Noble metal and enzymatic catalysts for plating and biofuel cell

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NANYANG
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NOBLE METAL AND ENZYMATIC CATALYSTS
FOR PLATING AND BIOFUEL CELL

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SCHOOL OF MECHANICAL AND AEROSPACE
ENGINEERING

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**NOBLE METAL AND ENZYMATIC CATALYSTS
FOR PLATING AND BIOFUEL CELL**

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School Of Mechanical and Aerospace Engineering

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Abstract

This project reports a facile and highly active Pd-P electrocatalyst that is capable of substituting the conventional Pd-Sn electrocatalyst employed in the current electroless nickel deposition process. For hypophosphite oxidation in nickel bath solution, the resulting Pd-P nanoparticles exhibited comparable specific activity and higher mass activity, compared to conventional Pd-Sn electrocatalyst.

The project also presents a novel centrifugation compression method developed and demonstrated to be effective in the immobilization of enzyme and redox mediator within the carbon matrix for glucose oxidation and oxygen reduction reaction. The enhancement between interactions of enzyme, redox mediator and carbon matrix was proved through the increased in catalytic activity of the bioelectrodes. The centrifuge compression method was further modified to create stacked immobilization structure for enzyme. Direct electron transfer was established with the multiple-stacking for carbon material and enzyme. The catalytic activity of the stacking biocathodes exhibited higher catalytic activity than the mixing biocathode. The fabricated bioelectrodes were able to display substantial power density in environment similar to physiological condition; low glucose content, low oxygen content and close to neutral pH condition and also when implanted in grape. This signifies the potential of these bioelectrodes to function in other *in vivo* system.

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List of Abbreviations

Abbreviation	Explanation
EBFC	Enzymatic Biofuel Cell
GBFC	Glucose Biofuel Cell
ET	Electron Transfer
ELD Ni	Electroless Nickel Deposition
MET	Mediated Electron Transfer
DET	Direct Electron Transfer
SAMs	Self-Assembled Monolayers
NAD	Nicotine Adenine Dinucleotide
PQQ	Pyrroloquinoline Quinone
FAD	Flavin Adenine Dinucleotide
GOx	Glucose Oxidase
CNTs	Carbon Nanotubes
SDS	Sodium Dodecylsulfate
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
OCP	Open-circuit Potential
OCV	Open-circuit Voltage
GDH	Glucose Dehydrogenase
ADH	Alcohol Dehydrogenase
AlDH	Aldehyde Dehydrogenase
FDH	Formate Dehydrogenase
ORR	Oxygen Reduction Reaction
MCOs	Multi-Copper Oxidases
LAC	Laccase
BOD	Bilirubin Oxidase
TNCs	Tri-nuclear Coppers
SPGE	Spectrographic Graphite Electrodes

RDE-GC	Rotating Disk Electrode - Glassy Carbon
CV	Cyclic Voltammetry
CA	Chronoamperometry
ECSA	Electrochemically Active Surface Area
ICP-MS	Inductively Coupled Plasma-Mass Spectroscopy
TEM	Transmission Electron Microscopy
FESEM	Field Emission Scanning Electron Microscopy
EDS	Energy-Dispersive X-ray Spectroscopy
UV-vis	Ultraviolet – Visible light
COOH-MWCNTs	Carboxylic Multi-wall Carbon Nanotubes

Chapter 1: Introduction

1.1 Background and Motivation

1.1.1 Electroless Ni Deposition

Electroless plating is an autocatalytic process that involves the chemical reduction of metallic ions by the reducing agent present in the solution. The reduction process of the metallic ions can only occur on catalytic surfaces rather than throughout the solution, thus a catalysed substrate must be present. In 1947, Brenner and Riddell reported the method of depositing nickel on steel or nickel surface without supplying electric current [1]. The reducing agents including sodium hypophosphite, hydrazine, borohydrides, amines boranes and some of their derivatives can be used in the bath for chemical reactions.

Electroless nickel deposition (ELD Ni) has several advantages that accounts for its wide application in current industry. ELD Ni can form uniform deposition on the objects even if their shapes are irregular or having isolated surfaces (solution must have access of these surfaces). The nickel coating by electroless deposition create less porosity compared to that electroplating, hence having better corrosion resistance. ELD Ni is also used on non-conductive substrates to construct a conductive layer on the surface. On the other hand, electroplating is not applicable in this process as electric current cannot pass through the non-conductive surfaces. Lastly, the ELD Ni process can be carried out in bulk quantity, which is crucial for scale-up production in industrial mass production [2].

ELD Ni involves several steps: (i) cleaning of the substrate surface, (ii) surface modification of the substrate to allow catalyse species to be deposited (e.g. etching of surface), (iii) sensitization and catalysing of the substrate

surface and (iv) activation (acceleration treatment, not compulsory) as shown in Figure 1 [3].

Figure 1 Schematic representation of the electroless deposition process.

1.1.2 Fuel Cell

The growing demands for power and depleting fossil fuels have always been a global concern. Many renewable energy systems (e.g. wind, solar) have been explored as alternative to produce power for sustaining global consumption and fuel cell is one of the potential options. The attractiveness of fuel cell is in its high energy efficiency and low operating cost compared to other renewable energy systems [4, 5]. The principle of fuel cell is to convert chemical energy via redox reactions into electrical energy, the by-products are usually non-toxic, leading to lesser environmental impact, unlike burning of fossil fuels.

Fuel cell reaction can be broken down into two half-cell reactions: oxidizing of the fuel at the anode and reducing of oxidant in the cathode. The oxidation of the fuel will generate ions and electrons at the anode, the ions will transverse to the cathode through the electrolyte (testing solution) while the electrons will flow to the cathode via the wire through the connected load. The reduction reaction will take place at the cathode (with the ions and electrons) to form the by-products from the oxidant [4, 6]. The operation of the fuel cell is illustrated in Figure 2.

Figure 2 Typical diagram of a fuel cell system.

1.1.3 Enzymatic Biofuel Cell

Enzymatic biofuel cell (EBFC) is a sub-class of fuel cell where redox processes employ enzymes to catalyse the reaction. The advantages of enzymes are their ability to operate efficiently in mild conditions (20-40 °C, within pH range 5-8) and the high selectivity for reaction which eliminates the need to separate fuel and oxidant. This allows simpler cell design (without separating membrane) while having high catalytic ability in ambient conditions.

Glucose enzymatic biofuel cell (GBFC) is a subclass of EBFC that operates on consuming glucose and oxygen as fuel and oxidant (Figure 3). The enzymes capable of oxidizing glucose to form d-glucono-1,5-lactone and give electrons are glucose oxidase (GOx) and glucose dehydrogenase (GDH) at the bioanode. As for the biocathode, laccase and bilirubin oxidase (BOD) are widely known enzymes for oxygen reduction reaction to form water molecules by consuming the electrons.

Figure 3 Diagram of GBFC with enzymes. [7]

Implantable glucose biofuel cell was first introduced by Drake et al. in 1970 where they used noble metals as catalyst for glucose oxidation and oxygen reduction [8]. The best performing catalyst for operation was platinum for both reactions but platinum is not compatible as both glucose and oxygen are present in the environment. This means that platinum can catalyse both the glucose oxidation and oxygen reduction reaction at the same time. The first implanted biofuel cell in dog with platinum electrodes delivered low power of $2.2 \mu W cm^{-2}$ with open circuit voltage (OCV) of 0.5 V [8]. Another implantation was conducted in vein of sheep delivered power of $40 \mu W cm^{-2}$ but it quickly decline in an hour [9]. The abiotic (e.g. platinum, palladium) catalysts exhibited

low catalytic activity in neutral pH and its non-selective nature for reactions render them inadequate for operation in physiological condition despite having better stability compared to enzymes. Implantable GBFC with enzyme catalysts were explored for achieving higher power output. Crespilho's group demonstrated the implantation of biofuel cell using glucose oxidase (bioanode) and platinum nanoparticles (biocathode) into the vein of a rat [10]. The implanted GBFC achieved $95 \mu\text{W cm}^{-2}$ and OCV is 0.125 V. They also observed that implantable devices can cause inflammatory reaction when implanted, thus affecting the enzymes immobilized and reducing the lifetime of the device. Later, Cinquin's group demonstrated implantation of GBFC (glucose oxidase and laccase as enzyme catalysts) into the abdominal cavity of rat [11]. To enhance the biocompatibility of the GBFC, the bioelectrodes were wrapped in dialysis membranes before stitching them into a Dacron® sleeve for implantation. The implanted GBFC exhibited $38.7 \mu\text{W}$ ($193.5 \mu\text{W cm}^{-2}$) with OCV of 0.57 V. The use of porous dialysis membranes inside the Dacron® bag prevents the external leakage of CNTs to the environment and also prevents the biological tissues to the GBFC. The *in vivo* GBFC was also successfully used to power a light-emitting diode and a digital thermometer (separately).

1.1.4 Enzyme Catalyst

Despite the advantage of utilising enzymes as catalysts, there are also limitations. In general, the electron transfer (ET) rate depends on the interfacial ET rate (between enzyme and supporting material) and the turnover number of the enzyme (maximum amount of reactants converted into products for a given concentration of enzyme per unit time) [12]. For efficient enzymatic catalysis, there must be fast interfacial ET between redox active site and the supporting

electrode. To achieve high ET rate, a notable guideline is to have redox sites away from the electrode surface with distance ≤ 1.4 nm [12, 13]. Since the redox active sites of enzymes are usually hidden deep in their protein matrix, slow ET rate is common between the enzymes to the electrode. This results in low catalytic activity in the enzymatic bioelectrodes.

Another problem is that enzymes have low stability compared to abiotic catalysts. Enzyme stability signified the enzyme's capability to retain its catalytic activity for reactions. Temperature is an important environment factor for GBFC; higher temperature in general will increase the catalytic activity of enzymes but enzyme stability may be affected as enzyme can be denatured under high temperature [14]. The *in vivo* study for stability of enzymatic catalysts is vital for implantable biofuel cell as its application in the subject should be long term. Martin's group demonstrated implanting biocathodes (adding chitosan/genipin to carbon nanotubes/laccase electrode) into rats for oxygen reduction [15]. The biocathode was covered with thin film of chitosan/genipin (as biocompatible interface) before implantation. The designed biocathode showed minimised inflammation to the implanted site and the biocathode was still operational (*ex vivo* measurement) after 6 months of implantation. The chitosan film has successfully formed a separation barrier for the biocathode.

1.1.5 Material for Electrode

Effective immobilization of enzymes is important for achieving high catalytic activity in enzymatic bioelectrodes. The enzymes are usually large molecules (20 – 850 kDalton) with diameter of 5 – several tens nano-meters [16]. The redox active sites of enzymes are hidden deep in these protein

matrixes, usually more than 1.4 nm, this strongly hinder ET between the redox active sites to electrode surface. Thus, materials with high surface area, high electrical conductivity and thin diameter are preferred for the immobilization of enzymes. Carbon nanomaterials (e.g. carbon nanotubes, graphene) are suitable materials for electrodes and had been widely used for immobilising enzymes [17-23]. Higher current outputs were observed with the used of carbon nanomaterials. Despite the use of carbon nanomaterials, EBFCs' electrodes still exhibited low enzyme loading on electrode surface, limited electrochemical active sites for reaction and poor ET rate from the enzymes' active sites to electrode surface [24, 25]. To resolve these issues, three-dimensional carbon materials were adopted. Xie's group three-dimensional graphene networks as substrate for immobilizing laccase and its redox mediator [26]. The 3D-laccase electrodes exhibited higher catalytic activity compared to 2D-laccase, indicating that the three-dimensional graphene networks are better substrate for laccase and its mediator immobilization.

Beside three-dimensional carbon materials, nickel foam can act as 3D-skeleton frame for deposition of carbon nanomaterials, enzymes and redox mediators. Nickel foam, with its porous structure, has low cost, high electrical conductivity and large electroactive surface area [27, 28]. These properties are advantageous towards higher enzyme loading and increasing electrochemically active sites. Dong's and Ma's group had successfully applied nickel foam as bioelectrodes for GBFCs and power output of $> 200 \mu\text{W cm}^{-2}$ were achieved [29, 30].

In consideration for designing electrodes to implantable biofuel cell, biocompatibility is an important factor as it will affect the stability of the

bioelectrodes. Carbon nanomaterials and nickel are toxic materials that are harmful to human body [31-34]. The main cause of toxicity of nickel and carbon nanomaterials is the accumulation of materials in the organs of organism (through oral, inhalation or blood flow), leading to failure in organs' function (e.g. lungs, liver, kidney and heart). Implanted biofuel cells of carbon nanotubes had been demonstrated in rats by concealing them in biocompatible materials like Dracon® bag [11] and chitosan film [15]. Thus, it is important to take into consideration the concealment measures for the bioelectrodes when using materials that may cause inflammation or harm to the body.

1.1.6 Motivation

Electroless nickel deposition: ELD Ni is widely applied in semiconductor industries and also other engineering which require coating process. In the fabrication of nickel foam as frame for bioelectrodes, ELD Ni can be applied to coat the nickel layer on the organic foam, before eliminating the organic foam to form the porous nickel foam [35, 36]. In conventional ELD Ni process, tin (II) chloride (SnCl_2) was used as the reducing agent to yield Pd nanoparticles on the surface. However, the stability of the SnCl_2 solution is poor due to chemical reactions which may occur such as hydrolysis or Sn (II) and Sn (IV) complex formation. This results in the need for tin recovery process and the renewing of reducing agent. The addition steps cause negative environmental and economic impact, which is undesirable to the industrial applications. Furthermore, the surface reduced by SnCl_2 will form tin oxides/complex layer that blocks the Pd catalysts. Supplementary step to perform acceleration treatment is required to remove the tin oxides/complex and reveal the Pd catalyst for hypophosphite oxidation. Therefore, it is necessary to investigate

alternative method in reducing Pd catalyst, so that ELD Ni process is more cost and material efficient.

Implantable Biofuel Cell: The application of implantable devices is widespread and growing in recent years. The primary goal for implantable biofuel cell is to supply power to operate other implantable devices (e.g. cardiac pacemakers, nerve stimulators, active drug administration and active biosensors). Implantable devices such as pacemakers, stimulators and sensors have peak power consumption around 100 – 200 μ W [37, 38], which is possible to achieve for biofuel cell. The current power source for implanted devices is lithium-ion battery which is capable of providing power for at least 5-10 years [39, 40], depending on the implanted devices. Surgical operation will be necessary to replace the drained battery of these implanted devices. There is high risk involved especially for patients with weak physical as their conditions cannot undergo these operations. Turning to implantable biofuel cell for powering the implanted devices, it is envisioned to have a longer operational lifetime than lithium-based batteries, thus reducing the possibility of conducting surgical operation since the implanted GBFC can deliver power provided that glucose and oxygen are replenished. In addition, implantable biofuel cell can also be used to power devices controlling locomotion of insects [41-46] and animals. Such controllers require higher power consumption and light-weighted power source so that the targeted organisms' mobility is not obstructed and the desired operation can be elongated. The long-term operation in implantable GBFC is a merit compared to batteries, thus there is a need to develop highly stable enzymatic bioelectrodes.

1.2 Aims and Objectives

Electroless Nickel Deposition: Alternative procedures are needed to deposit Pd nanoparticles which may be more stable and simple (without any acceleration treatment) while having as high catalytic activity as conventional Pd catalyst reduced by Sn (II) in alkaline bath.

- To substitute the method of reducing Pd catalyst by tin (II) chloride while maintaining high catalytic activity for hypophosphite oxidation;
- To reduce the cost and chemical needed for ELD Ni process.

Implantable Biofuel Cell: Implantable biofuel cells operating *in vivo* was first mention in 1970 [8] but they are still challenging to design and implement. There were several reported implanted biofuel cells operating *in vivo* in living organism: rats [10, 17, 18, 47, 48], lobsters [49], rabbits [50], mollusks [51, 52] and insect (cockroach) [53]. The goal is to develop a biofuel cell that is portable and implantable for operation in close to neutral pH environment with low glucose and oxygen content. The biofuel cell is expected to have considerable lifetime and high power output.

- To fabricate electrodes capable of catalysing the reaction of glucose oxidation and oxygen reduction reaction;
- To achieve superior catalytic activity in the respective reaction for the bioelectrodes;
- To achieve high and stable power output for the constructed biofuel cell operating *in vitro*;
- To achieve *in vivo* power output from the physiological fluid after implantation.

1.3 Scope

Electroless Nickel Deposition

- To establish alternative synthesis of Pd nanoparticles using different reducing agents;
- To investigate the Pd nanoparticles reduced by different reducing agents for the hypophosphite oxidation reaction;
- To evaluate the Pd NPs catalytic/specific/mass activity with comparison to conventional Pd-Sn catalyst.

Implantable Biofuel Cell

- To develop effective immobilisation method for enzymes catalysts on the electrode for catalysing reactions (glucose oxidation and oxygen reduction).
- To investigate the capability of the electrodes based on the respective half-cell (anodic/cathodic) reactions.
- To investigate the *in vitro* power output of the biofuel cell by combining the anode and cathode at mild conditions (low glucose and oxygen content).
- To investigate the possibility of biofuel cell operating *in vivo* and its power output after implantation.

1.4 Organisation of the report

This confirmation report is organised into 5 chapters. Each chapter begins with a short note reflecting the main contents of that chapter.

Chapter 1 is an introductory chapter and it gives an overview about the present status of the problem, aims, objectives, scope and organization of this report.

Chapter 2 contains a comprehensive literature review that has been carried out for this project. The theory, principle and process of electroless nickel deposition and the electrochemical assessment for the electrocatalyst applied. This literature review also contains the current advances on the synthesis, mechanism and application of cathode/anode in implantable enzymatic biofuel cell.

