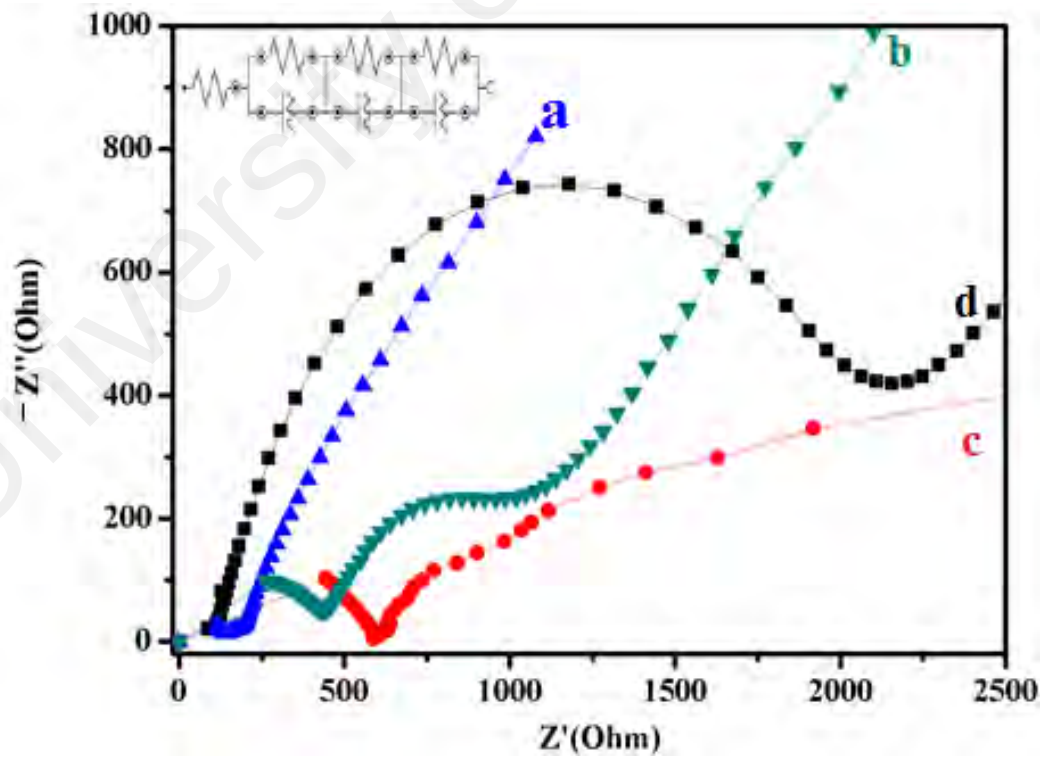

Figure 4.14: Nyquist plots of impedance spectra obtained in 0.1 M PBS (pH 7) (a) Bare GCE; (b) 3DG/GOx modified GCE; (c) CNT/GOx modified GCE and (d) CNT/3DG/GOx modified GCE

4.2.4 Polarization Curve

In order to evaluate the performance of fabricated bio-anode, the polarization curve is measured in this part. The OCP of 0.68 V observed which is much less than the reported OCPs for CNT aggregated electrodes employing DET. As it has been mentioned before, the fabricated GBFC polarized by connecting the fabricated electrode to a range of the resistors. Figure 4-15 represents the potential and power density of cell as the function of the current. It indicates that CNT/3DG/GOx modified GCE was able to supply electricity with the power density of approximately 253 $\mu W\ cm^{-2}$ at the potential of 0.3 V. This higher reported power density compared to the previous study indicates the critical role of CNT/3DG in enzyme immobilization and electron transfer which both improve the performance of the biofuel cell.

