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Development of glucose-oxidising enzyme electrodes for application in glucose-oxygen powered enzymatic fuel cells

Submitted by:
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Thesis is submitted for the Ph.D Degree by Research of
The National University of Ireland

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Month and Year of Submission: February 2016
Primary Supervisor: Professor Dónal Leech
Secondary Supervisor: Dr. Paul Kavanagh
Head of School: Professor Paul Murphy
Declaration

This thesis is the result of my own work carried out in the School of Chemistry & Ryan Institute, National University of Ireland Galway. The content of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or any other university.

Isioma Osadebe
February 2016
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Abstract

The objective of this thesis is to investigate different strategies for the optimisation of biocatalytic anodes with a view to developing a semi- or fully implantable, miniature, membrane-less enzymatic fuel cell via enzymatic oxidation of glucose coupled to the enzymatic reduction of dissolved dioxygen capable of operating under \textit{in vivo} conditions.

Chapter 2 describes the synthesis and characterisation of a range of osmium polypyridyl complexes. The complexes were characterised electrochemically and using a range of analytical techniques with the resulting osmium polypyridyl complexes used in the synthesis and electrochemical characterisation of redox polymers. The redox polymer is further characterised on electrode surface via the co-immobilisation with a glucose oxidising enzyme in the presence of carbon nanotubes. The immobilisations of enzyme electrodes are compared using glutaraldehyde vapour with a sodium borohydride and poly(ethylene glycol) diglycidyl ether crosslinking methodologies.

Chapter 3 focuses on comparing the performance of enzyme electrodes for glucose oxidation as a function of the amount of added multi-walled carbon nanotubes. The films were prepared based on FAD dependent glucose dehydrogenase, or glucose oxidase, as a glucose-oxidising enzyme co-immobilised with an [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$(polyvinylimidazole)$_{10}$Cl]$^+$ redox polymer on graphite electrodes. The enzyme electrodes are co-immobilised using poly(ethylene glycol) diglycidyl ether as a crosslinker. Glucose oxidation is highest for films with 68% w/w multi-walled carbon nanotubes, and a decrease is observed with larger amounts; this decrease is related to a decrease in retained enzyme activity. Enzymatic electrodes provide 4.2 mA cm$^{-2}$ current density at 0.12 V vs Ag/AgCl for GOx-based electrodes, compared to 2.7 mA cm$^{-2}$ for FADGDH-based electrodes in 50 mM phosphate-buffered saline containing 150 mM NaCl at 37 °C. Current densities of 0.52 and 1.1 mA cm$^{-2}$ are obtained for FADGDH and GOx-based electrodes, respectively, operating at physiologically relevant 5 mM glucose concentrations.
Thereby showing promise for application as anodes in enzymatic fuel cells for *in vivo* or *ex vivo* power generation.

In Chapter 4, graphite electrodes are modified with a redox polymer, \([\text{Os}(4,4\text{'-dimethoxy-2,2\text{'-bipyridine})_2(\text{polyvinylimidazole})_{10}\text{Cl}]^+\), crosslinked with glucose oxidising enzymes and various amounts of multi-walled carbon nanotubes are investigated for current generation in the presence of glucose in physiological buffer solutions. Enzyme electrodes based on glucose oxidase and FAD dependent glucose dehydrogenase are compared in the presence and absence of oxygen. The highest glucose oxidation currents are produced from enzyme electrodes containing 68% w/w multi-walled carbon nanotubes in the deposition matrix. The FAD-dependent glucose dehydrogenase and glucose oxidase enzyme electrodes provide similar current density of 0.8 mA cm\(^{-2}\) in de-oxygenated 50 mM phosphate-buffered saline at 37 °C containing 5 mM glucose concentration. Current densities under the same conditions, but in the presence of oxygen are 0.50 mA cm\(^{-2}\) and 0.27 mA cm\(^{-2}\) for glucose dehydrogenase and glucose oxidase enzyme electrodes respectively, with decreased currents as a result of oxygen reduction by the redox polymer in both cases, and oxygen acting as a co-substrate for the glucose oxidase-based electrodes.

Application of the anodes in membrane-less enzymatic fuel cells is demonstrated by connection to cathodes prepared by co-immobilisation of the \([\text{Os}(2,2\text{'-bipyridine})_2(\text{polyvinylimidazole})_{10}\text{Cl}]\text{Cl}\) redox polymer, *Myrothecium verrucaria* bilirubin oxidase and multi-walled carbon nanotubes on graphite electrodes. Power densities of up to 270 µW cm\(^{-2}\) are achieved, showing promise for *in vivo* or *ex vivo* power generation under these conditions.

Chapter 5 focuses on the optimisation of individual components such as multi-walled carbon nanotubes, FAD dependent glucose dehydrogenase and \([\text{Os}(4,4\text{'-dimethoxy-2,2\text{'-bipyridine})_2(\text{polyvinylimidazole})_{10}\text{Cl}]^+\) using a response surface methodology in order to maximise current capture on the enzyme electrode surface. The design of experiment model is developed and validated for enzyme electrode performance in pseudo-physiological conditions (5 mM glucose, 50 mM phosphate-buffered saline, pH 7.4, 37 °C). The components were co-immobilised using PEGDGE on graphite electrode surface as previously reported in chapters 3 and 4 with an optimised value of 1 mA cm\(^{-2}\) under pseudo physiological conditions. The optimised amounts are
further used as bioanodes for EFC application in pseudo-physiological conditions, artificial plasma and human plasma.

Chapter 6 summarises the contribution made towards the scientific body of knowledge by this thesis, and outlines other research undertaken during the course of the PhD, and some opinion on the direction such research may take in the future.
Abbreviations

Γ \text{os}  \quad \text{Osmium surface coverage}

\text{t}^{\frac{1}{2}}  \quad \text{Time until 50\% of current remains}

\text{Ag/AgCl}  \quad \text{Ag/AgCl (3 M KCl) reference electrode}

\text{ANOVA}  \quad \text{Analysis of variance}

\text{AspGDH}  \quad \text{Glucose dehydrogenase sourced from } Aspergillus \text{ sp.}

\text{AuNP}  \quad \text{Gold nano-particles}

\text{Bpy}  \quad \text{2,2\'-bipyridine}

\text{BOd}  \quad \text{Bilirubin oxidase}

\text{CDH}  \quad \text{Cellobiose dehydrogenase}

\text{CNT}  \quad \text{Carbon nanotube}

\text{DET}  \quad \text{Direct electron transfer}

\text{Dmbpy}  \quad \text{4,4\'-dimethyl-2,2\'-bipyridine}

\text{Dmo}  \quad \text{4,4\'-dimethoxy-2,2\'-bipyridine}

\text{DoE}  \quad \text{Design of experiment}

\text{EFC}  \quad \text{Enzymatic fuel cell}

\text{FAD}  \quad \text{Flavin adenine dinucleotide}

\text{FADGDH}  \quad \text{FAD-dependent glucose dehydrogenase}

\text{FWHM}  \quad \text{Full-width-half-maximum}

\text{GA}  \quad \text{Glutaraldehyde}

\text{GA(R)}  \quad \text{Glutaraldehyde with sodium borohydride reduction}

\text{GDH}  \quad \text{Glucose dehydrogenase}

\text{GOx}  \quad \text{Glucose oxidase}

\text{MBCO}  \quad \text{Multi-blue-copper oxidase}
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>MET</td>
<td>Mediated electron transfer</td>
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<tr>
<td>MFC</td>
<td>Microbial fuel cell</td>
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<tr>
<td>MtL</td>
<td><em>Myceliophthora thermophila</em> laccase</td>
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<tr>
<td>MWCNT</td>
<td>Multiwalled carbon nanotube</td>
</tr>
<tr>
<td>MvBOd</td>
<td><em>Myrothecium verrucaria</em> bilirubin oxidase</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NHE</td>
<td>Normal hydrogen electrode</td>
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<tr>
<td>Os(bpy)PVI</td>
<td>$[\text{Os}(2,2'\text{-bipyridine})<em>2(\text{poly-vinylimidazole})</em>{10}\text{Cl}]^+$</td>
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<td>Os(dmbpy)PVI</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PDB</td>
<td>Protein databank</td>
</tr>
<tr>
<td>PEGDGE</td>
<td>poly(ethylene glycol) diglycidyl ether</td>
</tr>
<tr>
<td>PEM</td>
<td>Proton exchange membrane</td>
</tr>
<tr>
<td>PQQ</td>
<td>Pyrrolo-quinoline quinone</td>
</tr>
<tr>
<td>PVI</td>
<td>poly-vinylimidazole</td>
</tr>
<tr>
<td>rCtCDH</td>
<td>Recombinant <em>Corynascus thermophilus</em> CDH</td>
</tr>
<tr>
<td>rGcGDH</td>
<td>Recombinant <em>Glomerella cingulata</em> GDH</td>
</tr>
<tr>
<td>ScL</td>
<td><em>Streptomyces coelicolor</em> Laccase</td>
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<tr>
<td>RSM</td>
<td>Response surface methodology</td>
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<td>SWCNT</td>
<td>Single walled carbon nanotube</td>
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Chapter 1: Introduction