Chapter 3 outlines the work done so far related to the experimental design, procedure, results obtained and discussion for the electroless nickel deposition.

Chapter 4 describes the work done so far related to experimental design, procedure, results obtained and discussion for the implantable biofuel cell.

Chapter 5 is the last chapter of this thesis report. It gives the overall summary of the benefits of this project. Suggestions for future work are also described here.

Chapter 2: Literature review

Section A: Electroless Nickel Deposition

2.1 Electrochemistry

Electrochemistry is the study of chemical processes that cause electrons to move. Electricity is generated by movements of electrons from one element to another in a reaction known as an oxidation-reduction (redox) reaction [54]. Electrochemistry can be divided into two processes; (i) electrolytic processes where electricity is applied to force the chemical reaction (e.g. electroplating) and (ii) galvanic processes where electrical energy can be generated from chemical redox reactions (e.g. fuel cell). The chemical reactions can be decomposed into two half-cell reactions: oxidation and reduction reactions. In a galvanic process, the electrons will be generated from the oxidation reaction (at the anode) via the oxidation of the species. These electrons will be utilised to drive the reduction reaction (at the cathode) where the reactants will receive electrons from the electrode and be reduced [55, 56].

For electrons to be transferred from the anode to the cathode, there must be energy potential that makes this phenomenon favourable. The energy potential derives from the potential for species to be oxidised at the anode and the potential for species to be reduced at the cathode. The cell potential is the potential for the overall redox reactions:

$$E_{cell} = E_{Red,Cathode} - E_{Red,Anode} \quad (1)$$

The change in free energy (ΔG) is the measure of the maximum amount of work of redox reaction and it can be represented by:

$$\Delta G = w_{max} = nFE_{cell} \quad (2)$$

where n is the number of moles of electrons and F is the Faraday's constant representing the amount of electric charge carried by one mole of electrons ($F = 96\,485.33 \text{ C mol}^{-1}$) [55, 56].

For the reaction to be thermodynamically feasible, ΔG must be negative. Thus from Eq. (2), E_{cell} must be positive for feasible reactions.

The standard condition is defined to be at 273 K and 1 atmospheric pressure. In order to calculate cell potential in non-standard condition, Nernst equation can be applied (derived from Gibbs free energy under standard conditions):

$$\begin{aligned} \Delta G &= \Delta G^o + RT \ln Q \\ -nFE_{cell} &= -nFE_{cell}^o + RT \ln Q \\ E_{cell} &= E_{cell}^o - \frac{RT}{nF} \ln Q \end{aligned} \quad (3)$$

where R is the ideal gas constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$), T is the temperature and Q is reaction quotient of the redox reaction.

Faraday's laws of electrolysis relate the quantity of chemical change at an electrode to the quantity of current passing through the electrode and current (I) is defined as the rate of change of charge. Thus the rate of reaction can be derived as [56]:

$$N = \frac{q}{nF} \quad (4)$$

$$I = \frac{dq}{dt} \quad (5)$$

$$\text{Rate of Reaction} = \frac{dN}{dt} = \frac{1}{nF} \frac{dq}{dt} = \frac{I}{nF} \quad (6)$$

where N is the number of mole of species involved in the reaction and q is the amount of charge in the reaction. From Eq. (6), the rate of reaction is proportionally to the current exhibited during reaction, thus the current can be a direct measure for the rate of the electrochemical reaction.

2.2 Nickel Metallizing Principles

In ELD Ni, nickel is continuously reduced and build-up to form a thin film/layer on the substrate by immersing in the nickel bath solution. The reducing agents in the nickel bath will supply the electrons from converting Ni^{2+} complexes to Ni metal.

Nickel will only be formed on the catalytic sites on the substrate and the deposited nickel further catalyses the Ni reduction for ELD Ni to be continuous. Marton et al. showed that nickel deposition starts at specific activated sites (Pd nanoparticles) on the surface, then with the continuous growth of nickel on these sites, islands around the nucleation sites are formed by using the electron transmission microscope [57]. These islands will gradually grow and merge with other islands, and eventually form a continuous film on the surface. The film mass thickness growth rate in ELD Ni is linear when the continuous film was established [57].

There were four mechanisms proposed to account for the electroless deposition process; (i) the ‘pure electrochemical’ mechanism [58-61] where electroless deposition was considered to result from mixed anodic and cathodic reactions. The electrons formed from oxidation of hypophosphite were involved in the cathodic reaction nickel reduction and formation of phosphorus

molecules. The hydrogen gas was produced as secondary reaction in the mechanism. (ii) 'hydride ion' mechanism [62, 63] where the hydride ions (H^-) were generated from the oxidation of reducing agent and these hydride ions can react with nickel ions to form nickel deposit or water to form hydrogen gas. (iii) 'metal hydroxide' mechanism [64, 65] proposed based on the experimental observations. In this mechanism, water ionization took place on the catalytic surface forming hydrogen and hydroxide ions (H^+ & OH^-). A hydrolysis reaction takes place when nickel ions react with hydroxide ions to form nickel hydroxo-complexes, these complexes will be reduced by reducing agent to form nickel deposit. Hydrogen gas was formed from the hydrogen radicals, created in the reaction process. (iv) 'atomic hydrogen' mechanism [1, 66-68] where the atomic hydrogen (H_{ad}) generated by hypophosphite reacting with water molecules, reduced nickel ions on the catalytic surface and hydrogen gas was formed when two atomic hydrogen molecules reacted.

The proposed mechanisms were able to describe most of the features of the electroless deposition process but they were unable to account for consistent description for all the electroless deposition processes. In the case of 'pure electrochemical' mechanism, the mechanism was contradictory to the reported observation that hydrogen gas emerged from the reductant [66, 68]. The 'metal hydroxide' mechanism cannot be applied to explain the process when the anodic and cathodic reactions were separated into different cells [58, 69]. Lastly, the 'hydride ion' and 'atomic hydrogen' mechanisms failed to explain why hydrogen gas produced were not equivalent to the metal deposition as observed by Pearlstein [70].

Based on the similarities of the four proposed mechanisms, van den Meerakker proposed a uniform (universal) mechanism that accounts for both the electrochemical and the catalytic nature of the process [71]. The uniform mechanism was established in consideration of the shared features in all electroless depositions: (i) ELD occurred only at catalytic surfaces; (ii) hydrogen gas is always generated in the process; (iii) poisons of catalysed reactions act as stabilizers in all electroless processes; and (iv) the deposition rate increases with an increase in pH [72, 73]. The electroless deposition process occurred in stages for all reducing agents, RH. For the anodic stages:

As for the cathodic stages in alkaline medium:

For the case of electroless deposition in acidic medium, stages 4 and 6 are:

The reducing agent, RH was dissociated into radical ($\dot{R}$) and atomic hydrogen ($\dot{H}$) on the catalytic surface. The oxidation of the radical and atomic hydrogen will generate the electrons needed for reduction of metal. However, the uniform mechanism failed to account for some processes. It failed to explain electroless plating occurring on non-conductive surfaces, and formation metal particles in the metal bath solution. The proposed mechanisms can explain

majority of the characteristic of the electroless deposition but some features which cannot be explained. Thus, further experimental studies are necessary to develop or prove the possibility of a general mechanism for electroless deposition.

With regards to the ‘pure electrochemical’ mechanism of ELD Ni, Paunovic [74] employed the mixed-potential theory with foundation from the Wagner-Traud mixed-potential theory [75] to analyse this mechanism. According to mixed-potential theory, the overall reaction can be broken down into one cathodic reaction Eq. (14) and one anodic reaction Eq. (15):

The two partial reactions occurred at a single electrode in the nickel bath solution. The equilibrium potential for oxidation of reducing agent must be more negative compared to the equilibrium potential of metal reduction so that the reducing agent can be oxidised and act as electron donor to the metal ions (electron acceptors) for metal reduction to occur based on Eq. (1).

The overall electroless deposition reaction can be described electrochemically based on the current-potential (I - V) curves for the overall and partial reactions. Curve 1 is the I - V curve for the overall reaction (summation of the partial anodic and cathodic reaction) represented by i_{tot} , the potential where the $i_{tot} = 0$ was designated as the steady-state mixed-potential (E_{mp}). Curve 2 represent the partial anodic reaction of the reducing agent (i_a) while Curve 3 represent the partial cathodic reaction of the metal (i_c).

In accordance to mixed-potential theory, the total current at any point can be represented by Eq. (16):

$$i_{tot} = i_a + i_c \quad (16)$$

Figure 4 I-V curves for a mixed-potential electrode [76].

The partial anodic and cathodic reactions are occurring simultaneously when the catalytic surface is brought to the bath solution. Each of these partial reactions strives to establish its characteristic equilibrium potential (E_{eq}). The steady-state mixed potential (E_{mp}) was developed when electroless metal deposition takes places. The characteristic equilibrium potentials of the cathodic and anodic partial reaction ($E_{eq,Red}$ and $E_{eq,M}$ respectively) will shift toward mixed potential E_{mp} [74]. The potential shift from $E_{eq,Red}$ and $E_{eq,M}$ to mixed potential are stated as the overpotential of the redox systems. The establishment of steady-state required the rate of reduction of M^{n+} , cathodic current density i_M be equal to the rate of oxidation of Red , the anodic current density i_{Red} . The rate of deposition, which can be represented by the current density of deposition, $i_{deposition}$ is:

$$i_{deposition} = i_{steady-state,M} = i_{steady-state,Red} \quad (17)$$

This means that at mixed potential, the electrons generated from the oxidation of reducing agent are completely used for the metal reduction process that is happening simultaneously. Thus, the net current is equal to zero during the electroless metal deposition process.

DeMinjer [58] was the first to verify the mixed-potential theory for ELD Ni as she investigated the possibility of having the anodic and cathodic reactions to occur at separate electrodes. She showed that the anodic oxidation of hypophosphite was observed at the Ni/Ni-P electrode when placed in a nickel-free hypophosphite solution, while nickel was reduced and deposited on a separate Ni/Ni-P electrode in a hypophosphite-free nickel electrolyte when both electrodes are connected. Later, Paunovic [77] confirmed the mixed-potential theory while using potentiodynamic method and Wagner-Traud and Evans diagram. The results showed that the computed rate of deposition and steady-state mixed potential from Wagner-Traud and Evans diagram methods were very close to the gravimetrically determined values, showing that mixed-potential theory is possible in predicting the electroless nickel deposition via polarization curves of anodic and cathodic processes.

Through the mixed-potential theory, it was assumed that the anodic partial reaction in the complete bath solution can be represented by the anodic polarisation curve obtained in the metal-free solution [76]. Thus, the rate of anodic oxidation of reductants was considered as a dominant factor in electroless deposition. Later, Cavallotti's group conducted computation study on ELD Ni with hypophosphite as reducing agent [78]. Their analysis suggested that nickel reduction proceed with the reaction of adsorbed nickel ions with hypophosphite ions and water molecules to form a nickel-

hydroxide/hypophosphite complex. Although the ELD Ni mechanism suggested was different, they also concluded that the oxidation of hypophosphite was the rate determining step of the process.

2.3 Electroless Nickel Baths

There are several components that are necessary for the establishment of a nickel bath, namely metal ions, reducing ions, complexants, accelerators (optional), stabilizers (optional), buffers, pH regulator and wetting agents. Table 1 shows the functions and examples of the components of the nickel bath solution.

The nickel bath can be categorized into two groups: acid bath and alkaline bath. The pH of the bath is an important factor that affects the deposition of the bath. In general, higher pH results lesser phosphorus content in the Ni-P deposit when using hypophosphite as reducing agent [79]. Chen's group observed that at low bath pH, the Ni film has higher phosphorus content (~25 % at pH 4) [80]. By increasing pH, the phosphorus reduction is slowed due to the formation of hydroxide ions in phosphorus reduction reaction in Eq. (22). The decrease in pH will decrease the deposition rate of reaction [79, 81]. Lowering pH will decrease nickel deposition as hydrogen ions are formed in nickel reduction based on Eq. (20). During ELD Ni, hydrogen ions are produced as nickel ions reduces, this means that the pH will tend to decrease. A pH buffering reagent must be included in the bath so that pH can be maintained throughout the process.

Figure 5 Effect on solution pH on deposition rate and phosphorus content of the coating. [79]

Another important factor in ELD Ni is the bath temperature. The increased in bath temperature (with all other factors constant) will exponentially increase the deposition rate of the ELD Ni [81]. In low temperatures, molecules have lesser energy for reaction which as well leads to slower deposition rates. In the case of high temperatures, the chemicals in the bath can be extremely active, which may result in ‘plate-out’ (nickel reduction occurs in bath solution) and low bath stability. The bath temperatures are usually capped between $60\text{ }^{\circ}\text{C} \leq T \leq 90\text{ }^{\circ}\text{C}$, alkaline baths can operate in low temperature (e.g. $40\text{ }^{\circ}\text{C}$) due to higher deposition rate in higher pH. In addition, the pH is difficult to control when the temperature is above $90\text{ }^{\circ}\text{C}$ for both acidic and alkaline bath. When the reaction is too fast, the pH buffer cannot maintain the pH as excessive hydrogen and hydroxide ions are formed quickly.

The most commonly used chemical reducing agent for electroless nickel bath is sodium hypophosphite. The ELD Ni by reduced hypophosphite ions has reactions:

Table 1 Components of Electroless Nickel Baths. [82]

Components	Function	Example
Metal ions	Source of metal to be plated.	Nickel Chloride, Nickel Sulfate, Nickel acetates
Reducing ions	Reducing agents	Sodium Hypophosphite, Hydrazine, Borohydrides, Amine boranes, and their derivatives
Complexants	Form Ni complexes and stabilise nickel ions, preventing nickel metal to precipitate.	Monocarboxylic acids, Dicarboxylic acids, Hydrocarboxylic acids, Ammonia, Alanolamines, etc
Accelerators	Activate the reducing agent and accelerate deposition; opposes stabilizers and complexants	Anions of some mono- and dicarboxylic acids, fluorides, borates
Stabilizers	Prevent solution decomposition by shielding catalytically active nickel nuclei	Pb, Sn, As, Mo, Cd, or Th ions, thioures, etc.
Buffers	For long-term pH control	Sodium salt of certain complexants, choice depends on pH range used.
pH regulators	For subsequent pH adjustment	Sulfuric and hydrochloric acids, Soda, caustic soda, ammonia.
Wetting agents	Increase wettability of surfaces to be coated.	Ionic and nonionic surfactants

2.4 Activation of Non-catalytic Surfaces

As mentioned previously, ELD Ni is an autocatalytic process. This means that the reduction of Ni can only occur on suitable catalytic surfaces. Ohno [83] conducted experiments to investigate the anodic oxidation behaviour of the reductants on different metal surfaces. The polarization experiment was carried out for gold, platinum, palladium, silver, copper, nickel and cobalt surfaces for appropriate nickel bath with reducing agents: sodium hypophosphite, formaldehyde, sodium borohydride, dimethylamine borane, and hydrazine. The result for the sodium hypophosphite bath showed that only Au, Pt, Pd, Ni and Co were capable in catalysing the reducing agent. The catalytic activity of the metals for the hypophosphite oxidation decreases from Au, Ni, Pd, Co, and Pt. This also means that ELD Ni with sodium hypophosphite can only occur on Au, Ni, Pd, Co and Pt surfaces but ELD Ni is not merely applied on these surfaces, hence the activation of non-catalytic surfaces are required.

The activation step is to deposit catalytic nuclei on the non-catalytic surfaces, so that ELD Ni process can be initiated. There are two types of activation used; electrochemical and photochemical.

2.4.1 Electrochemical Activation

In electrochemical activation, the catalytic nuclei of a metal are achieved by electrochemical reduction-oxidation reaction on the non-catalytic surface. A suitable combination of metal-reducing agent must be chosen for this reaction.

The preferred catalytic metal deposited is usually Pd as Pd^{2+} ions can be easily reduced on the non-catalytic surface. While the favoured reducing agent

is Sn^{2+} ion from SnCl_2 and the use of stannous chloride had been extensively investigated. The following reaction will occur when palladium (II) chloride and stannous chloride are applied:

The standard potential for Eq. (23) is -0.15 V and for Eq. (24) is 0.987 V. This means that E_{cell}^0 (based on Eq. (1)) of the overall reaction is 0.837 V (> 0 V) which suggest that Sn^{2+} can reduce Pd^{2+} to Pd. Electrochemical activation by Sn^{2+} and Pd^{2+} can be achieved by either a two-step system or single-step deposition.

Two-step system: The two-step activation process is the conventional method of activation with Sn^{2+} and Pd^{2+} . In the first step, Sn^{2+} ions are adsorbed onto the non-catalytic surface from the SnCl_2 solution (sensitization) followed by the nucleation/activation step, where the Sn^{2+} -adsorbed surface is immersed in PdCl_2 solution and Pd nuclei are deposited on the surface. The nucleation process will deposit the Pd catalytic sites forming an island network on the surface and the islands are usually less than $10 \text{ \AA}$ [57].

Single-step catalysis system: The single-step catalysing method was developed to simplify the process. In this system, the sensitizing solution and the nucleating solution are mixed into a single solution. The reduction of Pd^{2+} ions will occur in the solution and later forming Pd-Sn complexes [84]. When the non-catalytic surface is immersed in this mixture, Pd-Sn complexes will be adsorbed on the surface.

These Pd-Sn complexes can have very low catalytic activity as the Pd/Pd-Sn nuclei are concealed by stannic hydroxide shell which is catalytically poor in hypophosphite oxidation.