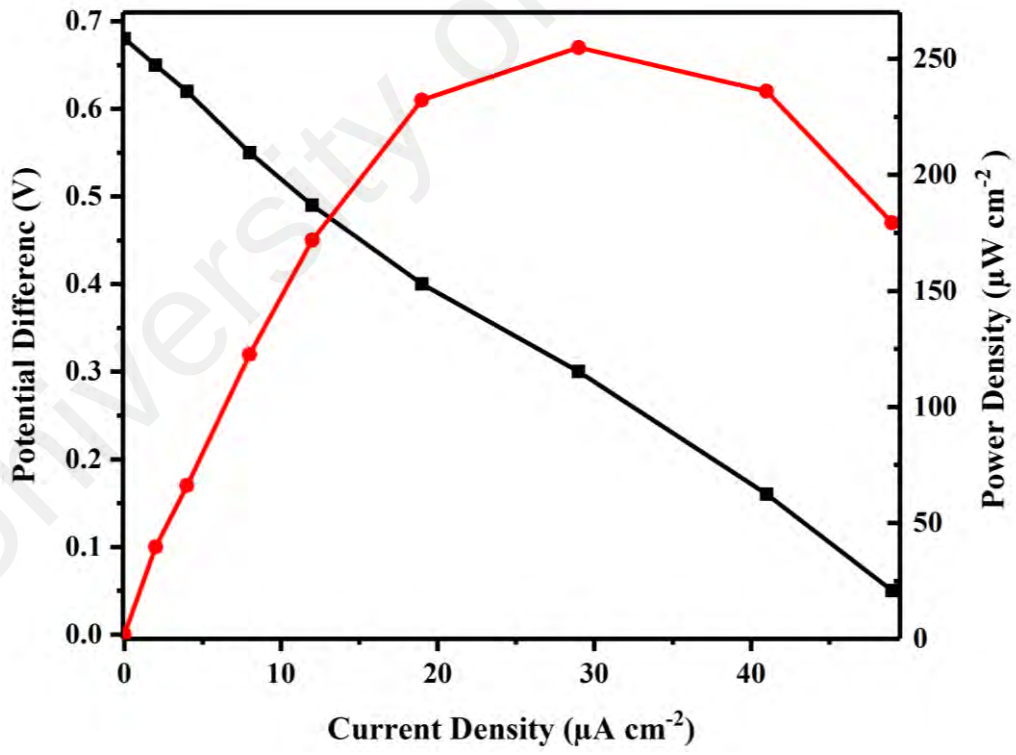

Figure 4.15: Polarization curve of the fabricated CNT/3DG/GOx modified GC electrode obtained in 0.1 M PBS (pH 7).

4.3 Discussion

Many researchers are focusing on the development of EBFCs using the immobilized enzyme on their bioelectrode(s). However, numerous problems are associated with the usage of enzyme-based EBFCs; Effective transport of electrons from the site of catalysis to the electrode surface, the durability of enzymes under operational pH ranges, biocompatibility of enzymes and electron mediators. The current study reports a significant solution to the aforesaid problems. In order to enhance the performance of the EBFCs, increase the lifetime of the enzymes, overcome the enzyme leaching challenge and finally improve the electron transfer between the enzyme active site and the electrode surface, novel CNT/3DG was considered. In this study, we synthesized the highly porous, conductive 3DG and utilized it to immobilize GOx and in order to wiring inside the enzyme active site, CNT nanowires employed. The biofuel cell operates at a power density of 164 $\mu W\ cm^{-2}$ at 0.44 V cell potential and pH 7. Our results show that the biofuel cell can also work on the O_2-containing solution which reveals that immobilized GOx is capable of maintaining its natural catalyst activity. Highly porous structure and excellent electrical conductivity of CNT/3DG can facilitate the DET between the active site of the GOx and modified GCE. All the above results demonstrate that constructed CNT/3DG/GOx/GCE can serve perfectly as a bioanode in the fabrication of the EBFCs. Furthermore, we believe that the synthesized CNT/3DG hybrid presented here is applicable to immobilize other enzymes both in the construction of EBFCs and the third generation biosensors. In conclusion, we have synthesized a novel CNT/3DG that can serve as an ideal biocatalyst support for the immobilization of GOx with efficient DET. This proposed membrane-less EBFC can be employed as a power source for biomedical devices in the laboratory. Finally, Table 5.1 presents a comparison between the fabricated EBFCs in this study and other studies.

Table 4.4: Comparison of different bioanode power density

Bio-Electrode Material	Anodic Enzyme	Cathodic Enzyme	Power Density (mW/cm^{-2})	Lifetime (Day)	Reference
Graphene	GDH	Laccase	22.50	33	(Karimi A., Othman A. *et al.*, 2015)
Graphene Nanosheets	GOx	BOD	24.3	14	(Liu C. *et al.*, 2010)
Graphene /Polypyrrole	GOx	Pt-cathode	78.3	56	(Liu C., Chen Z. F. *et al.*, 2011)
GO/Co(OH)2/chitosan	GOx	Laccase	517	-	(Uk Lee H., Young Yoo H. *et al.*, 2013)
3DGN	GOx	Laccase	112	-	(Zhang Y., Chu M. *et al.*, 2014)
SG/Frt	GOx	BOD	690	40	(Inamuddin, Haque S. u. *et al.*, 2016)
mesoporous materials	GOx	BOD	602	10	(Catalano P. N., Wolosiuk A. *et al.*, 2015)
Graphene /SWCNTs	GOx	BOD	190	50	(Campbell A. S., Jeong Y. J. *et al.*, 2015)
NG/AuNP	GDH	Laccase	1960	20	(Gai P., Ji Y. *et al.*, 2015)
3D-Graphene	GOx	Pt-cathode	164	230	This study
CNT/3DG	GOx	Pt-cathode	253.36	230	This study

CHAPTER 5: CONCLUSION AND FUTURE WORK

5.1 Conclusion

The aim of this study is to synthesize 3DG and CNT/3DG in order to immobilize the enzyme on the surface of the electrode which could be used during the EBFCs fabrication. 3-Dimensional graphene synthesized, combined with CNT and after characterization successfully employed in the fabrication of EBFC. The work explained here, with the successful power generation from glucose with the outstanding enzyme lifetime is a major step toward achieving this goal. In addition, the other advantage of fabricated EBFC is utilizing DET method to transfer the generated electron to the electrode surface and also a lack of need to the membrane. The characterization of synthesized 3DG and CNT/3DG show the high potential of these materials to utilize in the field of enzyme immobilization and electron transfer. Electrochemical characterization of fabricated electrode also shows a significant benefit of using 3DG in the fabrication process. The lifetime of the enzyme increased to more than 400 days while the power density stays in the acceptable range. 3DG holds a great promise for the development of implantable enzymatic biofuel cells. However, there is much more to do in order to develop this device in the case of mass industrial fabrication. Furthermore, suggested method and material in order to immobilize the enzyme on the surface of the electrode could be used in several other applications especially as highly sensitive long lasting biosensors. In addition, the fabricated EBFC have the potential to be implanted in the living organisms body and use metabolites such as blood sugar (which is also glucose) to generate sufficient power for other implantable devices. Collected results from the fabricated bioelectrode, point to some advantage of using a CNT/3DG hybrid to immobilize the GOx enzyme and increase the amount and stability of power generation.