1.1 Introduction

Miniaturised devices are increasingly important in the advancement to medical device technology. For example, there has been a growing trend towards the integration of implantable and semi-implantable devices such as pacemakers, insulin pumps etc for controlling and monitoring various medical conditions. These biomedical devices implanted in the body require a source of electrical power, for them to function [1]. As these medical devices become more sophisticated they require smaller, more portable and more lightweight power sources that are able to sustain operation over time. Currently, devices utilise lithium and alkaline electrolyte-based battery technology, providing electrical power from chemical reactions within the battery. Due to the toxic and corrosive nature of the battery components they require protective casings, membranes and seals to ensure isolation from the surrounding environment but also isolation of each electrode and electrolyte from each other to avoid unwanted chemical or electrochemical side reactions.

Ideally, a power source for implanted devices would be able to operate from reactions of chemical fuels presents in vivo and thus continues to generate power throughout the lifespan of the host. Electrocatalysis of fuels and oxidants, such as glucose and oxygen present in the body, in a fuel cell could power an implanted device [1-5]. Enzymatic fuel cells utilise enzymes as catalysts in order to improve specificity of the catalysis at fuel cell electrodes [1, 4]. Enzyme specificity can permit removal of the membrane and casings present in a fuel and therefore provide a route for miniaturisation of the device [4]. Although such enzymatic fuel cells (EFC) show promise towards miniaturised implantable devices, there are still a number of challenges to be addressed, such as obtaining improved current, power density and increased stability of power production.
1.2 Fuel cells

A fuel cell, previously known as a ‘gaseous voltaic battery’ [6], is an electrochemical device that converts chemical energy continuously to electricity for as long as the fuel and oxidant are supplied [7]: this operates through the oxidation of fuels at the anode and the reduction of oxidants at the cathode. The Welsh judge and physical scientist Sir William Robert Grove demonstrated the first use of a fuel cell in 1839 using platinum electrodes and sulphuric acid electrolyte, with hydrogen and oxygen as reactants [6, 8]. The power output (P_{CELL}) of a fuel cell is the product of the cell voltage (V_{CELL}) and the current produced by the cell (I_{CELL}) (equation 1.1). The cell voltage is the measure of the difference in potential between the anode (E_a) and the cathode (E_c), taking into consideration irreversible energy losses in the voltage (termed an overvoltage, η) as a result of kinetic limitations of the electron transfer processes at the electrode interfaces, ohmic resistances and mass transport limitation (equation 1.2) [7, 9].

\[
\text{P}_{\text{CELL}} = \text{V}_{\text{CELL}} \times I_{\text{CELL}} \quad \text{Equation 1.1}
\]
\[
\text{V}_{\text{CELL}} = (E_c - E_a) - \eta \quad \text{Equation 1.2}
\]

Fuel cells are similar to batteries in that they convert chemical energy to electrical energy by means of electrochemical oxidation-reduction (redox) reactions. The difference however is that batteries contain the active materials needed for electricity generation while a fuel cell does not: it is an open system into which fuels and oxidants are supplied externally. In principle a fuel cell can function indefinitely for as long as there is continuous supply of fuel and oxidant [7]. An example of the operational principle of a fuel cell, the hydrogen-oxygen proton-exchange membrane fuel cell (PEMFC) is illustrated in Figure 1.1. Equations 1.3 and 1.4 show the anodic and cathodic half reactions taking place within the PEMFC.
Anode: $H_2 \rightarrow 2e^- + 2H^+$  \hspace{1cm} \text{Equation 1.3}

Cathode: $\frac{1}{2} O_2 + 2e^- + 2H^+ \rightarrow H_2O$ \hspace{1cm} \text{Equation 1.4}

At the anode hydrogen gas is oxidised via a metal catalyst (typically platinum), releasing electrons and protons. The protons migrate through the proton exchange membrane towards the cathode, whilst the electrons travel through the external circuit producing current. At the cathode oxygen is reduced by the metal catalyst (again typically platinum) and combined with the protons and electrons to produce water. The standard potential difference ($\Delta E^0$) between the anode and cathode of the $H_2 \parallel O_2$ fuel cell is 1.23 V [10]. The platinum metal catalyst is highly effective under specific electrolyte conditions but both expensive and non-specific towards fuel or oxidant. This non-specificity of the catalyst necessitates the use of membranes and protective casings to separate the fuel and oxidant, thereby preventing migration of the fuel and oxidant to opposing electrodes.
1.2.1 Biofuel cells

Biofuel cells use biocatalysts to convert fuels and oxidant to electrical energy via electrochemical reactions involving biochemical pathways. Biofuel cells can be classified into: microbial fuel cells, organelle based fuel cells and enzymatic fuel cells [11]. Microbial fuel cells involve the use of entire microorganisms capable of catalysing the conversion of fuel and oxidant to electrical power, whereas enzymatic fuel cells involve the use of enzymes isolated from organisms for their conversion process. In both instances the biocatalyst can either be confined to an electrode surface or free in solution. The concept of microbial fuel cells will only be briefly presented as the work carried out within this thesis focuses on research aimed at enhancing power output of enzymatic fuel cells.

1.2.1.1 Microbial fuel cells

Microbial fuel cells (MFCs) harness the power of respiring microbes to generate electrical power from organic substrates (such as organic waste) as fuels. A typical MFC consists of anode and cathode compartments separated by a membrane and connected by an external circuit [11-13]. The oxidation of fuel by a microorganism occurs at the anode generating electrons and protons. The electrons are transferred to the cathode through the external circuit, while the protons are transferred through an ion exchange membrane to the catholyte. As in a PEMFC, protons and electrons combine with oxygen at the cathode to produce water. A metal catalyst is usually used to catalyse reduction of oxygen at the cathode [11, 12]. The first MFC was demonstrated by Potter in 1912, using yeast fed with glucose to produce electricity [14, 15]. Figure 1.2 presents a schematic cartoon of an MFC.
1.2.1.2 Organelle based fuel cells

An organelle based fuel cells utilises organelles such as mitochondria for the oxidation of compounds (fuels) found in metabolic pathways and serves as the anode of a biofuel cell. In biocatalysis, the two main types of organelles used are mitochondria and thylakoid. Mitochondria contain metabolic enzymes for the Krebs cycle whereby pyruvate is completely oxidised to carbon dioxide [16-18]. The first organelle-based fuel cells utilises electrode-bound mitochondria to drive the complete oxidation of pyruvate and serves as the anode of the biofuel cell where these organelles convert chemical energy into electrical energy [18-20].

1.2.1.3 Enzymatic fuel cells

An EFC produces electrical energy using enzymes, instead of a metal catalyst or microorganism, at electrodes. Enzymes used in EFCs are isolated from biological sources and are used to either oxidise a fuel, e.g. glucose at the anode, or reduce an oxidant, e.g. oxygen at the cathode [3, 13, 21-25]. Figure 1.3 presents the basic operating principle of a glucose-fuelled EFC. Yahiro et al. first reported enzymatic fuel cell results in 1960, using glucose as a fuel and enzyme electron transfer by a flavoprotein glucose oxidase system [26]. In the early 1990s interest in developing...
this technology was renewed, with the advent of improvements in enzyme electrode production and understanding [20, 27-29]. For example, it was recognised that immobilisation of enzymes on catalytically inert electrode surfaces both increases local concentration of catalyst and permits removal of separating membrane between anode and cathode as no cross-reactions should occur. This has led to renewed interest in recent years focused on the development of potentially implantable or portable, miniaturised, membrane-less EFCs operating on carbohydrates as fuel [30-36]. The most commonly described enzymatic fuel cell prototype in the literature consists of a glucose oxidising anode and an O₂ reducing cathode [37-39], although other systems utilising fuels such as methanol, fructose, alcohol (to name a few) and hydrogen peroxide as oxidant have been reported [40-43]. There remain, however, significant issues with the proposed technology, such as low power/current outputs, instability of enzymes and incomplete oxidation of fuels, which have thus instigated a range of studies to improve the performance of EFCs [44-46].