An additional phase, the acceleration treatment, must be performed for both the two-step system and single-step deposition in order to remove the stannic hydroxide surrounding the Pd/Pd-Sn nuclei and expose the active sites for hypophosphite oxidation. The acceleration treatment can be achieved either by immersing the deposited surface in acidic/alkaline solution or by electrochemical method using a potential greater than 1.0 V vs RHE [85]. Five different accelerating solutions (NaOH, HCL, H₂SO₄, NH₄OH and NH₄BF₄) were investigated by comparing the TEM and electron diffraction analysis [86]. It was identified that NaOH accelerator will result in finest Pd-Sn particles.

Figure 6 Acceleration treatment of Pd-Sn catalyst.

2.4.2 Photochemical Activation

The photochemical activation process is mainly classified into two kinetic schemes: photo-electrochemical and photoelectron kinetic. The photo-electrochemical scheme comprises of a reaction thru photochemical process which is then finished off with an electrochemical reaction. The reducing agent is generated from the photochemical reaction and the metal nuclei are then

reduced by the reducing agent through electrochemical reaction. As for photoelectron kinetic, the semiconductor absorbed photons incident on it, resulting in the generation of electrons and holes. The electrons generated are then used to reduce the metallic nuclei on the surface. The reduced metal acts as catalyst for ELD Ni.

2.5 Electroless Deposition Kinetics

The electroless deposition process will proceed towards its steady-state deposition at the mixed-potential, E_{mp} . The non-steady-state span when the process is shifted to steady-state is call induction period.

Induction period: The induction period is defined as the time required for the deposition process to reach steady-state metal deposition at E_{mp} . The induction period is determined by measuring the time from the point where the catalytic surface is immersed in the nickel bath solution, until the point where the measured potential reached steady-state mixed potential.

Figure 7 Induction period of electroless deposition for Cu electrode. [87]

Steady-state kinetics: The steady-state kinetics can be determined by two methods; (i) the rate of deposition is ascertained as the current coordinate of the intersection of the overpotential in the polarization curves for partial cathodic

and anodic process, at rest potential (Figure 7). To achieve steady-state, the anodic current (i_{Red}) from the oxidation of reducing agent should be equal to the cathodic current of metal reduction (i_M),:

$$i_{M,deposition} = (i_M)_{E_{mp}} = (i_{Red})_{E_{mp}} \quad (26)$$

Paunovic [77] verified the mixed-potential theory while using the Faraday's Law, the deposition rate for nickel may be obtained using the equation:

$$rate = (i_M \times 1.09) \text{ mg/h}\cdot\text{cm}^{-2} \quad (27)$$

where i_M is the cathodic/anodic current at E_{mp} in mA/cm^{-2} .

Figure 8 Plot of Wagner–Traud for electroless deposition. [77]

(ii) the low-overpotential method where the deposition rate is determined from the Le Roy equation. The Le Roy equation [88, 89] states that the deposition rate is:

$$i_{dep} = \frac{\sum_{j=1}^n i_j E_j}{\sum_{j=1}^n E_j^2} \quad (28)$$

$$E_j = 10^{\eta_j/b_a} - 10^{\eta_j/b_c} \quad (29)$$

where i_j and η_j are current density and overpotential, at the j^{th} point on the polarization curve; and b_a and b_c are the anodic and cathodic Tafel slopes, respectively.

2.6 Electrochemical Characterization of Pd Electrocatalyst

As explained in Section 2.2, the anodic polarization of reducing agent is dominant in the electroless deposition process. Thus, the electrocatalysts prepared are usually evaluated in a metal-free bath solution which comprises of the reducing agent, complexing agent, pH buffer/regulator with the targeted pH. Herein, since palladium (Pd) is the noble catalyst of interest, the anodic oxidation of hypophosphite with Pd was investigated.

2.6.1 Anodic Polarization of Hypophosphite

Khaldeev [90] studied the hypophosphite oxidation on a Pd electrode. It was noted that the presence of hypophosphite ions significantly affects the shape of the voltammetry curves of Pd electrode. Anodic currents were observed for both anodic and cathodic potential scan in the hypophosphite-containing solution. In the anodic potential sweep, two maximum peaks of anodic current at $E_1=0.32$ V and $E_2=1.00$ V were observed. The first maximum was caused by the oxidation of hypophosphite while the second maximum was due to the simultaneous anodic process of Pd oxidation ($Pd \rightarrow PdO$) and hypophosphite oxidation. In the cathodic potential sweep, when the potential fell below 0.7 V, desorption of oxygen from Pd surface was occurring, thus hypophosphite oxidation was initiated. The maximum anodic current in cathodic scan was then observed in the potential region of 0.32 – 0.35 V, showing that hypophosphite oxidation is an irreversible process. In Khaldeev's point of view, the first maximum current peak in the anodic potential sweep

was used to evaluate the effect of different concentration of hypophosphite ions and varying scan rate. The result was that the peak current was insignificantly affected by the scan rate of the voltammetry curve but the concentration of hypophosphite ions affects the peak current substantially.

According to Tian [91], the current of hypophosphite oxidation is correlated to the catalytic activity of the electrocatalyst. This is in agreement with the results of Khaldeev where the current of hypophosphite oxidation increases when the concentration of hypophosphite increases. Thus, it is possible to evaluate the catalytic activity of the electrocatalysts using the current obtaining in the anodic polarization of hypophosphite ions.

Figure 9 Voltammetry curve for Pd electrode in 0.12 M sodium acetate solution containing sodium hypophosphite at pH 4.5. Scan rate is 10 mV s⁻¹, 293 K. [90]

2.6.2 Open-Circuit Potential in Nickel Bath

The open-circuit potential (OCP) of the electrocatalysts were conducted to study the induction period necessary for each electrocatalyst to reach steady-state mixed-potential, E_{mp} . Abrantes et al. suggested that the induction time is the time required for the adsorption of hypophosphite ions to the catalytic surfaces and the cleavage of the hydrogen bond to form the hydrogen radicals [92]. This means that the deposition metal can only starts after the induction

period. The induction period measurement was proved by the results from Electrochemical Quartz Crystal Microbalance (EQCM) [93, 94]. The results from EQCM revealed that the time required before deposition of metal begins were the same as the induction period acquired in OCP measurements. This means that the shorter the induction period, the higher the catalytic activity of the catalysts in hypophosphite oxidation [91].

Section B: Implantable Biofuel Cell

2.7 Electron Communication of Electrode in EBFCs

One major challenge for developing high performance EBFCs is establishing an efficient electron-transfer communication between the redox sites of enzymes and the conductive support. The protein matrix that surrounds the redox active sites of enzymes acts as an electrical insulation between the redox centre and the support resulting in poor electron transfer. For the past few decades, several methods have been suggested and tested to establish the efficient electron transfer. In general, electron transfer can be categorised into two mechanisms: mediated electron transfer (MET) and direct electron transfer (DET).

2.7.1 Mediated Electron Transfer

MET describe an approach where a low molecular weight redox active species is introduced as a mediator to shuttle the electrons between the redox sites and the electrode surface. The mediators must have reversible electrochemistry that allows them to react directly with the enzyme or its cofactor and subsequently react with the support to complete the electron transfer. Figure 10 shows the schematic of a general MET reaction.

Figure 10 Schematic of MET process of bio-electrode. [95]

Mediators are generally classified into two subclasses, namely metal-based mediators and azine mediators which are further broken down to immobilized or solution type. Azine mediators enable electron transfer at low overpotential while having high reaction rate. For instance Kulys et al. immobilized methylene green into the carbon paste electrode, the electrode has a redox potential for NADH oxidation at $-0.122\text{ V vs. Ag/AgCl}_{sat}$, reducing the overpotential by 0.398 V [96].

Trifonov [97] discovered the immobilisation of redox mediators by encapsulating the mediators in the pores of mesoporous carbon and entrapped them inside the mesoporous carbon by blocking the pores with the respective enzyme. This was an innovative system as it can allow mediated electron transfer between enzyme and electrode while preventing the redox mediator from diffusing into the environment.

Figure 11 Schematic illustration of the GOx-capped, Fc-MeOH/mesoporous carbon electrode for glucose oxidation. [98]

This immobilization system paved a route for applying MET in implantable biofuel cells as the enzymes capping the pores of mesoporous carbon prevent the leaking of redox mediators into the physiological environment of the living organism. The mediator-CNP system was further augmented by introducing

bienzyme-functionalised mesoporous carbon [98]. The application of two enzymes is to improve the performance of the biofuel cell either by allowing simultaneous reactions or successive reactions to take place.

2.7.2 Direct Electron Transfer

The DET mechanism requires a direct electronic communication between the active redox site of the enzyme and the electrode surface, enabling the transfer of electrical charge from the redox site to the electrode surface (or vice versa). In this way, the enzymatic and electrode reactions cannot be considered as separate reactions, but as stages in an enzyme-catalysed electrode process.

The criteria for achieving DET between electrode surface and redox protein as described by Armstrong [99] were (i) the redox active site must be close to the perimeter of the protein matrix, (ii) the active site should be facing towards the electrode surface, (iii) deformation will not deactivate the enzyme, and (iv) an electron pathway is introduced by a second redox site or modification.

2.8 Enzyme Structure Promoting Direct Electron Transfer

The structure of enzymes plays an important role in promoting DET between the enzyme and the electrode surface. Small redox proteins that have metallocenters (e.g. hemes, copper clusters) in their active sites close to the protein surface [100] are more likely to display direct interaction with the electrode surface due to short electron tunnelling distance. For instance, laccase (Figure 12) belongs to a multi-copper enzyme family, composed of a mononuclear redox site (blue) and a trinuclear redox site (yellow). The mononuclear redox site is located near the surface of the protein and is responsible for transferring electrons to the trinuclear copper cluster so that

oxygen reduction reaction can take place. The structure below shows the ease in achieving DET with the appropriate orientation.

Figure 12 Structure of *Trametes versicolor* laccase III. [101]

2.9 Electrode Modification Promoting DET

The DET between dissolved enzymes on metal surface is usually unachievable due to the slow diffusion of large proteins and also considerable steric hindrance of the active centres. Establishment of DET communication requires interaction of enzymes and electrode surfaces that have an established film of proteins, in an ideal case, a monolayer.

In order to achieve a stable protein film, many novel electrode modifications have been explored by researchers throughout the years. One novel modification was to make use of self-assembled monolayers (SAMs) to achieve the preferred enzyme orientation. Cooney stated that the electrode surface must be functionalised with a “binding functional group that allows electron transfer by alternating π -electrons and a second function head group for oriented attachment of the redox protein” [95] to enable tunnelling of electrons thru SAMs. Willner [102, 103] reported a novel method to use gold

electrode surface functionalised with cystamine as SAMs. The base layer of cystamine was reacted with pyrroloquinoline quinone (PQQ), subsequently with *N*⁶-(2-aminoethyl)-flavin adenine dinucleotide (FAD) to yield PQQ/FAD diad layer. Further treatment with apo-glucose oxidase resulted in a glucose oxidase enzyme (GOx) monolayer on the gold electrode surface. The reported GOx monolayers displayed current density of $300 \pm 100 \mu\text{A cm}^{-2}$ (measured in 0.01 M phosphate buffer and 0.1 M sodium sulfate, pH 7.0, 35 °C), thus showing that all or most of reaction centers are connected.

Figure 13 Process step for fabrication of Au-NP-reconstituted GOx electrode. [104]

Xiao et al. later synthesized a reconstituted Au-NP-GOx monolayer electrode through two synthesis routes [104]. The two synthesis routes (Figure 13) require a spacer ((2), (3) & (4)) that bridges the Au electrode surface to the Au-NP functionalised with FAD, reconstituted with apo-GOx. The synthesized Au-NP-GOx electrode displayed a uni-molecular electron transfer rate constant of 5000 s^{-1} , which is seven times that of oxygen as electron acceptor ($\sim 700 \text{ s}^{-1}$)

[105]. It was demonstrated that the presence of oxygen at low glucose concentration show little (or no) influence to the response of electro-oxidation of glucose due to the much faster electron transfer rate of Au-NPs. Moreover, investigation was done to evaluate the effect of the structure of spacers. It was concluded that the electron tunnelling barrier between the electrode and the Au-NPs was lower for more conjugating bridging units and distortion of π -conjugation in (3) cause higher tunnelling barrier than (4).

Another modification is the use of conductive polymers or matrixes to entrap the biomolecules and establish an electrical communications between redox centres and electrode surface. Conductive polymers such as polyaniline and polypyrrole [106, 107] had been used to achieve DET through the provision of conjugated π -electrons. Sol-gel composites with graphite particles [108] and conductive carbon inks [109] are conductive matrixes that can entrap enzymes. The entrapment of enzymes is not favoured because it usually exhibit low sensitivity and low electron transfer rate which limit their applications.

2.9.1 Carbon Nanotubes and Graphene Immobilisation

A popular and effective method in achieving electron transfer is by modifying the electrodes with carbon nanotubes (CNTs). CNTs have diameters of a few nano-meters range, high chemical and electrochemical stability and good electrical conductivity. These properties make CNTs ideal conductive nanowires for achieving DET between the redox centers that are hidden deep inside the proteins and the electrode surface. Different approaches have been investigated to immobilise enzymes on CNTs, they are covalent binding [110, 111], entrapment [23, 112] or physical adsorption [20, 113].

Figure 14 Assembly of the SWCNT electrically connected glucose oxidase. [111]

Patolsky et al. immobilise the GOx on Au electrode through the use of covalent binding approach to achieve structural alignment CNTs with GOx on electrode (Figure 14) [111]. The SWCNTs were chemically shortened by oxidation in a mixture of concentrated sulphuric and nitric acids while sonicating for 8 hours. This process gave shortened SWCNTs with broad length distribution carboxylic groups at the terminal ends. The SWCNTs suspension was sonicated with sodium dodecylsulfate (SDS) as surfactant. The Au electrode with mixed monolayer of thioethanol/cystamine was exposed to the shortened SWCNTs in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Then FAD cofactor (1) was coupled to the carboxyl ends of SWCNTs on the electrode, followed by apo-GOx reconstituted on the FAD cofactors. The electrochemical performance of the synthesized GOx-CNT functionalised Au electrode was measured by the cyclic

voltammetry (CV) and calibration curve in different concentrations of glucose in 0.1 M phosphate buffer (pH 7.4). The electrode with 25 nm CNTs assembly exhibit a saturated current of 60 μA for glucose concentration larger than 160 mM and with the surface coverage of GOx-CNT units, the turnover rate of electron transferred to the Au electrode was estimated to be 4100 s^{-1} . This electron transfer rate value is six times higher than that of active sites of GOx to its natural oxygen acceptor. Furthermore, the relation between the electron transfer rate and the length of CNTs were studied. The conclusion obtained was that the shorter the length of CNTs, the larger the electron transfer rate.

A study of entrapping the enzyme in the CNTs structure was conducted by Zebda et al. [23]. In this study, the bio-electrodes were fabricated by mixing laccase (cathode) or GOx (anode) with MWCNTs and water to form a homogenous composite, the mixture was then compressed by a hydraulic press to form a disk structure. For bioanode, catalase was added to prevent the possible parasitic regeneration of free GOx by oxygen, decomposing the hydrogen peroxide which may deteriorate GOx. Insulating water-repellent glue was used to cover the unused area of the electrode. In this case, DET was achieved from the redox active sites of enzymes to the electrode which is the CNT matrix itself. The bio-cathode performance was evaluated and it was found the open-circuit potential (OCP) of the laccase-CNTs electrode is 0.58 V in air-saturated 0.1 M phosphate buffer, pH 7.

The electrochemical performance was evaluated for a time period of 30 days, no decrease in current density and OCP were observed, this indicates the high stability of the entrapped laccase in CNTs electrode. In the case of the bioanode, an anodic current response was observed when glucose was added at

multiple instances into the testing solution when the GOx-CNTs electrode was held at 0.1 V vs SCE. There was no increase in current when glucose was added for the CNTs electrode without GOx. This means that glucose oxidation was occurring for GOx-CNTs and the electron transfer mechanism between the redox active sites of GOx and CNT can only be DET. The evolution of OCP and current density was investigated in relation with the catalase/GOx ratio. It was shown that at the ratio 1/1, OCP and current density was at their maximum, meaning that the decomposition rate of hydrogen peroxide by catalase is not affecting the glucose oxidation of GOx at this particular ratio. In addition, the performance of the bioanode decreased by 25% of its initial values after 30 days, showing higher stability compared to other reported bioanodes [114, 115]. The two electrodes were employed in a glucose/dioxygen biofuel cell. The obtained open-circuit voltage of 0.95 ± 0.05 V was one of the highest obtained values. The biofuel cell could obtain a maximum power density of 1.3 mW cm^{-2} at 0.6 V and only had a 4 % decrease in maximum power density after 30 days storage in the phosphate buffer solution.

Graphene and its nanocomposites are known to have excellent conductivity and large surface area. They demonstrated their significant potential immobilization of enzyme to construct bioelectrodes in EBFCs [19, 116, 117]. Graphene can provide higher surface area and electro-catalytic activity, and it can also be modified with different functional linkers which assists the immobilization of enzymes [117]. The 2D and 3D graphene nanostructures allows the immobilized enzymes to have better accessibility to the fuel/oxidant, increase enzyme loading and facilitate DET between the enzyme redox active sites and the carbon material [118].

2.10 Applicability to Implantable Biofuel Cell

For implantable biofuel cell, MET is usually not applicable as the presence of mediator molecules in the living organism is unpredictable. The mediators may not be biocompatible to the living organism and the diffusion of mediators away from the electrodes is hard to suppressed. But the immobilisation of redox mediator in CNP may be possible if the leaking of mediator is negligible. The application of DET-biofuel cell is more reliable and safe in this context.