5.2 Recommendations for future work

As stated in this study and in line with the literature, EBFCs is one of the best energy harvesting alternative devices. They have lots of potential due to their substrate (glucose or other biofuels) in the case of powering implantable devices. Their performance has been increased significantly using the carbon-based nanomaterials. Despite the potential to work in normal temperature and pH and increasing the lifetime of the immobilized enzyme, the main obstacles towards the commercialization of EBFCs are the low current density. It is highly suggested that future studies target toward the complete oxidation of biofuels (glucose) may be by using the group of enzymes. The ultimate goal in all these processes is to provide a device which can exhibit optimum power density to replace the existing batteries of the implantable devices.

REFERENCES

A. K. Shukla, P. Suresh, S. Berchmans, & Rajendran, A. (2004). Biological fuel cells and their applications. *CURRENT SCIENCE, 87*(4), 455-469.

Alwarappan, S., Liu, C., Kumar, A., & Li, C.-Z. (2010). Enzyme-doped graphene nanosheets for enhanced glucose biosensing. *The Journal of Physical Chemistry C, 114*(30), 12920-12924.

Arechederra, R., & Minteer, S. D. (2008). Organelle-based biofuel cells: immobilized mitochondria on carbon paper electrodes. *Electrochimica Acta, 53*(23), 6698-6703.

Babadi, A. A., Bagheri, S., & Hamid, S. Bee A. (2016). Progress on implantable biofuel cell: Nano-carbon functionalization for enzyme immobilization enhancement. *Biosensors and Bioelectronics, 79*, 850-860.

Baby, T. T., Aravind, S. S. J., Arockiadoss, T., Rakhi, R. B., & Ramaprabhu, S. (2010). Metal decorated graphene nanosheets as immobilization matrix for amperometric glucose biosensor. *Sensors and Actuators B: Chemical, 145*(1), 71-77.

Back matter. (1986). *Journal of the Chemical Society, Faraday Transactions 1: Physical Chemistry in Condensed Phases, 82*(5), P055-P066.

Bahartan, K., Amir, L., Israel, A., Lichtenstein, R. G., & Alfonta, L. (2012). In Situ fuel processing in a microbial fuel cell. *ChemSusChem, 5*(9), 1820-1825.

Baker, R., & Zhang, J. (2011). Proton exchange membrane or Polymer Electrolyte Membrane (Pem) fuel cells. *Electrochemistry Encyclopedia*.

Bandodkar, A. J., & Wang, J. (2016). Wearable Biofuel Cells: A Review. *Electroanalysis, 28*(6), 1188-1200.

Bard, A. J., Stratmann, M., & Calvo, E. J. (2003). *Encyclopedia of Electrochemistry, Interfacial Kinetics and Mass Transport; Volume 2*: Wiley-VCh.

Barton, S. C., Gallaway, J., & Atanassov, P. (2004). Enzymatic biofuel cells for implantable and microscale devices. *Chem Rev, 104*(10), 4867-4886.

Barton, S. C., Gallaway, J., & Atanassov, P. (2004). Enzymatic biofuel cells for implantable and microscale devices. *Chemical Reviews, 104*, 4867-4886.

Bharathi, S., & Nogami, M. (2001). A glucose biosensor based on electrodeposited biocomposites of gold nanoparticles and glucose oxidase enzyme. *Analyst, 126*(11), 1919-1922.

Bhatia, R. B., Brinker, C. J., Gupta, A. K., & Singh, A. K. (2000). Aqueous sol− gel process for protein encapsulation. *Chemistry of Materials, 12*(8), 2434-2441.

Bhatnagar, D., Xu, S., Fischer, C., Arechederra, R. L., & Minteer, S. D. (2011). Mitochondrial biofuel cells: expanding fuel diversity to amino acids. *Physical Chemistry Chemical Physics, 13*(1), 86-92.

Blin, J. L., Gérardin, C., Carteret, C., Rodehüser, L., Selve, C., & Stébé, M. J. (2005). Direct One-Step Immobilization of Glucose Oxidase in Well-Ordered Mesostructured Silica Using a Nonionic Fluorinated Surfactant. *Chemistry of Materials, 17*(6), 1479-1486.

Bullen, R. A., Arnot, T., Lakeman, J., & Walsh, F. (2006). Biofuel cells and their development. *Biosensors and Bioelectronics, 21*(11), 2015-2045.