Figure 1.3: Operating principle of a glucose/oxygen EFC.

At the anode, β-D-glucose is catalytically converted to β-D-gluconolactone producing protons that travel through the electrolyte to the cathode and electrons that travel through the external circuit to the cathode combining with oxygen to produce water. The product of the cell voltage and the cell current is the power output of the cell. For a glucose/oxygen EFC operating on these in vivo available fuel and oxidant to be considered a realistic possibility it is imperative that the cell operates in
physiological conditions (i.e. pH 7.4 and temperature of 37 °C). Presently, the implanted device with the widest application in vivo is the heart pacemaker in which the lithium battery is located below the skin between the shoulder and the neck and is connected to electrodes in the heart muscle [47]. With a power density of 1 Wcm$^{-3}$, the typical lifetime of a 1 µW lithium battery exceeds ten years [48], whereas the lifetime of EFCs is, up to now, measured in days. An alternate approach to the utilisation of implanted EFCs is use as semi-implanted devices, for example in a sub-cutaneous cannula or patch, to allow for regular replacement yet providing power from glucose and oxygen present in vivo.

1.3 Enzyme catalyst
Enzymes, just like all catalysts, increase the rate at which a reaction occurs by lowering its activation energy. Enzymes are proteins and they consist of a unique three dimensional structure allowing them to be substrate-specific, thereby making them attractive for use in biosensors and biofuel cells [3, 4, 10, 11, 21, 49]. Advantages of the use of enzymes over other catalysts includes: their high substrate specificity, mild operating conditions such as pH, temperatures and also efficient substrate conversion [13]. Enzymes are available to catalyse a wide range of biochemical reactions, with substrate catalysis occurring in the active site of the enzyme, usually a groove or pocket of the enzyme protein structure. Unlike certain inorganic catalysts, enzymes can be less expensive to produce, renewable and with a range of enzymes available to specifically oxidise a wide range of fuels.

1.3.1 Enzyme anodes
There is a wide range of fuels that can be targeted for oxidation by enzyme anodes based on the availability of enzymes: methanol, ethanol, glucose etc. [21]. However, as the focus of the studies contained within this thesis is on EFCs for eventual in vivo operation the most suitable fuel in this context is glucose due to its relatively high concentration of 5-8 mM in human blood [50, 51]. Therefore, the following section focuses on introduction to enzymes that oxidise glucose: glucose oxidase and glucose dehydrogenase.

1.3.1.1 Glucose oxidase
Glucose oxidase (GOx, EC 1.1.3.4) is a dimeric oxido-reductase that catalyses the oxidation of β-D-glucose to D-gluconolactone, coupled to oxygen reduction to
hydrogen peroxide. GOx was discovered by Muller (1928) in *Aspergillus niger* extracts [52, 53]. To function as a biocatalyst, GOx requires a co-factor, flavin adenine dinucleotide (FAD). Figure 1.4 shows a representation of the crystal structure of one of the GOx subunits and Figure 1.5 presents FADH₂ structure and the relevant portion of the FAD active site in oxidised and reduced forms. In GOx-catalysed redox reactions, FAD functions as an initial electron acceptor during the oxidation of glucose and in turn is reduced to FADH₂, as shown in the right hand box of Figure 1.5. Finally, FADH₂ is oxidised back to FAD by oxygen (equation 1.6) or an artificial electron acceptor (mediator) which replaces oxygen in the electron transfer mechanism (*vide infra*).

![Crystal structure of the Aspergillus niger glucose oxidase (PDB ID: 3QVP, Kommoju et al. [54])](image)

**Figure 1.4:** Crystal structure of the *Aspergillus niger* glucose oxidase (PDB ID: 3QVP, Kommoju *et al.* [54]).
Figure 1.5: FADH\textsubscript{2} structure at left and at right is the reduction reaction of a simplified FAD structure to the FADH\textsubscript{2} counterpart at the active site of glucose oxidase upon oxidation of glucose.

$$\text{GOx (FAD)} + 2e^- + 2H^+ \rightleftharpoons \text{GOx (FADH}_2) \quad \text{Equation 1.5}$$

$$\text{GOx (FADH}_2) + O_2 \rightarrow \text{GOx (FAD)} + H_2O_2 \quad \text{Equation 1.6}$$

The utilisation of mediators to replace oxygen as the natural co-substrate allows for shuttling of the electron from the enzyme active site to an electrode surface (see section 1.4) and current generation at lower overpotentials, in comparison to systems based on reduction of oxygen or oxidation of peroxide. Also, the presence of mediators brings about competition between oxygen and the mediator as the final electron acceptor. This can lead to problems during development of in vivo enzyme anodes based on GOx, as oxygen, present in the fuel cell electrolyte, competes with the mediator. In addition, the catalytic oxidation of glucose to gluconolactone by GOx produces hydrogen peroxide when oxygen is the electron acceptor: hydrogen peroxide is a highly toxic product and can be detrimental to the activity of bioactive materials [55-58]. Nonetheless, GOx is extensively investigated for use in biosensors and biofuel cell applications due to its stability, substrate specificity, high electron turnover rate and commercial availability [59]. Although the FAD co-factor is deeply buried within the enzyme there are reports purporting direct electron transfer (DET) from GOx active site to electrodes, usually on nanocomposite electrode materials [60, 61]. In the case of GOx DET the active site of the enzyme is
reportedly directly connected to the electrode surface, although this is disputed [62] because of the large distance of the closest approach of the FAD active site to electrode material, further hampered by the requirement for orientation of enzymes on the electrode surface to enable DET to occur. The use of mediated electron transfer (MET) overcomes DET orientation and distance to active site limitations with co-immobilisation of enzymes and mediator on electrode surface providing a high density of mediators around the enzymes, effectively “wiring” it to an electrode. As noted above, however, mediated GOx-based anodes may prove disadvantageous due to oxygen competition with mediator or production of hydrogen peroxide when oxygen is the electron acceptor [56-58, 63]. Therefore, the use of enzymes that are insensitive to oxygen such as dehydrogenases may prove advantageous for application to glucose/oxygen EFCs.

1.3.1.2 Glucose dehydrogenase
Glucose dehydrogenase (GDH) is a class of oxidoreductase capable of oxidising glucose by transferring one or more electrons and protons to nicotinamide adenine dinucleotide (NAD) [64], a flavin such as FAD [65, 66] or a pyrrolo-quinoline quinone (PQQ) co-factor [65]. The use of GDH is gaining increased attention as a catalyst for glucose oxidation in biosensing and biofuel cell applications due to its ability to oxidise glucose without competition from oxygen as electron acceptor, and without producing hydrogen peroxide. The NAD⁺/NADH co-factor is not directly bound to the enzyme, although its presence is necessary in the bioelectrocatalytic function of the enzyme. The thermodynamic redox potential of NAD⁺/NADH is ~ −0.56 vs Ag/AgCl at neutral pH. However, the NADH co-factor itself is not a useful redox mediator for redox signalling because of the high overpotential and lack of electrochemical reversibility for the NADH/NAD⁺ redox process [28] and the interfering adsorption of the cofactor at electrode surfaces. Some glucose dehydrogenases use PQQ as co-factor instead of NADH (classified as EC 1.1.5.2). The PQQ has a redox potential of −0.16 V vs Ag/AgCl at pH 7.2 [67], although adoption of PQQ-dependent GDH in EFCs may be limited, at present, because to its relative instability when compared to GOx [27]. Nonetheless, the use of electrodes modified with PQQ-dependent GDH as glucose sensors and enzyme anodes has been investigated as a consequence of insensitivity to the presence of oxygen [27]. Alternate glucose dehydrogenases use FAD as co-factor (FADGDH; EC 1.1.5.9),
typically bound within the enzyme but can be removed, but are, unlike GOx, insensitive to the presence of oxygen. Yoshida et al. [68] reports on the structural analysis of fungus-derived FAD glucose dehydrogenase in comparison to GOx, and observed that the residues predicted to be associated with oxygen sensitivity in GOx are not conserved in FADGDH [68]. These differences may account for the oxygen insensitivity associated with FADGDH. In addition, increasing interest in the utilisation of FAD-dependent GDH expressed in an Aspergillus sp., is as a result of its high turnover rate, commercial availability and oxygen independence [69, 70].