In regards to promoting DET, the formation of SAMs on electrode surface for specific binding of cofactor or active sites is a possible option in biofuel. CNTs are notable in promoting DET in conjunction with SAMs as CNTs have high chemical stability which is essential when implanted into organism. Moreover, CNTs are cheap and have high electrical conductivity which is important in promoting DET. Manipulating noble metal nanoparticles (e.g. Au-NP, Pt-NP) for the relay of election transfer are also possible choices to achieve DET but more investigation must be conducted to verify if the presence of noble metals are harmful to the organism and noble metals are pricy.

2.11 Mechanism of Enzymatic Glucose Oxidation

2.11.1 Glucose Oxidase

Glucose oxidase (GOx) in *Aspergillus niger* extracts was first discovered by Muller [119]. It is a homodimer that consist of two identical subunits and two non-covalently bonded FAD. Wilson and Turner had shown that the FAD coenzyme is responsible for the electron transfer when catalysis takes place in GOx [120]. GOx has a molecular weight of ~ 160 kilo-Dalton (kDa) with each subunits weight ~ 80 kDa [121]. FAD is present in each subunit, as the redox

active sites for the catalysis reaction [122]. The oxidation of glucose required electrons to be transferred from the electrode substrate to the FAD but the FAD is hidden deep inside the carbohydrate shell of GOx, hindering this ET process [120, 123]. Thus, precise immobilisation of GOx enzyme is vital in establishing DET communication.

Figure 15 Glucose oxidase with its two subunits depicted as *dark* and *light blue*, while the FAD coenzyme is depicted as *pink*. [124]

GOx had been extensively studied for decades and two products had been identified when glucose was oxidised in the presence of GOx in a two-electron transfer process. The two products of the oxidation by GOx are D-glucono-1,5-lactone and hydrogen peroxide. Hydrogen peroxide is a by-product formed when oxygen molecules accept electrons from GOx-FADH₂ to oxidised back the glucose reacting GOx-FAD [125]. The activity of GOx will be hindered with the accumulation of hydrogen peroxide which eventually deactivating the enzyme. Thus, GOx-biocatalysts usually operate *in vitro* under anaerobic condition or in anodic solution with membrane separating the oxygen-containing cathode solution. To be applied in implantable biofuel cells, sophisticated and efficient assembly of enzyme on the electrode must be achieved to create the preferential electron transfer to the electrode than to the

oxygen molecules. This illustrates the importance of electrode modifications and enzyme immobilization mentioned in the previous section.

Figure 16 Glucose oxidation catalyze by GOx.

Another solution towards the formation of H_2O_2 is the addition of catalase to spontaneously decompose H_2O_2 . The presence of catalase can readily catalyze the H_2O_2 decomposition forming oxygen and water.

Figure 17 Decomposition of hydrogen peroxide in the presence of catalase.

Zebda et al. demonstrated this possibility by mixing catalase with GOx in CNTs entrapment to fabricate a bioanode for GBFC [23]. The polarization curves for the bioanode in the glucose solution, with and without catalase were verified the significance of having catalase (Figure 18).

Figure 18 Polarization curves of bioanode (red) without enzyme, (blue) with GOx and (black) with catalase/GOx in 0.05 M glucose solution. [23]

The addition of catalase decreased the OCP from -0.20 V to -0.43 V vs. SCE and increased the current density of the bioanode, showing that removing H_2O_2 surrounding GOx can enhance the activity of the enzyme under aerobic condition. This eliminates the negative effect of oxygen presence in bio-fluids.

2.11.2 Glucose Dehydrogenase

Glucose dehydrogenase (GDH), an oxidoreductases family enzyme, is an alternative to GOx for glucose oxidation. GDH transfer electrons via organic, non-protein redox cofactors (instead of oxygen) during glucose oxidation. This means that oxygen will not be able to react in the glucose oxidation reaction and hydrogen peroxide will not be formed. The literatures reported that three types of co-factors for GDH enzymes were mostly reported to enable glucose oxidation: Nicotine Adenine Dinucleotide (Phosphate)-dependent GDH (NAD(P)-GDH, E.C. 1.1.1.47), Pyrroloquinoline Quinone-dependent GDH (PQQ-GDH; E.C. 1.1.5.2) and Flavin Adenine Dinucleotide-dependent GDH (FAD-GDH; E.C. 1.1.99.10). The NAD(P)-dependent dehydrogenase oxidase glucose by transferring the electrons from the hydroxyl group of glucose to form NAD(P)^+ [7, 126]. NAD(P)^+ -cofactors are dissolved in solution when applied but this is not applicable for membrane-less implantable biofuel cell. The co-immobilisation of NAD^+ cofactor on electrode surface with GDH was reported with sophisticated and complicated procedures [127] which lack flexibility in loading the enzymes on the implantable electrode structures. During the oxidation of glucose to gluconic acid, NAD^+ cofactors are reduced to NADH and in order for the electrochemical oxidation of NADH back to NAD^+ , a special catalyst (e.g. methylene green, PQQ) must be present [47, 127]. This results in higher overpotential of the overall process due to the

catalysed reaction of NADH to NAD^+ . NAD-GDH has poor selectivity for the 2nd hydroxyl group, it can react with 2-amino-2-deoxy-D-glucose or glucosamine in the environment, hence hindering the glucose oxidation reaction [7, 128].

Figure 19 Schematic of the biofuel cell operating on multi-step enzyme kinetics and mediated electron transfer. [129]

An innovative application of NAD^+ -dependent dehydrogenases is to syntheses a multi-step/multi-catalysed system for deep oxidation of fuel [129, 130]. Kar et al. stimulated and demonstrated a multi-step enzyme catalysis system that oxidase methanol to carbon dioxide (Figure 19). The entire process require three oxidation reactions that require alcohol dehydrogenase (ADH), aldehyde dehydrogenase (AIDH) and formate dehydrogenase (FDH) respectively as catalyse in the presence of NAD^+ cofactors [129]. The results show that the multi-step enzyme catalysis system is viable and performance can be greatly enhanced with improved electrode design. It will be interestingly

significant if the same processes can be employed in implantable biofuel cells to increase the overall power generation.

PQQ belongs to the quinoprotein enzyme family [131] and it has high catalytic activity at physiological pH [132]. PQQ-GDH can be categorised into membrane-bound PQQ-GDH-A and water soluble PQQ-GDH-B [133-135]. PQQ-GDH-A have an affixing protein membrane that blocks the active sites of GDH, thus PQQ-GDH-A does not catalyse the oxidation reaction of disaccharides (e.g. sucrose and lactose) but only with monosaccharides (e.g. glucose, fructose and galactose) [136]. This means that PQQ-GDH-A has broad substrate specificity to monosaccharides. In the case of PQQ-GDH-B, PQQ disassociated from the active sites of GDH and mediate electrons between the enzyme active sites and the electrode during oxidation reaction [136-138]. The mediation of electrons in PQQ-GDH-B is less stable compared to membrane-bound GDH since PQQ-cofactor is soluble (detached from the GDH enzyme), thus external redox mediators may be necessary to facilitate the electron transfer. The electron transfer will be greatly affected if PQQ-cofactor cannot accept electrons from GDH readily.

Quinones can be used to improve the electrons tunnelling from the enzyme to the electrode [139]. In addition, quinones are also found to be able to help in orientating GDH enzyme when these molecules are adsorbed on the carbon matrix. PQQ-GDH-B was investigated to have better catalytic activity in glucose oxidation compared to GOx according to Flexer [140]. It was reported that PQQ-GDH produced about 2.3 times more current when using the same redox hydrogel matrix on both enzymes but it was also noticed that the rate of increased catalytic current (due to glucose oxidation) in term of applied

potential $\left(\frac{d}{d(Potential)}(current)\right)$ for PQQ-GDH is slower compared to that of GOx. This means that PQQ-GDH has mid-wave potential (mid-wave potential is defined as a potential at which wave current is equal to one half of diffusion limiting current) more positive than that of GOx. These behaviours were not explained by Flexer's group due to lack of information on the features in the electrochemistry of PQQ-GDH. PQQ-GDH-B has broad specificity for substrate; it can readily react with monosaccharides/disaccharides such as galactose and maltose, unlike GOx having high selectivity [133, 134]. PQQ-GDH is believed to be a promising alternative to replace GOx despite it being relatively more complex in immobilising the enzyme and having poor substrate selectivity (oxidise not just glucose).

Scherbahn et al. had reported to be successful in demonstrating DET of PQQ-GDH to electrode when immobilised on the modified vertical carbon nanotubes [141]. The PQQ-GDH deposited electrode exhibit a catalytic current of $590 \mu\text{A cm}^{-2}$ when tested in 10mM glucose + 5mM MES + 1 mM CaCl_2 , pH 6.5 buffers. This proves the capability of PQQ-GDH in oxidising glucose without the use of mediators.

FAD-GDH is a flavoprotein enzyme that usually classified to bacterial or fungus FAD-GDH. Bacterial FAD-GDH from *Burkholderia cepacia* has FAD as its cofactor in the catalytic subunit, it was verified to possess high thermal stability and is insensitive to oxygen [142-144]. The bacterial FAD-GDH consists of a catalytic subunit with FAD positioned, an electron transfer subunit that can bridge electron communication between the enzyme to the cofactor or even the electrode, a covalently attached multi-heme subunit, and another small subunit that is of unverified function. The native enzyme was proved to have

high catalytic activity but also broad substrate specificity that oxidise glucose, maltose and lactose [142, 145].

The first fungus FAD-GDH was first reported from *Aspergillus oryzae* in 1937 [146]. Fungus FAD-GDH was shown to have lower catalytic activity compared to bacterial FAD-GDH despite narrower substrate specificity. In general, FAD-GDH has the advantage of lower redox potential for glucose oxidation compared to other GDH [147-149].

2.11.3 Evaluating Catalytic Activity of Bioanode

2.11.3.1 Polarization Curve for Glucose Oxidation

Polarization curve in glucose solution was conducted in order to analyse the catalytic capability of the bioanode [23, 150]. Linear sweep voltammetry (LSV) was conducted from the measured open circuit to a specified potential for the bioanodes in absence or presence of glucose. From the polarization curve, the OCP can be identified when conducted in presence or absence of glucose. The negative shift in OCP (from absence to presence of glucose) meant that glucose oxidation reaction is occurring [23, 150]. The increase in current density can concurrently be used to evaluate the catalytic activity of the bioanode. Comparison between bioanodes can be analysed quantitatively by comparing the respective OCP and current density. Moreover, polarization curve is useful in verifying the content of the bioanode. To verify the presence of redox mediator immobilised in the bioanode, specific redox peaks for the employed redox mediator should be present in the polarization curve [151-153].

2.11.3.2 Chronoamperometric Response for Glucose Oxidation

Chronoamperometric response (CA) was also conducted to evaluate the catalytic capability of the bioanode at steady state [154, 155]. The bioanodes was held at 0.155 V vs SCE for a period of time. This potential selected may take reference from the polarization curve conducted so that glucose oxidation was verified to occur. The steady state current density can be obtained from the individual bioanodes and the catalytic activity of the bioanodes can be associated to the steady state current density.

2.11.3.3 Calculation of Apparent Michaelis-Menten Constant

The apparent Michaelis-Menten constant (K_m^{app}) represent the substrate concentration at which half of the maximum reaction rate occurs. This means that the smaller the K_m^{app} , the more efficient the catalyst reaction of the bioanode [154, 156]. The K_m^{app} of bioanodes were computed based of Lineweaver-Burke (double reciprocal) method (L-B method). CA was conducted for different concentration of glucose in the solution. The current responses from respective concentration of glucose were recorded. According to the Lineweaver-Burke method [157]:

$$\frac{1}{i} = \frac{1}{i_{max}} + \frac{K_m^{app}}{i_{max}} \times \frac{1}{[S]} \quad (30)$$

where i is the current obtained from the chronoamperometry responses, i_{max} is the current at maximum reaction rate and $[S]$ is the substrate concentrate (in this case is the glucose concentration). L-B plot can be plotted to obtain a straight line with gradient being $\frac{K_m^{app}}{i_{max}}$ and y-intercept being $\frac{1}{i_{max}}$.

Figure 20 L-B plot of enzyme kinetics by plotting $1/V_0$ as a function $1/[S]$. The slope is the K_m/V_{max} , the intercept on the vertical axis is $1/V_{max}$, and the intercept on the horizontal axis is $-1/K_m$. V is proportional to current of reaction [157].

The K_m^{app} represent the substrate concentration at which half of the maximum reaction rate occurs. This means that the smaller the K_m^{app} value, the more efficient the catalyst reaction of the bioanode.

2.11.3.4 Dependence of Power Density and Durability for GBFC

The bioanode was assembled with an auxiliary platinum (Pt) wire as cathode to form the GBFC. No membrane was used in the cell for power generation. This experiment can verify the power output of respective bioanode at low glucose and oxygen concentration. From the result, we can observe the open-circuit voltage (OCV) and also the maximum power output of the GBFC. These parameters can be used for comparison with other GBFC.

The GBFC was tested for consecutive 7 days to evaluate the durability of the GBFC, in other words, the durability of the bioanodes since Pt wire is assumed to be consistent. The durability test will evaluate the power output and OCV of the cell in each day.

2.12 Mechanism of Enzymatic Oxygen Reduction Reaction

Oxygen is a common fuel for many types of fuel cell, so oxygen reduction reaction (ORR) is of great interest for decades. The reduction of oxygen requires a catalyst to increase the electrode kinetics and traditionally, metal-based catalysts (e.g. Pt, Pd) are applied for fuel cells. In the case for implantable biofuel cell, metal-based catalyst may not be the best option for ORR. Metal-based catalysts are non-selective while the physiological bio-fluid comprises of many substances, thus it is highly possible for non-desired and incomplete reactions to take place and eventually result in fouling of the catalysts. Hence, catalytic activity and stability will be significantly affected. When operating in bio-fluid, enzyme catalysts are generally chosen due to its high selectivity. An instance is in direct methanol fuel cell, laccase was used instead of Pt minimized the oxidation of methanol (cross over from anode to cathode) and eventually improve the fuel efficiency [158].

Figure 21 A model of the mechanism depicting the role of trinuclear cluster asymmetry in the catalytic cycle of the MCO's. [159]

For ORR, enzyme catalysts employed are multi-copper oxidases (MCOs) such as laccases, bilirubin oxidases and polyphenol oxidases as they can reduce oxygen directly to water. These enzymes have redox active sites that composed of four copper centres: 1 mononuclear copper site (T1 Cu) and trinuclear copper sites (TNCs; T2/T3 Cu) as shown in Figure 21. The MCOs enzymes have high catalytic activity for ORR, this can be attributed to comparatively high reduction potential of the T1 copper sites, the ability to make use of the multi-atomic reaction sites (T1, T2 and two T3 copper sites) and the flexibility of both protein shells and Cu-Cu interatomic distances in the T2/T3 tri-nuclear copper sites [159-161]. The general mechanism for ORR is; (i) T1 copper site accept electrons from the electrode surface, (ii) the oxygen molecules will be adsorbed at the tri-nuclear copper sites; while the electrons transferred from T1 to tri-nuclear copper sites initiate the cleavage of the O-O bond and form water molecules as product. This mechanism is assumed that the enzymes are bonded to the electrode surface at the T1 copper site, but there are reported instances where the enzymes are immobilized via the tri-nuclear copper sites [162, 163]. In this case, the tri-nuclear copper sites can accept electrons from the electrode surface directly for ORR. In enzymatic ORR, laccases and bilirubin oxidases are identified to for their efficient enzymatic mechanism to achieve high-potential reduction of dioxygen at close to neutral pH.

2.12.1 Laccase

Laccase from *Trametes* had been widely studied as ORR catalysts due to its high reduction potential $\sim +0.58$ V vs. Ag/AgCl at the T1 copper sites. This potential is considerably higher compared to that of plant, bacterial and some fungal laccases with T1 potential between of $+0.23$ - $+0.51$ V vs. Ag/AgCl.

Despite the high reduction potential, fungal laccases (including *Trametes*) are inhibited by hydroxide [164, 165], fluoride [164, 166] and chloride (for MET) [166] ions (slighter extent). This restricts their application in implantable biofuel cells and led to the increasing focus in other fungal [167-169], bacterial [170, 171] and bilirubin oxidase (BOD) [172, 173]. It was found out that the orientation and DET with the electrode can help lessen the inhibition of chloride ion for laccase from *Trametes* [166, 174] by reducing the interaction of Cl^- with T1, thus minimizing competition with the redox reaction of T1 for ORR.

Figure 22 Electro-enzymatic system for creating an acidic local pH in the surrounding of a laccase-modified graphite electrode. [175]

Clot et al. proposed an innovative approach to control the local pH at laccases when coupled with GOx [175]. The magnet acts as the bioanode to hold the GOx-magnetic nanoparticles while the laccase-immobilized biocathode was placed in the centre of the magnet. Glucose oxidation occurred at GOx to form gluconic acid, while the hydrogen peroxide (by-product) was decomposed to oxygen and water molecules by the catalase enzyme (Cat) in the solution. The gluconic acid produced can lower the local pH around laccases and enhance the ORR since laccase has higher catalytic activity at more acidic pH. The local pH around laccase was measured to drop below 5 despite the

environment pH is higher than 6. This showed that the capability of this system to control its local pH.