Campbell, A. S., Jeong, Y. J., Geier, S. M., Koepsel, R. R., Russell, A. J., & Islam, M. F. (2015). Membrane/Mediator-Free Rechargeable Enzymatic Biofuel Cell Utilizing Graphene/Single-Wall Carbon Nanotube Cogel Electrodes. *Acs Applied Materials & Interfaces, 7*(7), 4056-4065.

Cannon, R. D. (2016). *Electron transfer reactions*: Butterworth-Heinemann.

Cao, H., Zhu, Y., Tang, L., Yang, X., & Li, C. (2008). A Glucose Biosensor Based on Immobilization of Glucose Oxidase into 3D Macroporous TiO2. *Electroanalysis, 20*(20), 2223-2228.

Catalano, P. N., Wolosiuk, A., Soler-Illia, G. J. A. A., & Bellino, M. G. (2015). Wired enzymes in mesoporous materials: A benchmark for fabricating biofuel cells. *Bioelectrochemistry, 106, Part A*, 14-21.

Chandan, A., Hattenberger, M., El-Kharouf, A., Du, S., Dhir, A., Self, V., . . . Bujalski, W. (2013). High temperature (HT) polymer electrolyte membrane fuel cells (PEMFC)–A review. *Journal of Power Sources, 231*, 264-278.

Chaubey, A., & Malhotra, B. D. (2002). Mediated biosensors. *Biosensors and Bioelectronics, 17*(6–7), 441-456.

Chen, Q., Kenausis, G. L., & Heller, A. (1998). Stability of oxidases immobilized in silica gels. *Journal of the American Chemical Society, 120*(19), 4582-4585.

Chen, T., Barton, S. C., Binyamin, G., Gao, Z., Zhang, Y., Kim, H.-H., & Heller, A. (2001). A Miniature Biofuel Cell. *Journal of the American Chemical Society, 123*(35), 8630-8631.

Damiano, L., Jambeck, J. R., & Ringelberg, D. B. (2014). Municipal solid waste landfill leachate treatment and electricity production using microbial fuel cells. *Applied Biochemistry and Biotechnology, 173*(2), 472-485.

Degani, Y., & Heller, A. (1987). Direct electrical communication between chemically modified enzymes and metal electrodes. 1. Electron transfer from glucose oxidase to metal electrodes via electron relays, bound covalently to the enzyme. *J. Phys. Chem.;(United States), 91*(6).

Deng, L., & Dong, S. (2015a). The Self-Powered Biosensors Based on Biofuel Cells. *Electrochemical Biosensors*, 371.

Deng, L., & Dong, S. (2015b). The Self-Powered Biosensors Based on Biofuel Cells *Electrochemical Biosensors* (pp. 371-387): Pan Stanford.

Devadas, B., Mani, V., & Chen, S.-M. (2012). A Glucose/O-2 Biofuel Cell Based on Graphene and Multiwalled Carbon Nanotube Composite Modified Electrode. *International Journal of Electrochemical Science, 7*(9), 8064-8075.

Dilgin, D. G., & Gökçel, H. İ. (2015). Photoelectrochemical glucose biosensor in flow injection analysis system based on glucose dehydrogenase immobilized on poly-hematoxylin modified glassy carbon electrode. *Analytical Methods, 7*(3), 990-999.

Du Toit, H., & Di Lorenzo, M. (2014). Glucose oxidase directly immobilized onto highly porous gold electrodes for sensing and fuel cell applications. *Electrochimica Acta, 138*, 86-92.

Du, Z., Li, H., & Gu, T. (2007). A state of the art review on microbial fuel cells: a promising technology for wastewater treatment and bioenergy. *Biotechnology Advances, 25*(5), 464-482.

Ekiz, F., Yuksel, M., Balan, A., Timur, S., & Toppare, L. (2010). Electrochemical Polymerization of (2-Dodecyl-4, 7-di (thiophen-2-yl)-2H-benzo[d][1,2,3] triazole): A Novel Matrix for Biomolecule Immobilization. *Macromolecular Bioscience, 10*(12), 1557-1565.

Fang, B., Zhang, C., Wang, G., Wang, M., & Ji, Y. (2011). A glucose oxidase immobilization platform for glucose biosensor using ZnO hollow nanospheres. *Sensors and Actuators B: Chemical, 155*(1), 304-310.

Fernandes, A. J. T. (2011). Insight into the multicopper oxidases stability.

Gai, P., Ji, Y., Chen, Y., Zhu, C., Zhang, J., & Zhu, J.-J. (2015). A nitrogen-doped graphene/gold nanoparticle/formate dehydrogenase bioanode for high power output membrane-less formic acid/O2 biofuel cells. *Analyst, 140*(6), 1822-1826.

Gao, F., Yan, Y., Su, L., Wang, L., & Mao, L. (2007). An enzymatic glucose/O-2 biofuel cell: Preparation, characterization and performance in serum. *Electrochemistry Communications, 9*(5), 989-996.

Ge, J., Yang, C., Zhu, J., Lu, D., & Liu, Z. (2012). Nanobiocatalysis in Organic Media: Opportunities for Enzymes in Nanostructures. *Topics in Catalysis, 55*(16-18), 1070-1080.

Gokhale, A. A., Lu, J., & Lee, I. (2013). Immobilization of cellulase on magnetoresponsive graphene nano-supports. *Journal of Molecular Catalysis B: Enzymatic, 90*(0), 76-86.