1.3.2 Enzyme cathodes

In fuel cells that depend on oxygen reduction at the cathode, a platinum metal catalyst has typically been employed. Although platinum is a highly efficient catalyst, it is non-specific and easily poisoned, thereby necessitating the use of a membrane to prevent the migration of fuel to the cathode [65, 71]. The utilisation of enzymatic biocatalysts offer substrate selectivity, compared to Pt, in favour of the oxidant over the fuel, may eliminate the requirement for compartmentalisation of the anode and cathode, thereby allowing miniaturisation [71]. At present, the use of multi-blue-copper oxidases (MBCO), such as laccase and bilirubin oxidase has received much attention due to their ability to reduce oxygen to water at relatively high reduction potentials and under mild conditions [63, 71-74].

1.3.2.1 Laccase

Laccases (EC 1.10.3.1) are MBCO polyphenol oxidases usually containing four copper atoms per monomer distributed in three redox, capable of oxidising a broad range of substrates [72, 75]. Substrate oxidation occurs at a type 1 (T1) single “blue” copper site, at which point the catalytically produced electrons are transferred intramolecularly to the oxygen reduction site, ~1.5 nm away, consisting of a trinuclear cluster of a type 2 (T2) copper site and a coupled pair of type 3 (T3) copper sites [72].
Laccases are isolated from various sources such as plants, fungi and microorganisms [77, 78]: laccases isolated from plants have a low T1 potential of $\sim +0.23$ V vs Ag/AgCl, whereas T1 potential of fungal laccases range from mid- ($\sim +0.27$ V to +0.51 V vs Ag/AgCl) or high range ($\sim +0.58$ V vs Ag/AgCl) [79]. High range potential fungal laccases are inhibited by hydroxyl ions, with maximal activity between pH 4 to pH 5. This inhibition limits their use as cathodes in an EFC operating in physiological conditions [37]. However, use of a laccase sourced from the *Streptomyces coelicolor* bacterium and a laccase sourced from the *Myceliophthora thermophila* fungus has been reported for EFCs operating in physiological conditions [80, 81]. As the focus of this thesis is on the development of EFCs to be utilised *in vivo*, cathodes that operate under physiological pH conditions based on bilirubin oxidase, instead of laccases, are selected.

1.3.2.2 Bilirubin oxidase

Bilirubin oxidase (BOd) is a MBCO that catalyses the oxidation of bilirubin to biliverdin, with the concomitant reduction of $O_2$ to water in a four electron reduction
The enzyme active site contains 4 redox active copper atoms; the T1 copper site in which the substrate oxidation occurs and a tri-nuclear T2/T3 copper cluster in which oxygen reduction occurs [72]. A *Myrothecium verrucaria* bilirubin oxidase (MvBOd) is the first reported BOd-based oxygen-reducing cathode [83-85]. The MvBOd was adsorbed onto carbon felt electrodes with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in solution phase for mediated reduction of oxygen in phosphate buffer pH 7.0 [83]. Steady-state current density of 0.85 mA cm$^{-2}$ for oxygen reduction at potentials of ~ +0.2 V vs Ag/AgCl in oxygen saturated phosphate buffer at pH 7.0 with rotation at 1400 rpm is reported by Tsujimura *et al.* [86] using a direct electron transfer approach (see section 1.4) through the immobilisation of MvBOd enzyme in poly(L-lysine) layers at carbon electrodes containing a high density of crystal edges [86]. MacAodha *et al.* [87] co-immobilised a MvBOd and an osmium redox polymer, prepared by substitution of a Cl ligand of Os(2,2'-bipyridine)$_2$Cl$_2$ with poly(vinylimidazole), in the presence of multi-walled carbon nanotubes on 3 mm diameter graphite electrodes to produce an oxygen reducing current density of up to 0.83 mA cm$^{-2}$ at an applied potential of 0 V vs Ag/AgCl [86]. In addition, Mano *et al.* [88] co-immobilised BOd and a different osmium redox polymer, prepared by substitution of a Cl ligand of Os(4,4'-dichloro-2,2'-bipyridine)$_2$Cl$_2$ with imidazole units of a co-polymer of poly(vinylimidazole) and polyacrylamide, on carbon cloth fibres to yield oxygen reduction current densities of 0.7 mA cm$^{-2}$ at a potential of +0.3 V vs Ag/AgCl in non-stirred phosphate buffered saline at 37 °C [88]. An increase to 6.25 mA cm$^{-2}$ is achieved when electrodes are rotated at 4000 pm [89].
1.4 Electron transfer between enzymes and electrodes

In any fuel cell the efficiency of electron transfer between the catalyst and electrode surface is of great importance. The electron transfer between biocatalysts and electrode surfaces can be achieved, as already mentioned, by either direct electron transfer (DET) or mediated electron transfer (MET), as depicted in Figure 1.8 [21, 86, 91]. In DET electrons are transferred directly to the electrode surface from the enzyme active site. When using this approach various factors are to be considered such as the thickness of the protein shell surrounding the redox centres of the enzyme and the requirement of correct enzyme orientation at electrodes for efficient electron transfer kinetics [62, 92].
Figure 1.8: Schematic depicting (A) direct electron transfer and (B) mediated electron transfer oxidation of the substrate between an enzyme active site and an electrode.

Compared to MET, current densities achieved by DET are often quite low and therefore it is necessary in most cases to introduce an electron transfer mediator to shuttle electrons between the electrode and the redox active sites in the enzyme. To have an effective electron exchange during MET the thermodynamic redox
potentials of the enzyme and the mediator should be matched in order to provide a driving force for electron transfer [21, 93]. For biocatalytic electrodes it has been proposed that mediators must have redox potentials approximately 50 mV downhill from the redox potential of the enzyme [2, 94]. The tuning of these redox potentials is a compromise between the need to have a high cell voltage and a high catalytic current.

1.4.1 Enzyme electrodes using redox mediators

Mediators form a link to transport electrons from the active site of enzymes to the electrode when active sites are buried within a proteinaceous insulating envelope too distant from the electrode surface to permit rapid electron transfer. In enzyme electrode technology mediators are preferably chemically stable in both oxidised and reduced states: this is important to permit continuous participation in the electrocatalytic cycle. Cass et al. [95] first demonstrated the use of ferrocene as a mediator for electron transfer from the GOx active site to carbon-based electrodes. Ferrocene derivatives were co-immobilised with GOX at a pyrolytic graphite electrode for glucose-sensing applications. Tamaki et al. [96, 97] reported high current densities (>1 mA cm⁻²) for glucose-oxidising anodes using vinylferrocene-based films on high surface area carbon electrodes as a mediator at a redox potential of > 0.4 V vs Ag/AgCl. The relatively high redox potentials required for oxidation of ferrocene derivatives could contribute to cell voltage loss if such mediators are used in anodes in an EFC [95]. In addition, the oxidised form of ferrocene, ferricenium, is unstable in aqueous solution as it undergoes hydrolysis [98] and is not readily soluble in its reduced form in aqueous solvents, which as a result may lead to difficulties for enzyme electrode assembly. The use of a range of tris(4,4ʹ-substituted-2,2ʹ-bipyridine) complexes of iron, ruthenium and osmium as mediators for GOx mediation were explored by Zakeeruddin et al. [99]. The resulting complexes display redox potentials spanning a wider range in comparison to that observed for ferrocene derivatives. The relative stability of osmium based polypyridyl redox complexes in their Os(II) and Os(III) oxidation states for redox cycling and the rapid self-exchange rate constants [37], has led to implementation of osmium-based redox polymers as mediators for biosensors and biofuel cell applications. Also, the redox potentials of the Os(II/III) transition in osmium based polypyridyl complexes can be tuned through selection of, and chemical modification of ligands complexed to the metal.
centre [100] making them suitable as mediators for utilisation in both anodes and cathodes, for biofuel cell applications [1-4, 31]. In 1990 Forster and Vos [101] developed the polyvinyl-imidazole (PVI)-bound osmium bipyridine series of redox polymers, of structural motif as shown in Figure 1.9.