2.12.2 Bilirubin Oxidase

Bilirubin oxidase is another popular copper-containing oxidase that catalyses the four-electron reduction of O_2 to water. BOD was discovered in 1981 by Tanaka and Murao [176] and it is a monomeric protein with molecular mass of 60 kDa. BOD is a potential catalyst for reducing oxygen directly to water because of their low over-potential needed to catalyse the reduction process [177]. With the same over-potential, BOD's catalyst turnover rate is three times higher compared to Pt nanoparticles [177]. The reduction potential of BOD for ORR is dependent on pH (around +0.6 V vs Ag/AgCl at pH 7.0) and reduction potential decreases with increasing pH. This is due to the presence of hydroxide ion inhibitors, resulting in poor electron transfer from T1 copper site to tri-nuclear copper site.

BOD's activity is greatly influenced by pH and temperature. BOD exhibited its highest catalytic activity at neutral pH and high temperature [178]. According to Tasca et al. [179], BOD can exhibit almost 100 % activity at temperature between 30 to 60 °C and pH between 7.0 to 8.5 (Figure 23). Its activity dropped to around 50 % when the pH is increased to 9.0.

Figure 23 Activity of BOD adsorbed SPGE at different pH and temperature. [179]

For BOD, two interconvertible forms of resting forms are observed: resting oxidised (RO) form and alternative resting (AR) form [180]. The AR form has reduction potential of +0.2 V vs Ag/AgCl for ORR while RO form has reduction potential of +0.6 V. This means that AR form can be activated for ORR only at sufficiently lower potential compared to RO form. The CVs of the AR-BOD deposited on spectrographic graphite electrodes (SPGE) showed that after the first scan (black curve), the reduction potential for ORR shifted from +0.4 V to +0.8 V vs NHE (from +0.2 V to +0.6 V vs Ag/AgCl) in Figure 24. This means that the AR form needs to be completely reduced in order to convert into RO which has superior catalytic activity for ORR. Similar result was observed when ABTS was used as redox mediator for ORR.

Figure 24 CVs of a polished SPGE (blue), first scan (black, 1 s), and second scan (red, 2 s) of the AR form of BOD adsorbed on SPGE. [180]

The inhibition of chloride was investigated extensively because of the presence of chloride ions in the physiological fluid. There have been contrasting conclusions on the chloride inhibition of BOD when different immobilisation was employed. Heller et al. reported that the BOD immobilization with osmium-redox polymers showed strong resistance against chloride inhibition of BOD [181, 182] while Tasca et al. reported substantial drop in activity of BOD when sodium chloride was added into the electrolyte [179]. By conducting

cyclic voltammetry scan for the chloride-inhibited BOD, activity recovered to 95 %. It was suggested that the addition of chloride ions result in BOD enzymes converting from the highly active resting oxidised (RO) form to the less active alternative resting (AR) form, thus reducing its activity. The cyclic voltammetry scan will allow the AR form to convert back to RO form, so enzyme activity was recovered [179]. Likewise to chloride, hydrogen peroxide can also hinder the activity of BOD enzyme. Slade et al. showed that BOD lost 90% of its activity in the presence of 0.02 mol L^{-1} hydrogen peroxide [183].

Figure 25 Chronoamperometry response of BOD adsorbed on SPGE. NaCl solution was added to the electrolyte at 1000 seconds. [179]

It has been reported that by controlling the binding orientation of the BOD enzymes, electron transfer from the electrode surface to the tri-nuclear copper sites can occur instead of transferring via T1 copper sites [184]. The intention is to bypass T1 copper site and eliminate the slow intramolecular electron transfer (IET) from T1 copper to tri-nuclear copper sites. The absence of IET was expected to increase the reaction rate for ORR. Dagys et al. had successfully immobilised laccase enzymes from *Didymocrea* sp. J6 (a small enzyme of 49 kDa) with DET contact for tri-nuclear copper sites and the electrode [184]. The biocathode exhibited high catalytic activity for ORR in

neutral and basic environment, differing from the previously reported laccase enzymes that were inactive in these conditions.

2.12.3 Evaluating Catalytic Activity of Biocathode for ORR

2.12.3.1 Cyclic Voltammetry for ORR

Cyclic voltammetry in oxygen-saturated electrolyte was conducted in order to analyse the catalytic capability of the biocathode [23, 97, 150]. CV was conducted between 0.65 V to -0.45 V vs SCE for the biocathodes in N₂-sat and O₂-sat electrolyte. The increase in current density can be used to evaluate the catalytic activity of the bioanode and identify ORR present. If the increase in current density is higher, it means that the rate of reaction is faster, thus the catalytic activity is higher. The onset potential can concurrently be evaluated for comparison between bioanodes, the more positive the onset potential, the higher the catalytic activity.

2.12.3.2 UV-vis Spectrometry

UV-vis was conducted to estimate the loss of BOD enzymes during the centrifuge process of the biocathode. The supernatants of the centrifuge were used for testing in UV-vis in order to investigate the amount of BOD in the supernatants. The testing solutions of each biocathode were also tested for the leakage of BOD enzymes after experiments. The ABTS redox mediators were added to the solutions so that there will be colorimetric change due to the presence of BOD enzymes and oxygen in the solutions. The colorimetric change was attributed to the activity of the BOD enzymes, thus an evaluation of the amount BOD enzymes can be computed [97, 151]. The scan of wavelength 450 to 400 nm was conducted and the peak at wavelength 420 nm was identified for the computation.

Chapter 3: Electroless Nickel Deposition

Section A: Experimental Procedure

3.1 Experimental Procedure

3.1.1 Stock Solution Preparation

An alkaline nickel bath solution was chosen as higher pH will allow higher deposition rate at low operating temperature (30 – 70 °C) but the higher pH of bath solution also decreases the stability of the bath, thus a nickel bath solution with pH 9 was chosen [185]. A hypophosphite-nickel bath solution (**Sol. 1**) containing 0.2 M sodium citrate (Sigma-Aldrich), 0.5 M boric acid (Strem Chemicals, Inc.), 0.06 M nickel (II) sulfate hexahydrate (BDH Prolabo Chemicals), 0.24 M sodium hypophosphite (Sigma-Aldrich), pH 9.0 was prepared for the OCP experiment [83]. Firstly, 29.41 g of sodium citrate, 15.46 g of boric acid and 7.5 g of nickel (II) sulfate hexahydrate was added to 400 ml of distilled water. The mixture was stirred for 30 mins to ensure all chemicals were dissolved and the complexing of nickel ions to complete. Next, 12.5 g of sodium hypophosphite was added and completely dissolved in the solution. The resulting solution was adjusted to pH 9.0 with 1 M sodium hydroxide and make up to a final volume of 500 ml. The nickel bath solution was left at room temperature for 24 hr before use.

A nickel-free bath solution (**Sol. 2**) was prepared for the CV experiment. The procedure for the preparation of this solution is similar to the hypophosphite-nickel bath solution (above) but excluding the nickel (II) sulfate

hexahydrate. The nickel-free bath solution was also left at room temperature for 24 hr before use.

The 20 mM solution of palladium (II) chloride (PdCl_2) (99% wt, Sigma Aldrich) was prepared by dissolving 0.3546 g of PdCl_2 into 99 mL of water with 1 mL of concentrated hydrochloric acid (HCl). The 2mM of PdCl_2 used in the electrochemical experimentation was prepared by diluting the 20 mM stock solution by 10 times.

The 0.2 M of sodium hypophosphite monohydrate (NaH_2PO_2 ; Sigma-Aldrich) solution, 0.2 M of hydrazine hydrate solution (N_2H_4 ; 50-60 wt%, Sigma Aldrich) and 0.2 M tin (II) chloride solution (SnCl_2 ; Alfa Aesar) was also prepared.

3.1.2 Stepwise Electroless Deposition of Pd Nanoparticles

The synthesis and deposition of Pd nanoparticles (NPs) electrocatalyst was carried out using the stepwise deposition method [186] on rotating disk electrode-glassy carbon (GC; 3mm diameter, Bioanalytical Systems, Inc.). The electrode was polished with Alumina slurry (0.05 μm , Bioanalytical Systems, Inc.), rinsed and sonicated in deionized (DI) water (Millie-Q Gradient A10) for 5 minutes. The GC was then air blow to dry the all surfaces before mounting onto the dipping machine.

The solutions containing respective reducing agent, 0.2 M sodium hypophosphite for Pd-P, 0.2 M hydrazine hydrate for Pd- N_2H_4 or 0.2 M tin (II) chloride for Pd-Sn, and 2 mM palladium (II) chloride (Sigma-Aldrich) were separately prepared and poured into vials set for our custom manufactured dipping-machine. The machine sequentially run the stepwise electroless deposition procedures to deposit Pd NP catalyst on GC; (1) dip the electrode in

the reducing agent solution for 20 s, (2) air blow to dry all surfaces, (3) transfer the electrode to dip in the PdCl_2 solution for 20 s, (4) wash the electrode in DI water to terminate the deposition reaction, and air blow the electrode. The process was repeated for 2 cycles for each reducing agent. There is an additional acceleration treatment for Pd-Sn after synthesis, where the Pd-Sn deposited on GC was immersed in 1.0 M NaOH for 3 minutes [85, 86, 187].

Figure 26 Stepwise electroless deposition process for Pd nanoparticles.

3.1.3 Electrochemically Active Surface Area of Pd Nanoparticles

Electrochemically active surface area (ECSA) is the active surface area that will be involved in the catalytic reaction of the electrocatalyst. Not all surface or facet of the electrocatalyst can participate in the catalysis process, thus ECSA is calculation for normalisation. The calculation of ECSA of Pd nanoparticles can be evaluated from the cyclic voltammetry sweep of the electrocatalyst in N_2 -saturated 0.5 M boric acid (pH 9) solution between $-0.625 - 0.6$ V vs $\text{Ag}/\text{AgCl}_{(\text{sat})}$. The potential sweep was conducted in the positive direction to fully oxidise the Pd active sites (to PdO) while the negative potential sweep will reduce the PdO (to Pd). The CV sweep was conducted for 10 consecutive cycles to ensure consistency. The required charge for complete PdO reduction to Pd in each electrocatalyst was obtained by integrating the cathodic peak

(between -0.1 – 0.1 V vs Ag/AgCl_(sat)) in the CV (Figure 27) . The reaction for ($PdO \rightarrow Pd$) is [188]:

The ECSA was calculated by assuming that the monolayer PdO reduction charge is 405 $\mu C\ cm^{-2}$ on the Pd surface [186, 188, 189]. This technique of calculating ECSA was confirmed by another technique using desorption of underpotential deposition hydrogen [190]. A polished Pd bulk electrode (geometric area of 0.0707 cm^2) was also used to calculate the ECSA and the obtained ECSA value was very close to the geometric area.

Figure 27 CV for Pd electrocatalyst in nitrogen-saturated 0.5 M boric acid (pH 9) solution. The shaded region representing the charge for reduction of PdO to Pd was tabulated by integration.

3.1.4 Cyclic Voltammetry for Anodic Hypophosphite Oxidation

CV was performed to analyse the electrocatalysts's activity in oxidising hypophosphite ions. The CV was conducted in a typical three-cell setup. The working electrode used was the RDE-GC deposited with respective electrocatalyst to be analysed while a platinum wire auxiliary electrode was

used as the counter electrode. The reference electrode used was Ag/AgCl (KCl saturated).

All CV was conducted in N₂-saturated **Sol. 2** at a scan rate of 50 mV s⁻¹. The CV was swept from open-circuit potential to 0.3 V vs Ag/AgCl_(sat) and vice-versa. The CVs were then normalised by ECSA and Pd load to obtain the specific activity and mass activity of the respective electrocatalysts. The specific activity represents the catalytic activity of the active site for hypophosphite oxidation in each electrocatalyst. In the case of mass activity, it represents the cost-effectiveness of the electrocatalyst in term of the catalytic activity. Thus, the activities of the electrocatalysts in hypophosphite oxidation were evaluated by comparing the specific and mass activity.

All the electrochemical measurements were conducted on a Princeton Applied Research VersaSTAT3-200 potentiostat.

3.1.5 Measuring Open-Circuit Potential of Electrocatalyst

OCP measurement was performed to determine the induction period of respective electrocatalyst in achieving steady-state mixed-potential (E_{mp}). The experimental set-up is similar to that of the CV measurement.

All OCP of electrocatalysts were measured in **Sol. 1** (preheated and maintained at 60 °C before experiment) for 120 seconds. Data points were plotted at 0.1 s per point. For respective electrocatalyst, the E_{mp} was first determined before analysing the time required for each OCP to reach E_{mp} .

3.1.6 Measuring Pd load Using ICP-MS

Inductively coupled plasma-mass spectroscopy (ICP-MS) was used to calculate the Pd load in each electrocatalyst. Firstly, the liquid sample is

converted to aerosol by the nebulizer before introducing the ICP torch. In the torch, the aerosol will undergo desolvation, vaporization, and ionization process when in contact with the plasma. The ions were directed to the mass spectroscopy where they will be dispersed in the mass analyser based on their mass-to-charge ratio (m/z) [191, 192].

After experiments with Pd electrocatalysts for hypophosphite oxidation, the Pd electrocatalysts were extracted for ICP-MS analysis. The RDE-GC surface was air blown dried followed by dripping 5 μL of concentrated nitric acid (HNO_3 , Merck Millipore) and left for 5 minutes. Subsequently, 4 μL of HNO_3 from the electrode surface was extracted and diluted to 500 μL with DI water. The sample was then analysed with inductively coupled plasma mass spectrometry, ICP-MS (Aligent 7700) with a 3rd generation He collision/reaction cell equipped to minimize the interferences. CV measurements were performed after the extraction to ensure that the Pd nanoparticles were completely extracted.

3.1.7 Transmission Electron Microscopy Sample Preparation

The transmission Electron microscopy (TEM) samples were prepared by using the stepwise electroless deposition on standard copper (Cu) specimen grid (Formvar/Carbon on 300 meshes, JEOL Asia Pte Ltd). The Cu grid was taped on a RDE-GC before performing the stepwise electroless deposition for Pd nanoparticles using the dipping machine. All TEM samples were allowed to dry overnight in a desiccator before performing TEM. The TEM sample was then observed using JEM-2010 microscope (JEOL) operated at 200 kV. Electron diffraction was conducted for the each electrocatalyst observe the Debye-Scherrer rings. The electron diffraction technique works by beaming electrons

to a sample and observing the resultant interference pattern due to the wave–particle duality characteristic of electrons [193, 194]. The Debye-Scherrer ring patterns can be used to study the crystal structure of the samples.

3.1.8 Field Emission Scanning Electron Microscopy Sample Preparation

The field emission scanning electron microscopy (FESEM) samples were prepared by using the stepwise electroless deposition on RDE-GC. The FESEM samples were allowed to dry overnight in a desiccator before conducting FESEM. FESEM (JEOL JSM-7600F) was used to observe the synthesized Pd nanoparticles. Energy-dispersive X-ray spectroscopy (EDS) was conducted for respective FESEM samples.

3.1.9 X-ray Diffraction Sample Preparation

The X-ray diffraction (XRD) samples were prepared by using the stepwise electroless deposition on glassy carbon plate. The glassy carbon plate was secured on the RDE-GC and performed the stepwise electroless deposition with the dipping machine. The samples were left to dry overnight in a desiccator before use. The XRD was conducted using PANalytical Empyrean diffractometer equipped with a Cu K α radiation source ($\lambda = 1.54060 \text{ \AA}$).

Section B: Results and Discussion

3.2 Result and Discussion

3.2.1 Physical Characterization

The Pd nanoparticles synthesized by the reducing agent (NaH_2PO_2 , N_2H_4 & SnCl_2) were characterized by TEM, SEM and XRD.

Figure 28 XRD profiles of Pd-P (A), Pd- N_2H_4 (B), Pd-Sn/BA (C) and Pd-Sn/AA (D).

XRD were conducted for the Pd nanoparticles (Pd-P, Pd- N_2H_4 , Pd-Sn/BA & Pd-Sn/AA) and shown in Figure 28. The XRD results presented that Pd- N_2H_4 exhibited defined Pd peaks: Pd(111), Pd(200), Pd(220) and Pd(311), showing that Pd- N_2H_4 nanoparticles are crystalline state. For Pd-P nanoparticles, it exhibited a wide peak at Pd(111) and no other Pd peaks were observed. This showed the amorphous structure of Pd-P nanoparticles. The XRD profiles also showed that Pd-Sn/AA has definite peaks indicating that SnO_2 and Pd are

present while Pd-Sn/BA has no peaks. This shows that the acceleration treatment removed the tin oxides complexes [187] on the surface and exposed the Pd nanoparticles underneath.

Figure 29 TEM of (A) Pd-P, (B) Pd-N₂H₄, (C) Pd-Sn/AA and Pd-Sn/BA. [195]

The TEM images of the various Pd nanoparticles are shown Figure 29. From the TEM images, Pd-P and Pd-Sn/AA (Pd-Sn after acceleration) nanoparticles are significantly smaller compared to that of Pd-N₂H₄. The Debye-Scherrer rings observed for Pd-P nanoparticles are diffused ring pattern and no discrete spots, this indicates that the amorphous structure of the Pd-P nanoparticles [194, 196]. This can be due to phosphorus co-deposited into the Pd lattice as confirmed by literatures [197, 198]. For Pd-N₂H₄, the observed Debye-Scherrer rings are made up of discrete spots [194], signifying that the Pd-N₂H₄ nanoparticles have polycrystalline structures. The TEM image of Pd-SN/BA showed that majority of the surface are covered with some materials

and its Debye-Scherrer rings showed diffused ring pattern. After acceleration treatment, these materials are significantly removed to expose the Pd nanoparticles; this showed that the removed materials were tin/tin-complexes. The electron diffraction pattern of Pd-Sn/AA shows that Pd-Sn/AA nanoparticles have polycrystalline structures which is in agreement with the result of Osaka and Nagasaka [86]. The electron diffraction results agreed to the conclusion of the XRD results.