Guarnera, S., Abate, A., Zhang, W., Foster, J. M., Richardson, G., Petrozza, A., & Snaith, H. J. (2015). Improving the long-term stability of perovskite solar cells with a porous Al2O3 buffer layer. *The journal of physical chemistry letters, 6*(3), 432-437.

Guiseppi-Elie, A., Lei, C., & Baughman, R. H. (2002). Direct electron transfer of glucose oxidase on carbon nanotubes. *Nanotechnology, 13*(5), 559.

Guo, H.-L., Wang, X.-F., Qian, Q.-Y., Wang, F.-B., & Xia, X.-H. (2009). A green approach to the synthesis of graphene nanosheets. *Acs Nano, 3*(9), 2653-2659.

Habermann, W., & Pommer, E. (1991). Biological fuel cells with sulphide storage capacity. *Appl Microbiol Biotechnol, 35*(1), 128-133.

Harnisch, F., & Schröder, U. (2009). Selectivity versus mobility: separation of anode and cathode in microbial bioelectrochemical systems. *Chemsuschem, 2*(10), 921-926.

Holzinger, M., Le Goff, A., & Cosnier, S. (2012). Carbon nanotube/enzyme biofuel cells. *Electrochimica Acta, 82*, 179-190.

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 (Online), 1*(1), 18-27.

Hubenova, Y., & Mitov, M. (2015). Mitochondrial origin of extracelullar transferred electrons in yeast-based biofuel cells. *Bioelectrochemistry, 106*, 232-239.

Hult, K., & Berglund, P. (2007). Enzyme promiscuity: mechanism and applications. *Trends in Biotechnology, 25*(5), 231-238.

Hummers Jr, W. S., & Offeman, R. E. (1958). Preparation of graphitic oxide. *Journal of the American Chemical Society, 80*(6), 1339-1339.

Inamuddin, Haque, S. u., & Naushad, M. (2016). Electrochemical studies of biocatalytic anode of sulfonated graphene/ferritin/glucose oxidase layer-by-layer biocomposite films for mediated electron transfer. *Enzyme and Microbial Technology, 87–88*, 29-36.

Ivanov, I., Vidakovic-Koch, T., & Sundmacher, K. (2010). Recent Advances in Enzymatic Fuel Cells: Experiments and Modeling. *Energies, 3*(4), 803-846.

Ivnitski, D., Branch, B., Atanassov, P., & Apblett, C. (2006). Glucose oxidase anode for biofuel cell based on direct electron transfer. *Electrochemistry Communications, 8*(8), 1204-1210.

Jia, W.-Z., Wang, K., Zhu, Z.-J., Song, H.-T., & Xia, X.-H. (2007). One-step immobilization of glucose oxidase in a silica matrix on a Pt electrode by an electrochemically induced sol-gel process. *Langmuir, 23*(23), 11896-11900.

Jiang, B., Yang, K., Zhao, Q., Wu, Q., Liang, Z., Zhang, L., . . . Zhang, Y. (2012). Hydrophilic immobilized trypsin reactor with magnetic graphene oxide as support for high efficient proteome digestion. *Journal of Chromatography A, 1254*(0), 8-13.

Jin, L., Yang, K., Yao, K., Zhang, S., Tao, H., Lee, S.-T., . . . Peng, R. (2012). Functionalized Graphene Oxide in Enzyme Engineering: A Selective Modulator for Enzyme Activity and Thermostability. *Acs Nano, 6*(6), 4864-4875.

Joshi, R. K., Gomez, H., Alvi, F., & Kumar, A. (2010). Graphene Films and Ribbons for Sensing of O2, and 100 ppm of CO and NO2 in Practical Conditions. *The Journal of Physical Chemistry C, 114*(14), 6610-6613.

Jusman, Y., Ng, S. C., & Abu Osman, N. A. (2014). Investigation of CPD and HMDS Sample Preparation Techniques for Cervical Cells in Developing Computer-

Aided Screening System Based on FE-SEM/EDX. *The Scientific World Journal, 2014*, 11.

Kane, R. S., & Stroock, A. D. (2007). Nanobiotechnology: protein-nanomaterial interactions. *Biotechnol Prog, 23*(2), 316-319.

Kang, X., Wang, J., Wu, H., Aksay, I. A., Liu, J., & Lin, Y. (2009). Glucose oxidase–graphene–chitosan modified electrode for direct electrochemistry and glucose sensing. *Biosensors and Bioelectronics, 25*(4), 901-905.

Karajić, A. (2015). *Development of electrode architectures for miniaturized biofuel cells*. Université de Bordeaux.

Karimi, A., Othman, A., Uzunoglu, A., Stanciu, L., & Andreescu, S. (2015). Graphene based enzymatic bioelectrodes and biofuel cells. *Nanoscale, 7*(16), 6909-6923.

Katz, E., & MacVittie, K. (2013). Implanted biofuel cells operating in vivo–methods, applications and perspectives–feature article. *Energy & Environmental Science, 6*(10), 2791-2803.

Kragl, U., Greiner, L., & Wandrey, C. (1999). Enzymes, immobilized, reactors. *Encyclopedia of Bioprocess Technology*.