![Figure 1.9: Structure of osmium redox polymer formed by the coordination of osmium “starting complex” Os(R,R'-2,2'-bipyridine)2Cl2 to polyvinylimidazole (PVI) in a ratio that is usually 1:9.](image)

The adsorption of a redox polymer-bound mediator on an enzyme electrode allows control over the electroactive nature of an electrode and can prevent oxidation and/or reduction of other species at the electrode. Additionally, a polymer-bound mediator provides for greater structural stability of the enzyme electrode. In electrode preparation procedures, redox polymers and enzymes are crosslinked using homobifunctional crosslinkers, resulting in a matrix of an enzyme-containing redox hydrogel on the electrode surface. In the early 1990s Gregg et al. [102] developed an approach to entrap enzymes and mediators on electrode surfaces using epoxide cross-linkers, forming a three dimensional matrix redox hydrogel. Redox hydrogels are crosslinked polymer network structures containing redox active substances that swell in water and represent an electron-conducting phase in which water-soluble chemicals dissolve and diffuse [1, 2, 4, 93, 103]. By enveloping enzymes and mediators, redox hydrogels electrically “wire” the active site of the enzyme to the electrode surface. It is proposed that electron conduction in redox hydrogels is performed by self-exchange of electrons or vacancies between rapidly reduced and oxidised redox functions [104, 105]. Electron transfer takes place by collisions between the reduced and oxidised forms of the mobile redox centres that are tethered
to the polymer backbone (Figure 1.10). Increased interest has been shown, over the past 2 decades, in the use of PVI-bound osmium redox polymer as mediators for biosensor [106-108] and biofuel cell [87, 109-111] applications, and use of such mediators are the major focus of the studies reported throughout this thesis.

**Figure 1.10:** Schematic illustrating proposed electron conduction mechanism in redox hydrogels results with electron transfer between reduced and oxidised redox centres tethered to the backbone of polymers, adapted from [2].

### 1.4.2 Immobilisation strategies

A major challenge in the utilisation of enzyme electrodes in EFCs for power generation is the requirement to extend the EFC operational life-time [4]. An approach to addressing this challenge is through immobilisation of the enzyme and other components on solid electrode surfaces. There are various immobilisation strategies such as physisorption, covalent attachment, cross-linking and entrapment in polymeric or inorganic gels [107] to name a few. Based on the application the immobilisation approach can often be a combination of these. For example, use of multilayers or other three-dimensional structures is favoured over monolayer configurations in order to improve current output and/or stability. The use of crosslinkers may introduce a degree of rigidity to the hydrogel that can affect electron transfer within the film [112], along with prolonging the hydrogel film structural integrity [112]. Strategies for crosslinking have been developed over the past few decades in order to improve connectivity and stability of electroactive films on electrode surfaces. Ohara et al. [113] demonstrated the use of di-epoxide crosslinker, poly(ethylene glycol) diglycidyl ether (PEGDGE) (Figure 1.11) to co-immobilise an osmium redox polymer and GOx on electrodes. This study showed that the redox hydrogels were permeable to glucose and allowed diffusion of electrons [113]. In addition, De Lumley-Woodyear *et al.* [112] compared PEGDGE
crosslinking to that using suberic acid bis(N-hydroxysuccinimide ester), dimethyl suberimidate or glutaraldehyde (Figure 1.11) in solution for co-immobilisation of enzyme with redox-polymer mediators. Although glutaraldehyde had a considerable negative impact on the resulting current density as compared to the current densities observed for the electrodes prepared using the other crosslinkers, the use of glutaraldehyde vapours as a crosslinking agent is recently reported on for enzyme immobilisation [69, 107, 114-116], thereby providing an alternate methodology to the di-epoxide solid-phase or glutaraldehyde solution phase crosslinking reaction.

![Figure 1.11: Structure of PEGDGE (A) and glutaraldehyde (B) crosslinkers](image)

**1.4.3 Nanomaterials in enzyme electrodes**

Incorporation of conductive nanomaterials, such as carbon nanotubes (CNTs) in enzyme electrodes may substantially enhance current densities, due to improved surface area and/or electrical connections, and signal stability, and is therefore an active area of research for enzyme electrode technology. For example, reports have shown that addition of carbon micro-, meso- and nanostructured materials to enzymatic electrodes show improved current signal over those prepared without addition of such materials [37, 64, 107, 117-121]. Since the discovery of CNTs in 1991 they have been the subject of intense research because they exhibit intrinsic electrical/mechanical properties which appear to be of technological significance [122]. For example, recent studies have shown that CNTs can promote electron transfer from proteins to electrode surfaces [123-126], which is attributed to their high electrical conductivity [127]. CNTs are graphene sheets that are rolled up to form tubular structures. Different types of CNTs can be created depending on the conditions of the nanotube synthesis, which include growth catalyst, temperature,
and concentration of starting material. Multi-walled CNTs (MWCNTs) consist of multiple rolled layers of concentric tubes of graphene [127], whereas single-walled CNTs (SWCNTs) consist of a single graphene sheet that is rolled up [128]. The conductivity of CNTs is directly related to the number of walls each carbon nanotube has [127]. For example, MWCNTs are more conductive than SWCNTs.

The addition of gold nanoparticles (AuNPs) to enzyme electrodes also improves electrical contact between enzyme and electrode, thereby promoting direct electron transfer between enzyme and electrode [129-131]. For instance, oxygen reduction utilising a BOD/AuNPs modified electrode has been developed by Murata et al. [131] to produce an oxygen reduction current density of 5.2 mA cm\(^{-2}\) when stirred at 4000 rpm, compared to little or no current signal in the absence of AuNPs. These enzyme electrodes were shown to retain 94\% and 90\% of initial current density after 24 h and 48 h, respectively, showing relatively high stability ascribed to the suppression of desorption of enzyme by adsorption into the mesopore of the AuNP assembly [131]. This BOD/AuNPs electrode was connected to a fructose dehydrogenase anode to form a membrane-less EFC, with carbon paper used as the electrode substrate, in O\(_2\)-saturated 100 mM acetate buffer solution at pH 6.0 containing 200 mM D-fructose. A maximum power density of 0.87 mW cm\(^{-2}\) operating at 0.3 V was achieved for the cell, in stirred electrolyte solution.

1.5 Enzymatic fuel cells

A potential application of enzyme electrode technology is as a power generating source operating in physiological conditions. It is however important to consider various physiological factors such as pH of fluids, temperature, velocity and concentration of substrates or inhibitors. In the human body the oxygen concentration in arteries and veins is ~ 2.14 × 10\(^{-4}\) M and 5.4 × 10\(^{-5}\), respectively, while the concentration of glucose in blood vessels in healthy adults is 5-8 mM [50, 132]. Blood is a buffered solution with a pH of 7.4 and the human body is thermostated at 37 °C. In blood vessels the velocity of blood is of the order of 1 to 40 cm s\(^{-1}\) [133]. Since the first demonstration of a biofuel cell by Yahiro et al. [26] recent advancements have focused on the development of membrane-less enzyme-based glucose oxidising/oxygen reducing fuel cells capable of operating in vivo [134, 135]. Such a membrane-less system requires catalysts specific to the fuel and oxidant to be immobilised at non-catalytic electrode surfaces. In the late 1990s Katz
et al. [136] reported on a membrane-less EFC that achieved 5 μW cm⁻² power generation at 0.064 V in 1 mM glucose. The EFC was composed of a reconstituted apo-GOx coupled to a (PQQ) relay conjugated to a thiol-modified gold anode and a cross-linked cytochrome c oxidase assembled on a gold cathode. In recent times, advancements in EFC research have led to the development of a miniature, membrane-less mediated EFC by the Heller group [94, 117, 137]. This EFC consists of GOx immobilised at the anode and laccase at the cathode, each electrode consisting of a carbon fibre and containing an appropriate redox polymer mediator. The EFC generated a power of 0.6 μW (137 μW cm⁻²) at 0.4 V cell voltage, in 37 °C chloride-free pH 5.0 citrate buffer containing 15 mM glucose [94]. Soukharev et al. [138] reported an EFC using GOx and fungal laccase, co-immobilised with osmium redox polymers on 7 μm diameter, 2 cm long carbon fibres, produces a power density of 350 μW cm⁻² in 15 mM glucose, pH 5 buffered, solutions. Under similar conditions, except in 5 mM glucose solutions and using GOx sourced from *Penicillium pinophilum*, an EFC was reported to produce a power density of 280 μW cm⁻², which is the highest operating EFC in 5 mM glucose reported to date [139]. In addition, Mano et al. [140] reported on a similar EFC using carbon fibre but operating under pseudo-physiological, pH 7.4 in chloride-containing, conditions, in 15 mM glucose, that produced 1.9 μW at 0.52 V cell voltage, where bilirubin oxidase was utilised instead of a laccase. The selection of BOD as oxygen reducing enzyme permitted operation under the physiologically relevant pH condition. Gao et al. [141] developed an analogue of this fuel cell, but used electrodes composed of nanotube wires, and produced a power density of 740 μW cm⁻² at 0.57 V cell voltage, under the same conditions.