Figure 30 FESEM images of (A) Pd-P, (B) Pd-N₂H₄, (C) Pd-Sn/AA and (D) Pd-Sn/BA. [195]

FESEM was conducted to show the distribution and size of the Pd nanoparticles on the glassy carbon electrode. The synthesized Pd-P nanoparticles are well dispersed on the electrode and the size of the nanoparticles varied from 5 to 50 nm. The EDS result shows that the Pd-P nanoparticles contain around 7.8 at% of phosphorus, indicating that phosphorus indeed was co-deposited in Pd lattice. The Pd-N₂H₄ nanoparticles are also well

dispersed and the EDS show that the nanoparticles synthesized are pure Pd. From Figure 30 (C & D), the Pd nanoparticles are found to be concealed under some materials and after acceleration; significant materials were removed (similar to the TEM results). The EDS result suggests that these materials are tin/tin-complexes. Comparing the result of XRD, tin oxides complexes are still present, this implied that the tin oxides complexes were not completely removed and probably leaving part of the Pd-Sn nanoparticles unexposed. Longer acceleration treatment was not conducted due to the fact that Pd-Sn nanoparticles might be lost as further removal of the tin oxide complexes are occurring [85].

Figure 31 EDS of (A) Pd-P, (B) Pd-N₂H₄, (C) Pd-Sn/AA and (D) Pd-Sn/BA.

3.2.2 Electrochemical Characterization

3.2.2.1 Anodic Polarization of Hypophosphite of Pd Electrocatalyst

Figure 32 CV of Pd-P in the solutions with (A) and without (B) sodium hypophosphite (pH 9). Scan rate is 50 mV s^{-1} . The inset is an enlarged scale of (B).

The anodic polarization curves of the Pd NPs were analysed to determine the electrocatalytic effect of the Pd NPs on hypophosphite oxidation. Figure 32 shows the CV of the Pd-P nanoparticles in solution with hypophosphite ions (**Sol. 2**) and without hypophosphite ions. In the presence of hypophosphite, the CV (**A; red**) shows a slight hump (P1) at approximately $-0.20 \text{ V vs. Ag/AgCl}$ followed by a broad peak (P2) at approximately 0.04 V while the backward scan shows an anodic peak (P3) at -0.23 V . The result indicates that P1, P2 and P3 correspond to the oxidation of hypophosphite in the solution. Comparing to Khaldeev's results [90], P1 is attributed to hypophosphite oxidation in the solution while P2 correspond to the simultaneous reaction of hypophosphite oxidation and palladium oxidation ($\text{Pd} + \text{H}_2\text{O} \rightarrow \text{PdO} + 2\text{H}^+ + 2\text{e}^-$). The CV without hypophosphite ions (**B; blue**) shows that the current output from palladium ionization is negligible, so the current output of P2 is likely to be a contribution of hypophosphite oxidation only. P3 is observed in the reverse

scan due to the reduction of PdO to Pd (Eq. (31)), thus the catalysis of hypophosphite oxidation on Pd surface occurs [90, 199].

Figure 33 Fig. S1 CV of (A) bare GC and (B) Pd-P in N₂-saturated **Sol.2**. Scan rate is 50 mV s⁻¹.

Figure 33 shows the CV for blank glassy carbon electrode and Pd-P electrocatalyst in **Sol. 2**, N₂-saturated condition. The results showed that the current output from Pd-P is significantly higher as compared to that of blank glassy carbon electrode. This indicates that the glassy carbon electrode has negligible effect on the polarization curve of Pd nanoparticles and the hypophosphite oxidation capability shown in the polarization curve is entirely dependent on the Pd electrocatalyst. The specific and mass activities of the electrocatalysts were calculated from the peak current of the respective P2 peaks shown in Figure 32.

The ECSA of each sample with Pd electrocatalyst deposited was measured before the anodic hypophosphite oxidation curve was conducted. The ECSA of Pd-Sn before and after acceleration was shown in Figure 34. The acceleration treatment exposes Pd reactive sites for hypophosphite oxidation as the PdO reduction peak can be observed only after the treatment. According to Osaka

and Nagasaka [86], the acceleration treatment removes the stannous and stannic oxides and hydroxides on the surface and exposes the bare active Pd nuclei. This was verified by XRD of Pd-Sn before and after the acceleration treatment as the Pd peak can be seen only after acceleration treatment.

Figure 34 CV of Pd-Sn electrocatalysts (A) before acceleration and (B) after acceleration. Scan rate 50 mV s^{-1} .

Table 2 Peak specific and mass activities of the electrocatalysts.

Electrocatalyst	Peak Specific Activity (mA cm^{-2})	Peak Mass Activity ($\mu\text{A mg}^{-1}$)
Pd-P	23.8	38.7
Pd-N₂H₄	13.8	10.5
Pd-Sn/AA	34.8	14.5

The CVs in the presence of hypophosphite with various electrocatalysts that were normalized by electrochemical active surface area and Pd loads shown in Figure 35 are the specific and mass activities for the oxidation of hypophosphite. In Figure 35-A, the specific activity for Pd-Sn/BA was not included as the ECSA of Pd nanoparticles was not computable with the negligible PdO reduction peak (as shown in Figure 34). The peak specific activity of Pd-P, Pd-N₂H₄ and Pd-Sn/AA are 23.8, 13.8 and 34.8 mA cm^{-2}

respectively (representing the ECSA normalised P2 peak in respective Pd electrocatalyst). Pd-Sn/AA exhibited higher specific activity compared to Pd-N₂H₄ indicates that the bimetallic interaction by having Sn incorporating into Pd has a positive effect on the catalytic properties. This is in agreement with other literature that studied the superior bimetallic properties compared to that of monometallic [200, 201]. Pd-P also exhibited higher specific activity compared to Pd-N₂H₄. There are two plausible explanation for this; (i) the phosphorus doped in the Pd lattice (as shown in EDS; Figure 31) caused an electronic effect that enhanced the catalytic activity of the Pd-P electrocatalyst [202-204] . (ii) the partially amorphous nature of Pd-P helps to improve the catalytic activity. The low degree of crystallinity in Pd-P contributes more abundant defects, kinks and edges, resulting in more low coordination sites that have high catalytic activity [186, 205].

The mass activities, the current normalized by the load of the precious metal (Pd in this study), of Pd-P, Pd-N₂H₄ and Pd-Sn electrocatalysts were compared as shown in Figure 35-B. The average peak mass activity of Pd-P, Pd-N₂H₄, Pd-Sn/BA and Pd-Sn/AA are 38.7, 10.5, 1.3 and 14.5 $\mu\text{A mg}^{-1}$ respectively. Pd-Sn/AA exhibited low mass activity possibly due to the surface having significant amount of reactive sites blocked by tin oxides (un-removed by the acceleration treatment) overlay and subsequently resulted in poor adsorption of hypophosphite ions on the Pd nanoparticles. This means that much of the Pd-Sn nanoparticles were embedded (wasted) under the tin oxide complexes and not participating in the oxidation process, causing the substantially poor mass activity. Another contributing factor to the poor mass activity is possibly due to the coagulation of Pd-Sn nanoparticles (shown in Figure 30), causing numerous reactive sites blocked or suppressed. The mass

activity for Pd-Sn/AA was noticeably higher compared to Pd-Sn/BA due to the increase in Pd-Sn nanoparticles exposed after partially removing the tin oxides complexes.

Figure 35 CV of various Pd nanoparticle electrocatalysts in solution (pH 9) with hypophosphite ions normalized by (A) electrochemically active surface area (specific activity) and (B) Pd load (mass activity). Scan rate is 50 mV s⁻¹.

Pd-P exhibited significantly higher mass activity compared to Pd-N₂H₄. This is probably due to the considerably higher loading of smaller nanoparticles (~5 nm) in Pd-P than Pd-N₂H₄ (shown in the inset of Figure 30). This implies that Pd-P nanoparticles have more reactive sites for hypophosphite oxidation and therefore significantly enhanced the oxidising capability compared to Pd-N₂H₄. Moreover, Pd-P and Pd-Sn/AA showed superior specific activity

compared to Pd-N₂H₄ compounding to the substantial difference (poor) in mass activity in Pd-N₂H₄.

3.2.2.2 OCP of Pd Electrocatalyst in Ni Bath Solution

The OCP measurements of the Pd electrocatalysts when immersed in the nickel bath solution (**Sol. 1**) at 60 °C [83, 91] were conducted. In Section 2.6.2, the induction time is shown to be the time required for the adsorption of hypophosphite ions to the catalytic surfaces and the cleavage of the hydrogen bond to form the hydrogen radicals. Nickel deposition only occurs after this induction time, thus the time taken to establish mixed-potential (E_{mp}) can signify the catalytic activity of the electrocatalysts [91]. From Figure 36, the E_{mp} is estimated to be around -0.94 V vs Ag/AgCl. Pd-Sn/AA (4.3 s) and Pd-P (4.5 s) have the lowest induction time compared to Pd-Sn/BA (10.4 s) and Pd-N₂H₄ (16.3 s), showing that the activity in hypophosphite oxidation is in the order of Pd-Sn/AA $\approx$ Pd-P > Pd-N₂H₄.

Figure 36 OCP of Pd electrocatalysts deposited electrodes as immersed in a nickel bath solution (**Sol. 1**), pH 9 at 60 °C.

3.3 Conclusion

The Pd electrocatalysts were evaluated for their catalytic activity in hypophosphite oxidation. Results from the anodic polarization curves and OCP indicate that Pd-N₂H₄ has the worst performance among the three electrocatalysts and the acceleration treatment improved the catalytic activity for Pd-Sn. It was observed that Pd-Sn and Pd-P have comparable activity but due to the tin oxides complexes blocking reactive sites and coagulation of Pd-Sn nanoparticles, Pd-Sn has much lower cost-effectiveness compared to Pd-P. In addition, it is noted that Pd-P exhibits competitive specific activity and the highest mass activity while not requiring any acceleration treatment unlike Pd-Sn which makes the nickel electroless deposition process simpler and less expensive.

In summary, Pd-P electrocatalyst has very high potential in substituting Pd-Sn for the nickel electroless deposition.

Chapter 4: Implantable Biofuel Cell

Section A: Experimental Procedure

4.1 Experimental Procedures

4.1.1 Enzymatic Glucose Oxidation

4.1.1.1 Stock Solution Preparation

The electrolyte used for in-vitro testing is 0.1 M phosphate buffer solution (PBS) at pH 6.5 (close to neutral pH condition). The PBS is prepared by mixing 8.578 g of sodium phosphate dibasic hepta-hydrate (Na_2HPO_4 ; Sigma-Aldrich) and 8.159 g of sodium phosphate monobasic (NaH_2PO_4 ; Sigma-Aldrich) to make up 1000 mL of PBS. The resulting solution was then adjusted to pH 6.5 using either Na_2HPO_4 or NaH_2PO_4 solution.

The enzymes used are dissolved in 0.1 M PBS, pH 6.5; glucose oxidase, *Aspergillus niger* (108 units mg^{-1} AMRESCO, CAS Number: 9001-37-0) was dissolved in PBS to make a concentration of 10 g L^{-1} and the GOx solution was stored at 4°C .

Viscous chitosan (CS) solution is prepared by dissolving 0.5 wt% of chitosan in 0.2 M acetic acid at 50°C under constant stirring for 30 minutes. Then, the dissolved CS solution was left under constant stirring at ambient temperature for 2 hours [15, 22]. Chitosan is used to disperse the carbon material as chitosan is a biocompatible polymer and has high permeability [15, 206]. The chitosan electrode can achieve higher stability for the enzyme activity and also minimize the inflammation responses when implanted [15, 207]. Thus, graphene nanopowders were added to the CS solution at 10 g L^{-1} concentration

under stirring for 15 minutes. The Graphene-CS matrix solution was placed in sonication bath for 30 minutes and subsequently, probe sonication (power ~60 W) for another 30 minutes before use.

The redox mediator solutions were prepared by; (i) hydroxymethylferrocene, 99 % (Fc-MeOH; Scientific Resources) was dissolved in water/ethanol (1:1 v/v) to make a concentration of 0.05 M. (ii) 2,2'-azino-bis(3-ethylbenzylthiazoline-6-sulfonic acid) diammonium salt, 98% (ABTS²⁻; Alfa Aesar, CAS Number: 30931-67-0) was dissolved in PBS to make a concentration of 0.05 M and stored at 4 °C. Fc-MeOH is the redox mediator of glucose oxidase for glucose oxidation and ABTS²⁻ is the redox mediator for bilirubin oxidase for oxygen reduction.

Glucose solution was prepared by dissolving glucose powder in 0.1 M PBS, pH6.5 and left overnight before use.

4.1.1.2 Electrode Preparation

A mixture solution containing 3 mL of Graphene-CS matrix solution, 2 mL of GOx from *Aspergillus niger* solution (10 g L⁻¹ prepared in pH 6.5 PBS) and 0.5 mL of hydroxymethylferrocene (Fc-MeOH, 0.05 M prepared in ethanol/deionized water in the ratio 1: 1) was placed in fridge at 4 °C for 1 hour.

Centrifuged Sample: The mixture solution was then subjected to centrifugation at 15 °C in the sequence of (i) 1500 rpm for 20 minutes, (ii) 5000 rpm for 20 minutes, (iii) investigated speed (5000 or 13500 rpm) for 1 hour and (iv) investigated speed (same as step iii) for 1 hour after removing the remaining solvent. The bioanode was constructed by collecting the centrifuged sample and mould to spherical structure. A Pt wire was pierced through the

centre to act as the electrical connection for the bioanode before leaving it to dry at 4 °C in air for 2 days before testing. The spherical bioanodes were measured to have diameter between 0.4 – 0.45 cm. Centrifuge sample of bioanode without Fc-MeOH (as redox mediator) was also prepared at 13500 rpm. All centrifuge of prepared mixture were conducted by Sorvall Legend X1R from Thermo Fisher Scientific [22].

Figure 37 Centrifuged bioanode connected to a platinum wire.

Casted Samples: Glassy carbon electrodes (GCEs, $d = 3$ mm, from Bioanalytical Systems, Inc) were polished with Alumina slurry (0.05 μm , Bioanalytical Systems, Inc.), rinsed and sonicated in ethanol, subsequently in deionized (DI) water (Millie-Q Gradient A10) for 5 minutes respectively. The clean GCEs were casted with 40 μL of mixture solution (Graphene-CS, GOx and Fc-MeOH) on the glassy carbon surface and allowed to dry at ambient temperature before storing in 4 °C [22].

4.1.1.3 Polarization Curve

All electrochemical measurements were obtained using Princeton Applied Research VersaSTAT3-200 potentiostat with Ag/AgCl (KCl-saturated) as reference electrode and Pt wire as counter electrode. Potentials were converted to saturated calomel electrode (SCE) for comparison with other literatures and they were represented by E vs SCE. All experiments were conducted at ambient temperature in 0.1 M phosphate buffer solution (PBS) with pH 6.5 as the support electrolyte.

LSV (for polarization curve) was performed to analyse the catalytic activity in glucose oxidation and oxygen reduction reaction for the bioanodes. The LSV were conducted in a typical three-cell setup, in nitrogen-saturated 0.1 M PBS (pH 6.5), in absence and presence of 50 mM glucose. The bioanodes were recorded from the measured open-circuit potential to 0.455 V vs. SCE. The scan rate for LSV measurements was 2 mV s⁻¹.

Cyclic voltammetry (CV) was conducted for auxiliary Pt wire in oxygen-saturated 0.1 M PBS (pH 6.5) to verify the catalytic capability of Pt wire for ORR.

4.1.1.4 Chronoamperometric Response

Chronoamperometry (CA) was conducted in nitrogen-saturated 0.1 M PBS (pH 6.5) absence and presence of 50 mM glucose to verify if glucose oxidation is occurring and also compare it steady-state current density.

CA were conducted for the centrifuged sample of 13500 rpm in air-saturated PBS (pH 6.5) at 0, 5, 10, 20, 40, 80 and 150 mM glucose till either the current stabilized or 45 minutes (for whichever condition was reached). The recorded CA results were used to plot the Lineweaver-Burke plot (Eq. 30), in order to calculate the K_m^{app} of the bioanodes.

4.1.1.5 Power Generation in Glucose Biofuel Cell

The GBFC was assembled by connecting the respective bioanodes with Pt wire as cathode without separating membrane. Power generation tests were conducted in air-saturated PBS (pH 6.5) in the presence of 10 mM glucose. The power density of the GBFC was recorded from the measured OCV to 0 V.

4.1.2 Enzymatic Oxygen Reduction Reaction

4.1.2.1 Stock Solution Preparation

The bilirubin oxidase (BOD) enzyme “Amano” 3 (~ 2.5 units mg^{-1} , Amano Enzyme Inc., CAS Number: 80619-01-8) was dissolved in 0.1 M PBS, pH 6.5 to make a concentration of 20 units mL^{-1} and stored at 4 °C before use.

The carboxyl multi-wall carbon nanotubes (COOH-MWCNTs, Sigma-Aldrich) suspension was prepared by dissolving the carbon materials in 0.1 M PBS, pH 6.5 at a concentration of 3 g L^{-1} under stirring for 15 minutes. The mixture was placed in sonication bath for 30 minutes and subsequently, probe sonication (power ~ 60 W) for another 30 minutes before use [97].