Krikštolaitytė, V. (2014). *Design and characterisation of the electrodes of enzymatic biofuel cells*. Vilnius University.

Lee, W., Lee, J. U., Jung, B. M., Byun, J.-H., Yi, J.-W., Lee, S.-B., & Kim, B.-S. (2013). Simultaneous enhancement of mechanical, electrical and thermal properties of graphene oxide paper by embedding dopamine. *Carbon, 65*(0), 296-304.

Li, X., Zhang, L., Su, L., Ohsaka, T., & Mao, L. (2009). A Miniature Glucose/O-2 Biofuel Cell With a High Tolerance Against Ascorbic Acid. *Fuel Cells, 9*(1), 85-91.

Lim, J., Cirigliano, N., Wang, J., & Dunn, B. (2007). Direct electron transfer in nanostructured sol–gel electrodes containing bilirubin oxidase. *Physical Chemistry Chemical Physics, 9*(15), 1809-1814.

Lin, J., Trabold, T. A., Walluk, M. R., & Smith, D. F. (2013). Bio-fuel reformation for solid oxide fuel cell applications. Part 1: Fuel vaporization and reactant mixing. *International Journal of Hydrogen Energy, 38*(27), 12024-12034.

Lin, Y., Lu, F., Tu, Y., & Ren, Z. (2004). Glucose biosensors based on carbon nanotube nanoelectrode ensembles. *Nano Letters, 4*(2), 191-195.

Liu, C., Alwarappan, S., Chen, Z., Kong, X., & Li, C.-Z. (2010). Membraneless enzymatic biofuel cells based on graphene nanosheets. *Biosensors and Bioelectronics, 25*(7), 1829-1833.

Liu, C., Chen, Z. F., & Li, C. Z. (2011). Surface Engineering of Graphene-Enzyme Nanocomposites for Miniaturized Biofuel Cell. *Ieee Transactions on Nanotechnology, 10*(1), 59-62.

Liu, Y., Wang, M., Zhao, F., Xu, Z., & Dong, S. (2005). The direct electron transfer of glucose oxidase and glucose biosensor based on carbon nanotubes/chitosan matrix. *Biosensors and Bioelectronics, 21*(6), 984-988.

Logan, B. E., & Elimelech, M. (2012). Membrane-based processes for sustainable power generation using water. *nature, 488*(7411), 313-319.

Logan, B. E., & Regan, J. M. (2006). MICROBIAL FUEL CE. *Environmental Science & Technology*.

Mano, N., Mao, F., & Heller, A. (2003). Characteristics of a Miniature Compartment-less Glucose−O2 Biofuel Cell and Its Operation in a Living Plant. *Journal of the American Chemical Society, 125*(21), 6588-6594.

Mattiasson, B., & Kaul, R. (1991). Determination of coupling yields and handling of labile proteins in immobilization technology. *Bioprocess technology, 14*, 161.

Meredith, M. T., & Minteer, S. D. (2012). Biofuel Cells: Enhanced Enzymatic Bioelectrocatalysis. *Annual Review of Analytical Chemistry, Vol 5, 5*, 157-179.

Misson, M., Zhang, H., & Jin, B. (2015). Nanobiocatalyst advancements and bioprocessing applications. *Journal of the Royal Society Interface, 12*(102), 20140891.

Moehlenbrock, M. J., & Minteer, S. D. (2008). Extended lifetime biofuel cells. *Chemical Society Reviews, 37*(6), 1188-1196.

Mohan, S. V., Velvizhi, G., Modestra, J. A., & Srikanth, S. (2014). Microbial fuel cell: critical factors regulating bio-catalyzed electrochemical process and recent advancements. *Renewable and Sustainable Energy Reviews, 40*, 779-797.

Mu, S., & Xue, H. (1996). Bioelectrochemical characteristics of glucose oxidase immobilized in a polyaniline film. *Sensors and Actuators B: Chemical, 31*(3), 155-160.

Najafpour, G. (2015). *Biochemical engineering and biotechnology*: Elsevier.

O'driscoll, K. (1976). [12] Techniques of enzyme entrapment in gels. *Methods in enzymology, 44*, 169-183.

Ó Conghaile, P., Falk, M., MacAodha, D., Yakovleva, M. E., Gonaus, C., Peterbauer, C. K., . . . Leech, D. n. (2016). Fully Enzymatic Membraneless Glucose| Oxygen Fuel Cell That Provides 0.275 mA cm–2 in 5 mM Glucose, Operates in Human Physiological Solutions, and Powers Transmission of Sensing Data. *Analytical Chemistry, 88*(4), 2156-2163.

Osman, M., Shah, A., & Walsh, F. (2011). Recent progress and continuing challenges in bio-fuel cells. Part I: Enzymatic cells. *Biosensors and Bioelectronics, 26*(7), 3087-3102.

Palmore, G. T. R., & Whitesides, G. M. (1994). Microbial and enzymatic biofuel cells: ACS Publications.

Pavlidis, I., Vorhaben, T., Gournis, D., Papadopoulos, G., Bornscheuer, U., & Stamatis, H. (2012). Regulation of catalytic behaviour of hydrolases through interactions with functionalized carbon-based nanomaterials. *Journal of Nanoparticle Research, 14*(5), 1-10.