Despite advances in miniaturisation of EFC, little has been reported on testing of such systems in blood or artificial plasma. MacAodha et al. [87] described an EFC consisting of an FAD-dependent GDH co-immobilised with an [Os(4,4'-dimethyl-2,2'-bipyridine)₂(polyvinylimidazole)₁₀Cl]⁺ (Os(dmbpy)PVI) redox polymer at the anode and a *Myceliophthora thermophila* laccase with an [Os(2,2'-bipyridine)₂(polyvinylimidazole)₁₀Cl]⁺ (Os(bpy)PVI) redox polymer at the cathode, each co-immobilised onto a graphite electrode in the presence of MWCNTs, producing a power density of 110 μW cm⁻² in 5 mM glucose in PBS that dropped to 60 μW cm⁻² in artificial plasma. Ó Conghaile et al. [142] assembled an EFC
consisting of *Streptomyces coelicolor* laccase co-immobilised with a redox complex [Os(2,2'-bipyridine)$_2$(4-aminomethylpyridine)Cl]$^+$ for oxygen reduction at the cathode, and PQQGDH co-immobilised with [Os(4,4'-dimethoxy-2,2'-bipyridine)$_2$(polyvinylimidazole)$_{10}$Cl]$^+$, (Os(dmobpy)PVI) for glucose oxidation at the anode tested in both physiological buffer and human serum. The EFC produced a power output of 66 μW cm$^{-2}$ in 5 mM glucose, 150 mM NaCl, phosphate buffer solution at 37 °C, and 37 μW cm$^{-2}$ in human serum, the highest reported power density to date in serum [142].

1.6 Design of experiments

Experimenters are typically interested in changing one or more process variables (or factors) in order to observe the effect the changes have on one or more response variables, but often each factor is evaluated by isolation, keeping all other factors constant. This experimental approach is termed one-factor-at-a-time (OFAT). However, the use of a systematic method through the selection of factorial experimental designs to determine the relationship between factors affecting a process and the output of that process is a more efficient type of experiment that may be carried out [143, 144]. The (statistical) design of experiments (DoE) is an efficient procedure that provides a structure to experimental planning so that the data obtained can be analysed to yield valid and objective conclusions [144-146]. After the execution of a purposefully designed experiment, cause-and-effect relationships in a process or system, between experimental output and experimental factors, are established. There have been various reports of the application of DoE in solving scientific and engineering problems [147-150]. For example, Flexer *et al.* [151] proposed and described the use of an experimental design methodology combined with relaxation simulation technique to simulate the amperometric response of self-assembled GOx and osmium poly(allylamine) electrodes to extract the unknown kinetic parameters from experimental data for the steady-state current at different glucose concentrations. The resulting simulated currents are shown to be in excellent agreement with the experimental results achieved. This same methodology was further used to extract case diagrams by systematically varying the film thickness, glucose concentration and redox potential and hence redox mediator concentration in the enzyme multilayer films [152]. The results obtained showed that the enzyme electrode wiring efficiency increases in proportion to the number of layers for film
thickness above 300 nm, approaching the behaviour of redox hydrogels [152]. More recently, Babanova et al. [153] reported on a DoE approach for optimisation of the performance of an air-breathing bilirubin oxidase-based EFC cathode.

When using DoE, the output is a mathematical equation that attempts to fit an empirical model to the data collected in the experiment [153]. The experimental output, $y$, may be described by the following equation in relation to the experimental factors:

$$ y = f(x_1, x_2, \ldots, x_n) + \varepsilon $$

Equation 1.7

Where $\varepsilon$ is experimental error, and thereby implies that the functional relationship between the chosen experimental factors $(x_1, x_2, \ldots, x_n)$ and $y$ may not be fully explained [144]. An example of a process is illustrated in Figure 1.12, describing a system with all of its associated variables such as operators, equipment, procedures, environmental conditions, raw materials etc. changing an input into an output and having one or more observable response variables. Process variables and material properties are either controllable $(x_1, x_2, \ldots, x_n)$, or uncontrollable $(z_1, z_2, \ldots, z_p)$.

![A model of a process or system.](image)

**Figure 1.12:** A model of a process or system.
Controllable input factors, or x factors, are those input parameters that can be modified in an experiment or process, while uncontrollable input factors are those parameters that cannot be changed. In the DoE, it is important to recognise these factors in order to understand how they may influence the response variable. Responses, or output measures, are the elements of the process outcome that gage the desired effect. When designing an experiment, the initial step is recognition of the knowledge gap or problem in the process that needs to be solved [144]. Following definition of the problem, there are three general stages of DoE, these are:

a) **Selection of factors**: Factors are selected on the basis that they may affect the result of an experiment.

b) **Choosing the design of the experiment and performing the experiment**: The experimental values for each factor are organised in a structure that minimises the effect of uncontrolled factors and allows for meaningful statistical comparisons.

3) **Data analysis**: Statistical model such as analysis of variance (ANOVA) is utilised to predict and validate the accuracy of the design through graphical or numerical optimisation of parameters for desired response.

To understand and optimise desired response, a response surface design methodology is often used to refine models after one has determined important factors using factorial designs. The types of response surface designs include: central composite design, Box-Behnken design and D-optimal design [143, 154]. As Chapter 5 in this thesis focuses on the use of DoE for the optimisation of the component selection amounts in preparation of enzyme electrode for application as a glucose-oxidising anode, this section will discuss the utilisation of Box-Behnken design in DoE. The Box-Behnken Design (BBD) is a rotatable second order design based on a three-level factorial design: these are less expensive to run in comparison to central composite design with the same number of factors and can efficiently estimate the first- and second-order coefficients. The Box-Behnken design requires an experiment number according to $N = 2k (k-1) + C_0$, [155] where $k$ is the number of factors, and $C_0$ is the number of central points. In this design, the treatment combinations are at the midpoints of edges of the process space and at the centre, and these designs are rotatable (or near rotatable). An illustration of the Box-Behnken design is shown in Figure 1.13 below.
Figure 1.13: A Box-Behnken design for three factors

The geometry of this design suggests a sphere within the process space such that the surface of the sphere protrudes through each face with the surface of the sphere tangential to the midpoint of each edge of the space. For three factors, the Box-Behnken design offers some advantage in that it requires a fewer number of runs in comparison to central composite design that can fit a full quadratic model [144, 150]. Kumar et al. [150] recently demonstrated the use of Box-Behnken design to improve the current output of enzyme electrodes prepared using multiple components (mediator, enzyme, MWCNTs) for application as anodes in an EFC [150]. According to the report, components used to construct enzyme electrodes were optimised and validated using DoE, with a resulting glucose oxidation current density of $1.2 \pm 0.1 \text{ mA cm}^{-2}$ in PBS, significantly higher by 32% than the previously observed current density for enzyme electrodes optimised by varying one factor at a time [149, 155]. The application of experimental design is therefore useful for the optimisation of component amounts in enzyme electrodes for application to EFCs.
1.7 Electroanalytical techniques
Electrochemical methods are utilised as techniques to determine electrical characteristics and properties of analyte/films. Throughout this thesis electroanalytical methodologies are utilised to characterise the systems under investigation. A brief description of the basic principles of electroanalysis is therefore presented in the following sections.