The redox mediator solutions were prepared by dissolving 2,2'-azino-bis(3-ethylbenzylthiazoline-6-sulfonic acid) diammonium salt, 98% (ABTS²⁻; Alfa Aesar, CAS Number: 30931-67-0) in PBS to make a concentration of 0.05 M, wrapped in aluminum foil (to prevent exposure to light) and stored at 4 °C [97].

The 2 mM palladium (II) chloride and 0.2 M sodium hypophosphate solution were prepared as mentioned in Chapter 3 [186, 195].

4.1.2.2 Electrode Preparation

Pd-P-modified RDE-GC: The Pd-P electrocatalyst was shown to exhibit high catalytic activity in electroless nickel deposition in Chapter 3 [195] and ORR at alkaline environment [186]. It was prepared and test for ORR in 0.1 M PBS, pH 6.5. The rotating disk glassy carbon electrode (RDE-GC) was used to perform stepwise electroless deposition using sodium hypophosphite (0.2 M) as the reducing agent and palladium (II) chloride (2 mM) as the metal solution.

The stepwise electroless deposition was conducted using the dipping machine and 3 cycles of Pd deposition were conducted.

Centrifuged Mixed Sample: A mixture solution containing 5 mL of COOH-MWCNTs (3 g L^{-1}) and 2 mL of BOD enzymes (20 units mL^{-1} prepared in pH 6.5 PBS) was placed in fridge at 4°C for 1 hour. The mixture solution was then subjected to centrifugation at 15°C in the sequence of (i) 5000 RPM for 20 minutes, (ii) 13,500 RPM for 30 minutes and (iii) 13,500 rpm for 30 minutes after removing the supernatant. The biocathode was constructed by collecting the centrifuged sample and mould to spherical structure. A Pt wire was pierced through the centre to act as the electrical connection for the biocathode before leaving it to dry at 4°C in air overnight before testing. The spherical biocathodes were measured to have diameter between $\sim 0.3 \text{ cm}$ after drying [22].

Figure 38 Schematic process of different stacking layer with stepwise centrifuge.

Centrifuged Stacked Sample: A stepwise centrifuged method was introduced to immobilise the enzyme between layers for carbon support. A

fixed amount of COOH-MWCNTs, BOD and ABTS was set for comparison – 5 mL COOH-MWCNTs suspension and an equivalent of 40 units BOD enzymes. The sequence of centrifuge for each biocathode was illustrated in Figure 38. For instance, in the case of SK4 biocathode, 1.8 mL of COOH-MWCNTs suspension was first centrifuged for 10 minutes at 5,000 rpm, then 5 minutes at 13,500 rpm to form the initial carbon layer. Subsequently, 1 mL of BOD solution (10 units mL^{-1}) was centrifuged for 10 minutes at 13,500 rpm, remove the supernatant, then 0.8 mL of COOH-MWCNTs suspensions was centrifuged for 5 minutes at 13,500 rpm. This was repeated for another 3 centrifuge to complete four BOD enzymes and four COOH-MWCNTs centrifugation. Finally, the stacked mixture was subjected to centrifuge for 30 minutes at 13,500 rpm after removing the supernatant. The biocathode was constructed by collecting the final centrifuged sample and mould to spherical structure. A Pt wire was pierced through the centre to act as the electrical connection for the biocathode before leaving it to dry at 4°C in air overnight before testing. The spherical biocathodes were measured to have estimated diameter of 0.3 cm after drying.

Pd-P-modified electrode: The Pd-P electrocatalyst [186, 195] was prepared and test for ORR in 0.1 M PBS, pH 6.5. The glassy carbon electrode was used to perform stepwise electroless deposition using 0.2 M sodium hypophosphite as the reducing agent and 2 mM palladium (II) chloride as the metal solution. The stepwise electroless deposition was conducted using the dipping machine and 3 cycles of Pd deposition were conducted.

4.1.2.3 Cyclic Voltammetry

For ORR, CV was conducted in oxygen- & nitrogen-saturated 0.1 M PBS (pH 6.5) at scan rates of 0.2, 2 and 5 mV s⁻¹. The CV was swept between 0.65 V to - 0.045 V vs SCE. The onset potential and increase in current density due to oxygen reduction was evaluated for the catalytic of the biocathode. A more positive onset potential represents the higher catalytic activity of the biocathode to reduce oxygen and the higher increases in current density represent the faster rate of reaction at the applied potential, thus higher catalytic activity [186, 202].

CV was also conducted in oxygen-saturated 0.1 M PBS (pH 6.5) with the addition of 0.3 mM ABTS (redox mediator of BOD for ORR) [21, 162, 163]. This electrochemical test was conducted to verify the degree of DET in the bioanode by comparing the difference between DET and MET result for ORR (MET/DET ratio). The result will show how efficient the immobilisations of enzymes are within the carbon matrix, leading to efficient DET between the redox active sites of enzymes and the carbon matrix.

4.1.2.4 Preparation for Ultra Violet-Visible Light Spectroscopy

The UV-vis was conducted by using filling a quartz cuvette with 2 mL of 0.1 M PBS (pH 6.5). The supernatant of the centrifuge samples was diluted by 25 times before testing. Then, 60 µL of the diluted supernatant was added into the cuvette. Following that, 10 µL of 5 g L⁻¹ of ABTS was added to the cuvette and the UV-vis was conducted after 120 seconds. The scan was conducted from 450 nm to 400 nm and peak at wavelength 420 nm was identified for evaluation. For calibration, different concentrations of BOD (2.85, 4, 7.5 U mL⁻¹) were used to plot a linear relationship between concentration of BOD and

absorbance. In the case of biocathode samples, the amounts of BOD were estimated by measuring the BOD amount in the supernatant of the centrifuge process. The BOD enzymes in the testing solution were also evaluated to estimate the amount of enzymes leached from the biocathodes. The UV-Vis spectroscopy was conducted using Shimadzu UV-2600.

4.1.3 Power Generation in GBFC and Implantation

The GBFC was assembled by connecting the bioanode (GOx/Fc-MeOH/COOH-MWCNTs; fabricated according to method mentioned in glucose oxidation section with COOH-MWCNTs replacing Graphene-Chitosan matrix) and biocathode (BOD/COOH-MWCNTs; mixing sample) without separating membrane. Power generation tests were conducted in air-saturated PBS (pH 6.5) in the presence of 5 mM glucose. The power density of the GBFC was recorded from the measured OCV to 0 V. The bioelectrodes were then implanted in grape for power generation test.

Section B: Results and Discussion

4.2 Result and Discussion

4.2.1 Anodic Half Cell – Glucose Oxidation

As mentioned previously, literature had reported the mechanical compression of sludge-like mixture into bioanode. This method enables immobilisation of enzyme and redox mediator within the carbon matrix [23, 150]. Herein, a novel fabrication method of a graphene-based bioanode utilizing ferrocenemethanol as mediator and glucose oxidase as enzyme catalyst via centrifugation of the mixture solution was proposed [22]. The purpose of the centrifuge compression method is that mixing of components (enzyme, redox mediator and carbon matrix) was conducted in higher volume of solution to ensure greater mobility of molecules within the mixture. This will facilitate uniform packing and interactions of the different components, then the centrifuge force applied can allowed better interaction of enzyme, redox mediator and graphene-chitosan (Graphene-CS) support under the applied centrifugal force. The immobilisation of enzymes and redox mediators within the Graphene-CS matrix during centrifuge will enable higher degree of connectivity between respective components within the matrix before constructing the spherical bioanode.

4.2.1.1 Analysis of the Polarization Curve and Chronoamperometry Response

The polarization curves of bioanodes were conducted to analyze their catalytic performance for glucose oxidation. Different rotation speeds were conducted for the fabrication of the bioanodes. By varying the rotational speed in centrifuging, the centrifugal force applied to compressed the mixture solution

can be varied and achieve controlled level of interaction of between the enzymes, redox mediator and carbon matrix. Two centrifuge speeds (5,000 and 13,500 rpm) were chosen to fabricate the bioanode samples (ctf-5000 and ctf-13500 respectively). For no centrifugal force applied sample, the casting method was used. From Figure 39, anodic peaks in the range of 0.24 – 0.27 V vs SCE were observed for the centrifuged samples. These anodic peaks were attributed to the redox oxidation peak for Fc-MeOH [97, 151-153]. This proves that Fc-MeOH mediators were electrically connected to the graphene-chitosan matrix.

Figure 39 Polarization curves of bioanodes: ctf-13500 (1), ctf-5000 (2) and casted (3) in the presence of 50 mM glucose solution. Polarization curve of ctf-13500 in absence of glucose (4) was included. Scan rate 2 mV s⁻¹. Electrolyte solution composed of 0.1 M PBS (pH 6.5) in nitrogen-saturated condition.

Glucose oxidation was detected when the synthesized bioanodes (ctf-13500, ctf-5000 and casted sample) were tested in the presence/absence of glucose. The ctf-13500 bioanode had an open-circuit potential (OCP) of 0.38 V in the absence of glucose (Figure 39; Curve 4). When glucose was introduced in the environment, all synthesized bioanodes exhibited more negative OCP and

increased current density. The negative shifting of OCP (~ 0.4 V) and increase in current density showed that the bioanodes indeed undergo glucose oxidation when glucose was present [154, 208, 209].

The OCP and current density for ctf-13500, ctf-5000 and casted sample were shown in Table 1. The centrifuged samples exhibited superior catalytic activity towards glucose oxidation compared to the casted sample. The catalytic current observed in the centrifuged samples (ctf-13500 and ctf-5000) were approximately 3 times higher compared to that of casted samples, thus showing the superior catalytic activity of centrifuged samples.

Table 3 Catalytic performances for the different bioanodes in Figure 39

Electrode	OCP (V vs. SCE)	I (mA cm^{-2}) @ 0.4 V vs. SCE
ctf-13500 (1)	-0.05	4.21
ctf-5000 (2)	-0.06	3.67
Casted (3)	0	1.23
Graphite/Fc/GOx [209]	0	~ 1.85

The polarization curves of ctf-5000 and ctf-13500 showed that the increased in current density due to higher centrifugal force was not very significant. Two conclusions can be drawn from this observation; (i) the interaction of the different components was already optimal or (ii) the increase in centrifugal force when increasing the speed to 13,500 rpm was not significant to achieve substantial increase in the interaction.

From Figure 40, the positive shifts of OCP and increase in current density (from Curve 3 $\rightarrow$ 2) were minimal. This result showed that there were no significant direct electron transfers (DET) between the redox active sites of GOx enzyme and the Graphene-CS matrix. With the addition of redox

mediators (Figure 40; from Curve 2 $\rightarrow$ 1), there was a very significant positive shift in OCP and the current density was increased by about 7 fold. These observations again proved that Fc-MeOH redox mediators were electrically connected to the Graphene-CS matrix and the mediators played a crucial role in transporting electrons between the redox active sites of GOx and the Graphene-CS matrix.

Figure 40 Polarization curves ctf-13500 with (1) and without (2) redox mediator at 50 mM glucose solutions. Polarization curve in absence of glucose (3) for centrifuged bioanode. Scan rate is 2 mV s⁻¹.

The chronoamperometric responses (CA) of the bioanodes were tested at 0.155 V vs SCE [154, 155] with presence of glucose. The bioanodes exhibited steady-state catalytic current of 0.98, 0.81 and 0.23 mA cm⁻² for ctf-13500, ctf-5000 and casted electrode respectively. Similar to the catalytic current density in the polarization curve, the steady-state currents of centrifuged samples were also $\sim$ 3 times higher compared to that of casted samples. The higher anodic current density in centrifuged sample showed that the electron transfer rate more efficient from the enzyme redox active sites to the carbon support via electron shuttling of the redox mediator. This shows that centrifuge can improve the interaction of the respective components.

Figure 41 Chronoamperometric response at 0.155 V vs SCE for bioanodes: centrifuged at 13500 rpm (1), centrifuged at 5000 rpm (2) and casted on GC electrode (3) in 0.05 M glucose, 0.1 M PBS (pH 6.5) in nitrogen-saturated condition.

4.2.1.2 Calculating Enzyme Affinity towards Glucose Oxidation

The apparent Michaelis-Menten (K_M^{app}) was investigated to understand the catalytic efficiency of the immobilized enzymes in the bioanode. The K_M^{app} represent the substrate concentration at which half of the maximum reaction rate occurs. This means that the smaller the K_M^{app} , the more efficient the catalyst reaction of the bioanode. The K_M^{app} of ctf-13500 bioanodes were computed based of Lineweaver-Burke method [156] from the CA experiments. The K_M^{app} values obtained were within the range of 60 – 80 mM. The high K_M^{app} values were similar to literature ($\cong 60$ mM) and this is possibly due to the slow diffusion of glucose molecules into the 3D bioanode [155, 210]. This implies that the GOx immobilized within the Graphene-CS matrix (not only the surface of bioanode) also contributed to glucose oxidation reaction and the slow diffusion of glucose into the matrix (reaching GOx enzyme deep inside) was hindering the catalytic efficiency of the bioanodes.

Figure 42 Chronoamperometric responses at 0.355 V vs SCE for ctf-13500 in 0, 5, 10, 20, 40, 80 and 150 mM glucose, at air-saturated condition.

Figure 43 Plot of current towards glucose concentrations for ctf-13500 (above) and Lineweaver-Burke plot for ctf-13500 bioanode (inset).

4.2.1.3 Power Generation and Durability of GBFC

The glucose/O₂ biofuel cells comprising the synthesized bioanodes (pairing with auxiliary Pt-wire as cathode for oxygen reduction reaction) were investigated without any separating membrane.

Figure 44 Assembly of GBFC with the synthesized bioanodes and auxiliary platinum wire as cathode.

Figure 45 Dependence of power density on operating voltage for ctf-13500 (1), ctf- 5000 (2) and casted (3) bioanodes against auxiliary Pt wire as cathode in 10 mM glucose solutions, 0.1 M PBS (pH 6.5) in air-saturated condition.

Power generation tests of GBFC were conducted in air-saturated PBS (pH 6.5) in the presence of glucose. The testing condition is to simulate the environment of low oxygen and low glucose content in implantable biofuel cell. The recorded open-circuit voltages (OCV) were 0.38 ± 0.02 V for centrifuged bioanode (ctf-13500 and ctf-5000) and 0.27 ± 0.01 V for casted bioanode (Figure 45). The OCP for Pt wire in oxygen reduction reaction (ORR) in oxygen-saturated PBS, pH 6.5, is ~ 0.30 V vs SCE (Figure 46), thus the

obtained OCV for the assembled glucose biofuel cell was in agreement with the theoretical OCV calculated based on anodic and cathodic half-cell reactions.

Figure 46 Polarization curve for ORR of platinum wire in 0.1 M PBS pH 6.5, oxygen-saturated.

The maximum power densities of the bioanodes are 69.1, 64.2, and 15.4 $\mu\text{W cm}^{-2}$ for ctf-13500, ctf-5000 and casted respectively. The results showed that the maximum power density of centrifuged bioanode was ~ 4 times higher compared casted bioanodes and the OCV of the biofuel for centrifuged bioanode was also superior. The power density at 10 mM glucose observed in centrifuged bioanodes was considerably higher compared to literatures (that uses Pt as cathode) [154]. This was in agreement with polarisation curve and CA results of centrifuging improve the electrical connectivity with the bioanodes, exhibiting higher catalytic activity in glucose oxidation.

The GBFC exhibited high power density and OCV even days after the preparation (Figure 47). The OCV of the GBFC remained relatively constant for the first 3 days which then decreased the following days after. The mean OCV recorded on Day 7 was 0.32 V, which is a decrease of ~ 0.07 V from Day 1. In regards to the maximum power density, it remained approximately the

same for the first 4 days [154, 211] and gradually decreases for the next 3 days. At Day 7, the maximum power density dropped to around 62 % of the first day.

Figure 47 Durability of GBFC against time of bioanodes centrifuged at 13500 rpm with Pt wire as cathode. The error bars represent the standard deviation of different trials with sample size 4.

The decrease in power output could be possibly due to a few reasons; firstly, the loss in enzyme activity, redox mediator or both from the bioanodes itself during testing [154], the loosely attached enzymes/mediators could be released to the solution and there is the likelihood of deactivation of enzyme throughout the electrochemical testing. Secondly, the electrical disconnection or rearrangement between the enzyme [155] and the graphene-chitosan matrix caused by the swelling effect of chitosan in aqueous solution [212], thus affecting the electron transfers process. Furthermore, the swelling effect may also disrupt the connectivity between the 3D bioanodes and the Pt wire (pierce in the spherical bioanode), hence causing the loss of electrons communication, resulting in decrease in power generation.

Nonetheless, the observed maximum power density in Day 7 was $\geq 34.1 \mu\text{W cm}^{-2}$; this value was higher than the reported maximum value in literature

[154]. This means that the ctf-13500 bioanode was able to retain substantial catalytic activity (60 % of maximum power generation) even after 7 days.

4.2.2 Cathodic Half Cell – Oxygen Reduction Reaction

The stepwise deposition of Pd-P electrocatalyst used for electroless nickel deposition [195] and ORR in alkaline environment [186] was tested in oxygen-saturated 0.1 M PBS (pH 6.5). The onset potential of Pd-P for ORR was identified to be 0.26 V vs SCE, this was comparatively more negative than the reported values of laccase/BOD (0.50 – 0.55 V vs SCE) in enzymatic ORR. This result showed that laccase/bilirubin oxidase (BOD) has higher catalytic activity compared to palladium electrocatalyst for ORR at close to neutral pH environment [12, 177].

Figure 48 Cyclic voltammetry corresponding to bioelectrocatalytic ORR of the Pd-P in oxygen-saturated PBS. Scan rate is 5 mV s^{-1} .