Pavlidis, I. V., Vorhaben, T., Tsoufis, T., Rudolf, P., Bornscheuer, U. T., Gournis, D., & Stamatis, H. (2012). Development of effective nanobiocatalytic systems through the immobilization of hydrolases on functionalized carbon-based nanomaterials. *Bioresource Technology, 115*(0), 164-171.

Periasamy, A. P., Chang, Y.-J., & Chen, S.-M. (2011). Amperometric glucose sensor based on glucose oxidase immobilized on gelatin-multiwalled carbon nanotube modified glassy carbon electrode. *Bioelectrochemistry, 80*(2), 114-120.

Prasad, K. P., Chen, Y., & Chen, P. (2014). Three-dimensional graphene-carbon nanotube hybrid for high-performance enzymatic biofuel cells. *Acs Applied Materials & Interfaces, 6*(5), 3387-3393.

Putzbach, W., & Ronkainen, N. J. (2013). Immobilization Techniques in the Fabrication of Nanomaterial-Based Electrochemical Biosensors: A Review. *Sensors (Basel, Switzerland), 13*(4), 4811-4840.

Rabaey, K., & Verstraete, W. (2005). Microbial fuel cells: novel biotechnology for energy generation. *Trends in Biotechnology, 23*(6), 291-298.

Ramanavicius, A., & Ramanaviciene, A. (2009). Hemoproteins in Design of Biofuel Cells. *Fuel Cells, 9*(1), 25-36.

Sahney, R., Anand, S., Puri, B., & Srivastava, A. (2006). A comparative study of immobilization techniques for urease on glass-pH-electrode and its application in urea detection in blood serum. *Analytica Chimica Acta, 578*(2), 156-161.

Shan, C., Yang, H., Song, J., Han, D., Ivaska, A., & Niu, L. (2009). Direct Electrochemistry of Glucose Oxidase and Biosensing for Glucose Based on Graphene. *Analytical Chemistry, 81*(6), 2378-2382.

Shao, Q., Wu, P., Xu, X., Zhang, H., & Cai, C. (2012). Insight into the effects of graphene oxide sheets on the conformation and activity of glucose oxidase: towards developing a nanomaterial-based protein conformation assay. *Physical Chemistry Chemical Physics, 14*(25), 9076-9085.

Sheldon, R. A. (2007). Enzyme immobilization: the quest for optimum performance. *Advanced Synthesis & Catalysis, 349*(8-9), 1289-1307.

Suberu, M. Y., Mustafa, M. W., & Bashir, N. (2014). Energy storage systems for renewable energy power sector integration and mitigation of intermittency. *Renewable and Sustainable Energy Reviews, 35*, 499-514.

Sun, J., Hu, Y., Bi, Z., & Cao, Y. (2009). Improved performance of air-cathode single-chamber microbial fuel cell for wastewater treatment using microfiltration membranes and multiple sludge inoculation. *Journal of Power Sources, 187*(2), 471-479.

Sur, U. K. (2012). Graphene: A Rising Star on the Horizon of Materials Science. *International Journal of Electrochemistry*.

Tasca, F., Farias, D., Castro, C., Acuna-Rougier, C., & Antiochia, R. (2015). Bilirubin Oxidase from Myrothecium verrucaria Physically Absorbed on Graphite Electrodes. Insights into the Alternative Resting Form and the Sources of Activity Loss. *Plos One, 10*(7), e0132181.

Topcagic, S., & Minteer, S. D. (2006). Development of a membraneless ethanol/oxygen biofuel cell. *Electrochimica Acta, 51*(11), 2168-2172.

Tsai, T.-W., Heckert, G., Neves, L. s. F., Tan, Y., Kao, D.-Y., Harrison, R. G., . . . Schmidtke, D. W. (2009). Adsorption of glucose oxidase onto single-walled carbon nanotubes and its application in layer-by-layer biosensors. *Analytical Chemistry, 81*(19), 7917-7925.

Uk Lee, H., Young Yoo, H., Lkhagvasuren, T., Seok Song, Y., Park, C., Kim, J., & Wook Kim, S. (2013). Enzymatic fuel cells based on electrodeposited graphite oxide/cobalt hydroxide/chitosan composite–enzymeelectrode. *Biosensors and Bioelectronics, 42*, 342-348.

Unnikrishnan, B., Palanisamy, S., & Chen, S.-M. (2013). A simple electrochemical approach to fabricate a glucose biosensor based on graphene–glucose oxidase biocomposite. *Biosensors and Bioelectronics, 39*(1), 70-75.

Wang, Z. L., & Wu, W. (2012). Nanotechnology-enabled energy harvesting for self-powered micro-/nanosystems. *Angewandte Chemie International Edition, 51*(47), 11700-11721.

Xian, T., Yang, H., Di, L., Ma, J., Zhang, H., & Dai, J. (2014). Photocatalytic reduction synthesis of SrTiO3-graphene nanocomposites and their enhanced photocatalytic activity. *Nanoscale Research Letters, 9*(1), 1-9.