1.7.1 Voltammetric and amperometric technique
Voltammetry is a very useful technique when performing electroanalysis: it includes a collection of methods in which the relation between electric current and voltage is observed during the electrochemical process. It is the measurement of variations in current produced by variations in potential of a working electrode. Polarography is the first voltammetry experiment, conducted by Heyrovsky (1922) using a dropping-mercury electrode [157-161]. Electron transfer in voltammetry occurs between electrodes, usually a solid or liquid, and electroactive species, usually in solution, once the applied potential is sufficiently oxidising or reducing. Cyclic voltammetry (CV) is the fundamental and most effective electroanalytical technique for studying redox systems. In voltammetry an initial potential is applied across the electrochemical cell, and is ramped linearly at rates of \(0.001 - 10^5 \text{ V s}^{-1}\) [10, 162, 163]. Cyclic voltammetry is usually undertaken in an unstirred solution, with semi-infinite diffusion and no migration or convection contribution, by cycling the potential applied at a working electrode and measuring the resulting current, with the potential of the working electrode controlled versus a reference electrode [162]. The potential waveform that is applied across the electrode/solution interface for CV is illustrated in Figure 1.14 and 1.15.
Working electrodes are generally made of relatively electrochemically inert carbon such as glassy carbon and graphite or inert metals such as platinum and gold. The reference electrode utilised is usually the silver/silver chloride (Ag/AgCl) electrode in 3 M chloride solution. It is simple to construct, inexpensive, and provides a stable potential. A saturated calomel electrode (SCE) may also be utilised as a reference electrode. A platinum wire is normally used as the counter electrode, with a large enough area to ensure the reaction at the counter electrode does not limit current flow through the working electrode [162].
Figure 1.15: A typical cyclic voltammogram for surface confined (A) and solution (B) electrochemistry.

An electrochemically reversible reaction is characterised by the rate of heterogeneous electron transfer reaction being fast enough to ensure that the relative concentrations of the reduced and oxidised species at the electrode surface are in thermodynamic equilibrium, and the potential is defined by the approximate Nernst equation [163] (Figure 1.15).

\[ E = E^{\circ} - \frac{0.059}{n} \log \frac{[\text{red}]}{[\text{ox}]} \]

Equation 1.8
Where \( E \) is the electrode potential relative to the \( E' \), the standard electrode potential, \( ox \) is the oxidised species, \( red \) is the reduced species and \( n \) is the number of electrons in the half reaction. For a reversible semi-infinite diffusion controlled electrochemical reaction, the voltage separation between the current peaks is:

\[
\Delta E = E_{pa} - E_{pc} = 59/n \text{ mV}
\]

Equation 1.9

Where \( E_{pa} \) is the anodic peak potential and \( E_{pc} \) the cathodic peak potential. Peak currents scale proportionally to the square root of scan rate (\( \nu \)) and the ratio of peak currents is equal to one. The peak current for an electrochemically reversible reduction or oxidation is given by the Randles-Sevčik equation (1.12).

\[
i_p = 0.4463 n F \sqrt{\frac{nF}{RT}} AD^{1/2} C^{1/2} \text{ mV}
\]

Equation 1.10

Where \( i_p \) is the peak current in Amperes, \( n \) is the number of moles of electrons being transferred, \( A \) is the electrode area (cm\(^2\)), \( D \) is the diffusion coefficient (cm\(^2\) s\(^{-1}\)), \( C \) is the concentration (mol cm\(^{-3}\)) and \( \nu \) is the scan rate (V s\(^{-1}\)). The CV is said to show irreversible or quasi-reversible behaviour if the rate of electron transfer is slow relative to the scan rate. For a system that is not reversible, electron transfer becomes the rate determining step and Nernstian equilibrium is not maintained, i.e. the peak to peak separation is greater than \( 59/n \text{ mV} \) and increases with increasing scan rate [162].

Characteristics of an ideal Nernstian system for CV of a monolayer of surface confined species, where there are no lateral interactions between neighbouring redox centres, are:

\[
FWHM = \frac{3.53 RT}{nF} = \frac{90.6}{n} \text{ mV}
\]

Equation 1.11

\[
E_{pa} = E_{pc}
\]

Equation 1.12

Where FWHM is the full width at half maximum of the anodic or cathodic wave [162, 165]. When the timescale of the voltammetric experiment is long enough to permit all of the species to be electrolysed this response may also be observed for both multilayer films of confined electroactive species or redox reactions in thin-layer cells. In this case an estimate of concentration of electronically addressed redox sites by the electrode is characterised as a surface coverage (\( \Gamma \), in moles/cm\(^2\)) for
confined species, and can be calculated from the integration of the Faradaic charge ($Q$) passed, usually under conditions of slow scan rate voltammetry from:

$$\Gamma = \frac{Q}{nFA}$$  
\text{Equation 1.13}

Where $Q$ is the charge passed (in coulombs), $n$ is the number of moles of electrons, $F$ is Faraday’s constant and $A$ is the area of the electrode (cm$^2$).

In amperometry, chronoamperometry or constant potential recording, a fixed potential is held at the working electrode, versus a reference electrode, and the current flow monitored with time.
1.8 Thesis proposition

The aim of this thesis is to investigate approaches to improve enzyme electrode performance for use as anode in an enzymatic fuel cell. Development was performed by focussing on benchmarking the performance of films prepared using novel methodologies against those prepared using more established methodologies. Chapter 2 is concerned with the synthesis and characterisation a range of osmium polypyridyl complexes. The complexes were characterised electrochemically and using a range of analytical techniques, including CHN microanalysis and mass spectrometry. The resulting osmium polypyridyl complexes are then used in the synthesis of redox polymers, such as an Os(dmbpy)PVI polymer. The electrochemical characteristics of films of the redox polymers on electrodes were evaluated using CV. The Os(dmbpy)PVI redox polymer is co-immobilised with GOx on graphite electrodes in the presence of MWCNTs using glutaraldehyde vapour with a NaBH₄ reduction crosslinking methodology. This methodology is used due to the fact that previous reports show that utilising glutaraldehyde vapour with a NaBH₄ reduction crosslinking strategy leads to improved glucose oxidation current density and stability of the enzyme electrodes [107]. Addition of different amounts of MWCNTs was undertaken to evaluate the effects of MWCNTs amount on enzyme electrode signal, using the same crosslinking method. Although the use of the glutaraldehyde vapour with a NaBH₄ reduction crosslinking strategy show improved current signals and/or stability, the NaBH₄ reduction approach in crosslinking methodology may affect the operation of enzyme electrodes due to less control in the borohydride reduction step, which could result in detachment of films on electrode surface. Due to difficulties in the control during enzyme electrodes preparation using GA(R) crosslinking, poly(ethylene glycol) diglycidyl ether PEGDGE, as the more widely accepted methodology, is utilised as a crosslinker in chapter 3, 4 and 5 of this thesis.

Chapter 3 reports on the effect of varying the amount of MWCNTs on the response of enzyme electrodes to glucose oxidation utilising PEGDGE as a crosslinker. The enzymatic electrodes were prepared by co-immobilisation on graphite of an Os(dmbpy)PVI redox polymer and either GOx or a FAD-dependent GDH. The Os(dmbpy)PVI was selected because of its lower redox potential (0.12 V vs Ag/AgCl) in comparison to an Os(bpy)PVI redox polymer (0.22 V vs Ag/AgCl)
used in previous reports [107]. This lower redox potential can contribute to an overall increased difference in voltage between anode and cathode of an assembled EFC leading to improved power output of the fuel cell. GOx was utilised as a benchmark for the performance of electrodes based on glucose-oxidising enzymes. However, it utilises FAD as cofactor which is reduced to FADH$_2$ that is then re-oxidised by the final electron acceptor which is naturally oxygen or redox polymer mediator in this case. The competition between oxygen and mediator on the enzyme electrode can affect current output in an EFC, as a result, comparison to anodes based on glucose dehydrogenase that do not donate electrons to oxygen, is of interest.

In Chapter 4, the use of an Os(dmobpy)PVI redox polymer co-immobilised with either GOx or FADGDH on graphite electrodes with varying amounts of MWCNTs is reported, in an attempt to improve the current and cell voltage of glucose|oxygen EFCs. The Os(dmobpy)PVI is utilised as it has a lower redox potential, −0.02 V vs Ag/AgCl, compared to 0.12 V vs Ag/AgCl, for the Os(dmbpy)PVI used in Chapter 3. The MWCNT amount added to Os(dmobpy)PVI enzyme electrodes is optimised for current output. Membrane-less EFCs operating in pseudo-physiological conditions (5 mM glucose, 50 mM phosphate buffered saline, pH 7.4, 37 °C) were assembled and compared using anodes based on either Os(dmbpy)PVI or Os(dmobpy)PVI as mediator combined with a cathode consisting of bilirubin oxidase (BOd) crosslinked with MWCNTs and Os(bpy)PVI redox polymer as mediator on graphite electrodes.