For the immobilization of enzyme, the centrifuge compression method was also adopted for the immobilization of bilirubin oxidase in COOH-MWCNTs matrix for ORR in cathode. Unlike the glucose oxidase enzyme, BOD has a smaller protein matrix (as mentioned in Chapter 2) and weighs about 60 kDa

(GOx is about 160 kDa). The redox active sites for BOD are also situated closer to the perimeter of the protein matrix. This makes the electrical connection between redox active sites to carbon matrix can be established with more ease.

4.2.2.1 Analysis of CV towards ORR

The centrifuge compress of BOD in carboxylic multiwall carbon nanotubes (COOH-MWCNTs) matrix was conducted by mixing BOD and COOH-MWCNTs solutions together. The fabrication of the biocathode was similar to the procedure mentioned for the bioanode previously.

Figure 49 Cyclic voltammetry corresponding to catalytic ORR of the mixed biocathode in oxygen-saturated PBS. Scan rate is 2 mV s^{-1} .

The onset potential for the mixed biocathode was found to be $\sim 0.46 \text{ V}$ vs SCE. The change in current density is $\Delta I_d = 0.53 \text{ mA cm}^{-2}$ at 0.2 V vs SCE. This result showed that ORR was present in the cell and this also proved that DET between redox active sites of BOD and carbon matrix was established. The centrifuge compression method was applicable to the immobilization of BOD enzymes in the carbon matrix even without using redox mediators.

In view of improving catalytic activity and immobilization of BOD, the stepwise centrifuge stacking method where the COOH-MWCNTs and BOD solution was conducted in a stepwise centrifuge procedure was introduced (as described in Figure 38). The first and last layer has to be COOH-MWCNTs so that BOD will always be sandwiched between the carbon matrixes. The number of BOD layers were noted for the labelling and SK1, SK2 and SK4 (Figure 38) were fabricated and investigated.

Figure 50 Cyclic voltammetry corresponding to catalytic ORR of SK1, SK2 and SK4 in oxygen-saturated PBS. Scan rate is 2 mV s^{-1} .

The onset potential for the SK1, SK2 and SK4 were found to be $\sim 0.49 \text{ V}$, $\sim 0.50 \text{ V}$ and $\sim 0.51 \text{ V}$ vs SCE respectively. The increase in current density is $\Delta I_d = 0.74 \text{ mA cm}^{-2}$, 0.96 mA cm^{-2} and 1.06 mA cm^{-2} at 0.2 V vs SCE . Comparing the result of mixed and stacked biocathodes, all stacked biocathodes had onset potential more positive and the increased in current density were also higher compared to mixed sample. The CV results showed that the catalytic activities for SK samples were higher compared to that of mixing samples.

Considering that the amount of BOD and COOH-MWCNTs used were equal in both samples, the improved catalytic activity can be associated to the

enhanced immobilization of BOD in COOH-MWCNTs matrix, leading to enhanced electrons transfer between the redox active sites and carbon matrix. This enhanced immobilization of BOD can be due to (i) the increased in binding of preferential T1 copper orientation through the biocathode by stacking centrifuge method. The preferential T1 copper orientation has more positive onset potential for ORR compared to the tri-nuclear copper sites (TNCs) orientation, resulting in higher current density in region of higher potential [21, 162]. (ii) The improved distribution of BOD throughout the COOH-MWCNTs matrix due to exposure of different areas of carbon matrix for BOD immobilization. The superior distribution can also prevent the overcrowding of BOD molecules in a region and allow fewer obstacles for immobilization. This may also result in higher loading of BOD within the SK samples. A trend of increasing catalytic activity when increasing the number of layer from the results of SK1, SK2 and SK4 was observed. This further showed that the stepwise centrifuge can improve the catalytic activity of the immobilized BOD in COOH-MWCNTs matrix for ORR.

Figure 51 Cyclic voltammetry corresponding to ORR of SK4 biocathode in oxygen-saturated PBS for multiple scans. Scan rate is 2 mV s^{-1} .

Another observation from CV of the biocathodes was the presence of mass transport limitation in the experiment (Figure 51). The result from the multiple scans showed that the 1st scan always has slightly higher catalytic current compared to the 2nd scan while the 3rd scan is almost equal to the 2nd scan. From this observation, we can conclude that the 1st scan exhibited slightly higher catalytic ORR because oxygen was filled within the spherical biocathode. But after the 1st scan, this initial oxygen content was used up or reduced to the ORR and new oxygen molecules were slow in diffusing into the carbon matrix, thus the 2nd and subsequent scan exhibited a slightly lowered catalytic ORR.

Figure 52 Cyclic voltammetry corresponding to ORR of mixed and SK4 biocathodes in PBS. N₂ and O₂ gas were vigorously bubbled to create agitation. Scan rate are A & B) 0.2 mV s⁻¹ (above) and C& D) 5 mV s⁻¹ (below).

The mixed and SK4 biocathode were then conducted at different scan rates and under agitation in solution to reduce the effect of mass transport limitation of oxygen (Figure 52). CVs were conducted 5 minutes after the immersion of biocathodes in solution. The CV conducted at 0.2 mV s⁻¹ showed that the

capacitance current of both samples are similar. By taking current density at 0.2 V vs SCE, the half-wave potentials for both samples were calculated. The half-wave potential for SK4 and mixing were 0.47 V and 0.43 V, the more positive half-wave potential in SK4 proved that SK4 exhibited higher catalytic activity compared to mixing. The CV conducted at 5 mV s⁻¹ showed the same conclusion as previous.

In order to verify if the higher catalytic activity in stepwise centrifuge stacking method enhanced immobilization of BOD in the carbon matrix, redox mediators were added into the testing solution. The MET/DET ratios of the biocathodes were evaluated to understand the immobilization of BOD enzymes within the biocathodes. The redox mediators (ABTS) in the solution helps to shuttle electrons (mediated electron transfer, MET) between the poorly immobilized BOD enzymes and carbon substrate, where DET connection were not established. The addition of ABTS will then result in an increase in catalytic current since ORR can now occur via the DET and MET pathway. The increase in catalytic current will be attributed to the MET due to ABTS, thus by comparing MET/DET ratio, the efficacy of BOD enzyme immobilization in the biocathodes can be evaluated [21, 162, 163]. In order to quantify the result, MET/DET ratio of the biocathodes were calculated, where [163]:

$$\frac{MET}{DET} ratio = \frac{mediated\ current - direct\ current}{direct\ current} \quad (32)$$

The COOH-MWCNTs-modified electrodes (without enzymes) were tested at different scan rates when 0.3 mM ABTS was added. Figure 53 showed that both scan rates have distinct ABTS reduction range, thus the working ranges of ABTS for mediation of electrons were identified. In 5 mV s⁻¹, the working

range for ABTS is 0.13 – 0.46 V vs SCE, while in 2 mV s^{-1} , the range is 0.24 – 0.47 V vs SCE. The 5 mV s^{-1} scan rate was chosen for evaluation because of its wider redox range and higher current output.

Figure 53 CV of Blank COOH-MWCNTs electrode in oxygen-saturated PBS and 0.3 mM ABTS. Scan rates are 2 (left) and 5 (right) mV s^{-1} .

Figure 54 CVs of ORR of SK4 biocathode in PBS and 0.3 mM ABTS, respective gas were bubbled vigorously. Scan rate is 5 mV s^{-1} .

The addition of ABTS in the electrolyte results in an increase of current density for SK4 biocathode. This increase was due to the mediated electron transfer via the ABTS^{2-} redox mediator pathway present in the solution, indicating that not all BOD enzymes are properly orientated within the carbon matrix and DET was not established for these poorly orientated enzymes. The MET/DET ratio calculated for SK4 biocathode has an average value of $0.15 \pm$

0.06; the MET/DET ratio of SK4 biocathode is very small, almost close to zero as compared to other literature [21, 162, 163]. According Lojou's group, the low MET/DET ratio strongly suggests that majority of the BOD enzymes are properly orientated to the carbon support for DET reaction [163].

In the case of mixed biocathode, no reliable MET/DET ratio was obtained. The possible reason for this situation was that there were significant amount of BOD leakage in the testing electrolyte, thus reacting with ABTS in the solution and forbidding the meditation of electrons between the immobilized enzymes and carbon substrate. The significant quantity of BOD in solution was proved by the UV-vis spectroscopy, showing there was considerable leakage of BOD enzyme in the testing electrolyte.

4.2.2.2 UV-vis Spectroscopy Result

Figure 55 UV-vis spectra for measurement of BOD content in the solutions: (blue) mix centrifuge supernatant, (red) stacked centrifuge supernatant, (green) testing electrolyte of mixed biocathode and (purple) testing electrolyte of SK4 biocathode. Inset: Calibration curve showing linear relationship between BOD concentration and absorbance in the UV-vis spectrometry.

In order to evaluate if the higher catalytic activity in SK4 sample is due to higher BOD loading compared to mixed sample, UV-vis spectrometry was conducted on the supernatant of the centrifuge sample. From Figure 55, the estimated amounts of BOD immobilized in the respective biocathodes were computed by back calculating the amount of BOD loss (in supernatant). The result from the UV-vis showed that SK4 biocathode had approximately the same amount of BOD enzymes immobilized as compared to the mixed samples. The higher loading in BOD was expected to be at least partly attributed to the higher catalytic activity in SK4 samples. The UV-vis analysis also showed that the testing solution of mixed biocathode exhibited significantly higher BOD activity compared to stacked biocathode. This implied that the mixed biocathode had high leakage of BOD enzymes from the carbon matrix. The BOD immobilization in the mixed biocathode was partially due to entrapment of enzymes within the carbon matrixes, thus BOD leakage occurred when immersed in the electrolyte. The negligible BOD enzyme leakage in SK4 biocathode suggested that BOD binding were more stable and likely more effective towards DET, thus exhibiting enhanced catalytic activity.

4.2.3 Biofuel Cell – Power Evaluation

Power generation tests of GBFC were conducted in air-saturated PBS (pH 6.5) in the presence of 5 mM glucose within the testing cell. The testing condition is to simulate the environment of low oxygen and low glucose content in implantable biofuel cell. The GOx/Fc-MeOH/COOH-MWCNTs bioanode and BOD/COOH-MWCNTs biocathode were assembled in the GBFC for the power generation test. The recorded open-circuit voltage (OCV) was 0.56 V and has a maximum power density of $\sim 280 \mu\text{W cm}^{-2}$. This GBFC exhibited a

wider OCV and 4 times higher maximum power density compared to the (GOx/Fc-MeOH/Graphene-CS || Pt wire) GBFC (mentioned in glucose oxidation section) even when operating in lower glucose concentration.

The bioelectrodes were then implanted in a grape to test for power generation. The sugar compositions in grape are mainly glucose and fructose. The sugar concentration is estimated to be $150 - 250 \text{ g L}^{-1}$ and for grape that are ripe, the ratio of glucose to fructose content is around 1:1 [213]. This means that grapes has high glucose content and the targeted fuel for power generation in the assembled biofuel cell is glucose, thus grape was chosen to demonstrate the capability of the bioelectrodes when operating in biological fluid. From Figure 56, the OCV of the assembled biofuel cell in grape remains at 0.56 V and has a maximum power density of $\sim 160 \mu\text{W cm}^{-2}$. The result showed that the bioelectrodes are capable of generating power even in biological fluid.

Figure 56 Dependence of power density assembled GBFC (without separating membrane) in 5 mM glucose, air-saturated 0.1 M PBS (pH 6.5) and when implanted in grape.

4.3 Conclusion

The results demonstrated that centrifugation compression was effective in the immobilizing of enzyme and redox mediator in the graphene-chitosan matrix for glucose oxidation. The centrifuged samples were proved to exhibited higher catalytic compared to the casted samples showing the advantage of centrifuge process improving the interaction between the respective components. The power generation of assembled GBFC with Pt wire as cathode also showed the similar conclusion of centrifuged samples generation higher power output compared to the casted samples. The fabricated bioanode produced a power output comparable with literature and was also able to retain ~60 % of its maximum activity after 7 days.

In ORR, the BOD-catalyzed biocathodes showed more positive onset potential and also higher increased in current density compared to the Pd-P electrocatalyst in pH 6.5. This means that the immobilized BOD has higher catalytic activity compared to Pd-P electrocatalyst in close to neutral pH environment. In comparing the mixed and stacked-centrifuged samples, the results showed that stacked samples were more catalytic active in oxygen reduction reaction. The SK4 samples showed the best configuration for the controlled condition when the content in each sample were held the same. Mass diffusion limitation was observed in the cyclic voltammetry of the bioanodes since the 1st scan always exhibited slightly higher current compared to the 2nd & 3rd scan, thus agitation via bubbling gas into the testing cell was implemented to enhance the diffusion of substrate. The value of MET/DET ratio obtained for SK4 biocathode was very small, suggesting that majority of the enzymes immobilized are properly orientated to the carbon support for DET reaction.

The MET/DET ratio of mixed biocathode was inconsistent due the significant amount of BOD enzymes in the testing solution interfering with the electron mediation. On the other hand, the UV-vis spectrometry result showed that SK4 biocathode had approximately same BOD loading as the mixed sample, thus the higher catalytic activity was attributed effective immobilization of BOD enzymes in SK4 biocathode.

The synthesized bioanode and biocathode were tested in both controlled cell experiment and implanted in grape. The assembled GBFC showed good power generation with a wide cell voltage of 0.56 V at very mild condition (low glucose and oxygen content, close to neutral pH). The implanted bioelectrodes also showed possible power generation and no drop in cell voltage, thus showing the possibility of operating these electrodes in biological fluids. The success of generating power when implanting in grape can leads to prospect of applying these bioelectrodes to living organism.

Chapter 5: Conclusion and Future Work

5.1 Conclusion

5.1.1 Electroless Nickel Deposition

In conclusion, the Pd-P electrocatalyst perform substantially well in electroless deposition of Ni. Pd-P electrocatalyst has comparable catalytic performance compared to conventional Pd-Sn electrocatalyst and more importantly, Pd-P exhibited significantly higher cost-effectiveness compared to Pd-Sn. This makes Pd-P electrocatalyst preferentially applied in industries as the amount of palladium used is a cost barrier in electroless nickel deposition, which is widely used currently all over the world.

The research shows that in terms of catalytic activity in hypophosphite oxidation, Pd-P electrocatalyst will be a promising substitute for Pd-Sn and the ELD Ni process can be substantially simplified when Pd-P electrocatalyst is employed. In-depth analysis on the physical characteristic of Ni film formed by using Pd-P electrocatalyst will be interesting to further show that Pd-P electrocatalyst is better compared to Pd-Sn.

5.1.2 Implantable Biofuel Cell

A novel centrifugation compression method developed and demonstrated to be effective in the immobilization of enzyme and redox mediator within the graphene-chitosan matrix for glucose oxidation. Moreover, the centrifuged samples displayed higher catalytic compared to the casted samples showing the enhancement between interaction of respective components. The fabricated bioanode (paired with Pt wire) produced substantial power output comparable

with literature and maintain this maximum activity for about 4 days. The stability of the GBFC remains at ~60 % of its maximum activity even after 7 days.

The centrifuge compression method was further modified to create stacked immobilization structure for BOD enzyme. Owing to the DET-promoting structure of BOD enzyme, where its redox active being closer to the perimeter of its protein matrix, stepwise centrifuge of carbon matrix and BOD solution was developed. The modified process creates a scenario for multiple layers/faces within the carbon matrix to interact with BOD enzyme and allow more efficient immobilization of the enzyme. The enhanced catalytic activity for ORR of the SK4 compared to the mixed biocathode proved the superior immobilization of BOD enzyme in the carbon matrix. The value of MET/DET ratio obtained for SK4 biocathode was very small, suggesting that majority of the enzymes immobilized are properly orientated to the carbon support for DET reaction.

The fabricated bioelectrodes were able to display substantial power density in environment similar to physiological condition; low glucose content, low oxygen content and close to neutral pH condition. This shows the capability of the bioelectrodes to operate in mild environment. Moreover, the bioelectrodes can still exhibit power generation when implanted in a grape; this proved the ability to operate in biological fluid and signify the potential of these bioelectrodes to function in other *in vivo* system like beetle or human.

5.2 Future work

5.2.1 Implantable Biofuel Cell

The research on implantable biofuel cell can be further demonstrated and improved. The evaluation of the biocathodes can also be more detailed for more definite conclusion. There are further areas of interest to be covered:

- a. Improving the power output of the biofuel cell. With the constraint in size and biocompatibility, the biofuel cell has to generate more power to maintain the operation of implantable devices. A direction is to manipulate the shape and design of the bioelectrodes as the results observed have mass transport limitation, this may be hindering the overall performance of the biofuel cell. Nickel foam can be used as frame for bioelectrodes to achieve higher enzyme loading and increasing electrochemically active sites, so that the power generation can increase.
- b. Investigating stability and operating time of biofuel cell. The stability of the biofuel cell is of great importance as it is intended for long time operation. There are improvements in stability of biofuel cell via coating a biocompatible layer around the bioelectrodes. Martin's group was able to demonstrate continuous operation of biofuel cell for 6 months in the implantation of rats via the inclusion of chitosan in bioelectrodes.
- c. Operation in human serum for the intention of implanting in human body. The physiological fluids of human contain many other organic molecules besides glucose. It is essential to investigate if there are other molecules that can inhibit or obstruct the operation of the biofuel cell.

Publications

International journal publications

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- 2) **Tan, D. C.**, Khezri, B., Amatyakul, W., Webster, R. D., & Sato, H. (2015). A facile synthesized highly active Pd nanoparticle electrocatalyst for electroless deposition process. *RSC Advances*, 5(108), 88805-88808.
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