Xie, T., Lv, W., Wei, W., Li, Z., Li, B., Kang, F., & Yang, Q. H. (2013). A unique carbon with a high specific surface area produced by the carbonization of agar in the presence of graphene. *Chem Commun (Camb), 49*(88), 10427-10429.

Yang, X., Hua, L., Gong, H., & Tan, S. N. (2003). Covalent immobilization of an enzyme (glucose oxidase) onto a carbon sol–gel silicate composite surface as a biosensing platform. *Analytica Chimica Acta, 478*(1), 67-75.

Zebda, A., Cosnier, S., Alcaraz, J. P., Holzinger, M., Le Goff, A., Gondran, C., . . . Cinquin, P. (2013). Single Glucose Biofuel Cells Implanted in Rats Power Electronic Devices. *Sci. Rep., 3*.

Zebda, A., Renaud, L., Cretin, M., Innocent, C., Ferrigno, R., & Tingry, S. (2010). Membraneless microchannel glucose biofuel cell with improved electrical performances. *Sensors and Actuators B: Chemical, 149*(1), 44-50.

Zeng, K., & Zhang, D. (2010). Recent progress in alkaline water electrolysis for hydrogen production and applications. *Progress in Energy and Combustion Science, 36*(3), 307-326.

Zhang, S., Wang, N., Niu, Y., & Sun, C. (2005). Immobilization of glucose oxidase on gold nanoparticles modified Au electrode for the construction of biosensor. *Sensors and Actuators B: Chemical, 109*(2), 367-374.

Zhang, Y., Chu, M., Yang, L., Tan, Y., Deng, W., Ma, M., . . . Xie, Q. (2014). Three-Dimensional Graphene Networks as a New Substrate for Immobilization of Laccase and Dopamine and Its Application in Glucose/O2 Biofuel Cell. *ACS Appl Mater Interfaces, 6*(15), 12808-12814.

Zhang, Y., Zhang, J., Huang, X., Zhou, X., Wu, H., & Guo, S. (2012). Assembly of Graphene Oxide–Enzyme Conjugates through Hydrophobic Interaction. *Small, 8*(1), 154-159.

Zhao, C.-e., Gai, P., Song, R., Chen, Y., Zhang, J., & Zhu, J.-J. (2017). Nanostructured material-based biofuel cells: recent advances and future prospects. *Chemical Society Reviews*.

Zheng, W., Zhao, H. Y., Zhang, J. X., Zhou, H. M., Xu, X. X., Zheng, Y. F., . . . Jang, B. Z. (2010). A glucose/O2 biofuel cell base on nanographene platelet-modified electrodes. *Electrochemistry Communications, 12*(7), 869-871.

Zhou, K., Zhu, Y., Yang, X., & Li, C. (2010). Electrocatalytic Oxidation of Glucose by the Glucose Oxidase Immobilized in Graphene-Au-Nafion Biocomposite. *Electroanalysis, 22*(3), 259-264.

Zhou, M., & Dong, S. (2011). Bioelectrochemical interface engineering: toward the fabrication of electrochemical biosensors, biofuel cells, and self-powered logic biosensors. *Accounts of Chemical Research, 44*(11), 1232-1243.

Zhou, M., Kuralay, F., Windmiller, J. R., & Wang, J. (2012). DNAzyme logic-controlled biofuel cells for self-powered biosensors. *Chem Commun (Camb), 48*(32), 3815-3817.

Zhou, M., Zhou, N., Kuralay, F., Windmiller, J. R., Parkhomovsky, S., Valdes-Ramirez, G., . . . Wang, J. (2012). A self-powered "sense-act-treat" system that is based on a biofuel cell and controlled by boolean logic. *Angew Chem Int Ed Engl, 51*(11), 2686-2689.

Zhu, B., Raza, R., Qin, H., Liu, Q., & Fan, L. (2011). Fuel cells based on electrolyte and non-electrolyte separators. *Energy & Environmental Science, 4*(8), 2986-2992.

List of The Publication and Papers Presented

Publication

Babadi, A. A., Bagheri, S., & Hamid, S. B. A. (2016). Progress on antimicrobial surgical gloves: A review. *Rubber Chemistry and Technology, 89*(1), 117-125.

Babadi, A. A., Bagheri, S., & Hamid, S. B. A. (2016). Progress on implantable biofuel cell: Nano-carbon functionalization for enzyme immobilization enhancement. *Biosens Bioelectron, 79*, 850-860.

Gheibi, A., Khoshnevisan, K., Ketabchi, N., Derakhshan, M. A., & Babadi, A. A. (2016). Application of Electrospun Nanofibrous PHBV Scaffold in Neural Graft and Regeneration: A Mini-Review. *Nanomedicine Research Journal, 1*(2), 107-111.

Mansouri, N., Babadi, A. A., Bagheri, S., & Hamid, S. B. A. (2017). Immobilization of glucose oxidase on 3D graphene thin film: Novel glucose bioanalytical sensing platform. *International Journal of Hydrogen Energy, 42*(2), 1337-1343.

Conference and seminar presented

Babadi, A. A., Bagheri, S., & Hamid, S. B. A. (2016). Performance Enhancement of Enzymatic Biofuel Cell. *International Seminar on Nanoscience and Nanotechnology* 2016, 3-4