Chapter 5 focuses on optimisation of individual component amounts in the preparation of enzyme electrodes. These components are MWCNTs, the FADGDH enzyme and Os(dmobpy)PVI mediator. Optimisation is achieved using a response surface methodology in order to maximise current density provided by the enzyme electrode. The DoE model is developed and validated for enzyme electrode performance in pseudo-physiological conditions (5 mM glucose, 50 mM phosphate buffered saline, pH 7.4, 37 °C). The components were co-immobilised using PEGDGE on a graphite electrode surface as previously reported in Chapters 3 and 4. The optimised enzyme electrode is used as an anode in an EFC for application to power generation in pseudo-physiological conditions, artificial plasma and human plasma.
Finally, Chapter 6 summarises the contribution made towards the scientific body of knowledge by this thesis, outlines other research undertaken during the course of the PhD, and provides opinion on the direction such research may take in the future. Also, an appendix is included providing a complete list of my publications as well as my oral and poster presentations I have made over the course of my PhD studies.
1.9 References


Chapter 2: Materials and Methods

2.1 Synthesis and characterisation of polypyridyl osmium complexes

There has been intensive research into the use of polypyridyl transition metal complexes for applications in rechargeable batteries [1, 2], sensors and biosensors [3-7], electrocatalysis [8, 9] and more recently fuel cells [10-14]. Polypyridyl transition metal complexes have been studied extensively due to their photochemical and photophysical properties, which have diverse application [15-19]. The group 8 transition metals have been extensively utilised, due to the fact that the metal ions form low-spin octahedral complexes with strong-field ligands such as 2,2’-bipyridine [20]. Metal complexes of osmium and ruthenium are of interest because of their relative stability in both the oxidised and reduced states [19, 21-26].

Although the photophysical and photochemical properties of the polypyridyl complexes of osmium (Os) are similar to those of analogous complexes of ruthenium (Ru), osmium possesses many added advantages over Ru and other group 8 metals due to differences in electronic structure [21] such as:

- Greater extension of the metal d orbitals, enhancing metal-ligand back-bonding and thereby providing increased complex stability.
- A lower third ionisation energy for Os, leading to a lower redox potential for complexes of Os(II) and consequent stabilisation of higher oxidation states.

In bonding interactions between ligand and the central metal ion as described by ligand field theory, the separation of orbitals refers to the ligand-field splitting parameter (ΔO), where the subscript O signifies an octahedral crystal field [20]. The ΔO for Os is shown to be larger than that of Ru; as a result, the energy of the excited state is much higher for the osmium complexes than for their Ru analogues. Therefore making osmium polypyridyl complexes more photostable than that of ruthenium, as all excited states involved in the photochemical processes are ligand based. The above advantages of Os over other group 8 metals has led to complexes of osmium being employed where low redox potentials are required for minimising the effects of interferences in biosensors [3-5] or as catalysts in bio-anodes of biocatalytic fuel cells [10-14].
The redox potential of a $M(n)/M(n-1)$ redox couple is affected by the nature of the ligands, as the amount of electronic charge localised on the metal centre (and thus, the tendency to lose an electron) is governed by the $\sigma$ ($\sigma$-donor orbitals localised on nitrogen atoms) and $\pi$ ($\pi$ donor and $\pi^*$ acceptor orbitals more or less delocalised on aromatic rings) properties of the ligands. For instance, the presence of electron withdrawing substituents on ligands of the same series results in an increase of redox potential of the complex, while the opposite occurs with electron donating substituents. A clear example of the effect of ligand substituent on redox potential is seen for the series of osmium polypyridyl complexes of 2,2'-bipyridine ($bpy$) and its derivatives, as presented in Figure 2.1. The presence of an electron-donating substituent such as $-\text{OCH}_3$ at the 4 and 4' positions of bipyridine leads to a shift in redox potential to $-0.27$ V, whilst the presence of an electron withdrawing substituent such as $-\text{Cl}$ at the 4 and 4' positions leads to an shift in redox potential of $0.2$ V [28].

A method was developed by Lever [29] that evaluates the ligand contributions to redox potential of such transition metal complexes. This model assumes that ligand contributions to redox potentials of $M(n)/M(n-1)$ couples are additive, therefore, the redox potential can be calculated according to equation 2.1

$$E_{\text{calc}} = S_M [\Sigma E_L(L)] + I_M$$

Equation 2.1

Where $E_L(L)$ is the ligand electrochemical parameter, $S_M$ and $I_M$ are the slopes and the $y$-intercept of a plot of $E_{1/2}$ versus $\Sigma E_L(L)$. The $S_M$ and $I_M$ values are specific for the spin state and redox couple of the metal, and the solvent used. In this model, the electrochemical parameters for the ligand were derived from a $[\text{Ru(bpy)}_nX_{6-2n}]^{2+}$ ($n = 0-3$) set of compounds where X may represent monodentate or polydentate ligands. The $[\text{Ru(bpy)}_3]^{2+}$ cation was first considered in this study: its Ru(III)/Ru(II) redox potential occurs at 1.53 V vs NHE in acetonitrile, LiClO$_4$ as supporting electrolyte [29]. By utilising Levers’ guidelines [29] a simplistic method for prediction of redox potentials has been established.
Since the 1940s the synthesis and characterisation of polypyrpyridyl complexes of both Os and Ru has been an active area of research [19, 21, 30, 31]. The first reported Os polypyrpyridyl complex was among the products of pyrolysis of mixtures of potassium hexachloroosmate and bipyridine [32]. Successive synthetic methods which have
been developed provide an efficient high-yielding synthesis of complexes of general formula \( \text{cis-}[M(N-N)_2\text{Cl}_2] \) (where \( N-N \) = polypyridyl ligand) [21, 33], see Figure 2.1.

In this chapter, the synthesis and characterisation of a range of osmium polypyridyl complexes is described, which can subsequently be used in biosensor and enzymatic fuel cell devices. The formal redox potentials (\( E^{\circ} \)) of the osmium complexes were manipulated through appropriate substitutions on the polypyridyl ligands. Complexes with osmium as the metal centre are utilised because of the chemical stability, and low redox potential, in comparison to Ru analogues. Also, osmium polypyridyl complexes have been extensively characterised in the literature and synthetic procedures are well-developed. Subsequent to the production of \( \text{cis-}[\text{Os}(N-N)_2\text{Cl}_2] \), one of the chlorine ligands is substituted with a pendant monomer of a poly(N-vinylimidazole) (PVI) polymer, as ligand (1:10 ratio of Os complex and monomer unit added) to form polymer bound complexes suitable for adsorption to solid electrode surfaces.

### 2.1.2 Materials and reagents

2,2′-bipyridine, 4,4′-dimethyl-2,2′-bipyridine, 4,4′-dimethoxy-2,2′-bipyridine, ammonium hexachloroosmate, ethylene glycol, acetonitrile, sodium dithionite, N-vinylimidazole, tetraethylammonium perchlorate, ethanol and diethyl ether were purchased from Sigma and used as received. Glucose oxidase from *Aspergillus niger* (GOx EC 1.1.3.4.) purchased from Sigma-Aldrich, MWCNTs (Sigma-Aldrich) was prepared by stirring under reflux in nitric acid for 6 h and isolated by filtration, glutaraldehyde solution (25%) (Sigma-Aldrich).

### 2.1.3 Ligand abbreviations

The following abbreviations for ligands are used: bpy = 2,2′-bipyridine; dmbpy = 4,4′-dimethyl-2,2′-bipyridine; dmobpy = 4,4′-dimethoxy-2,2′-bipyridine.
2.1.1 Instrumentation and techniques

Electrochemical tests were conducted using a CH Instrument 600 potentiostat in a three electrode cell containing 50 mM phosphate buffer (PBS) pH 7.4, 150 mM NaCl at 37 °C purged with nitrogen, using Ag/AgCl (3 M KCl) reference electrode, abbreviated to Ag/AgCl throughout the thesis, 3 mm graphite working electrode and a platinum mesh counter electrode (Goodfellow). Cyclic voltammograms (CV) of the cis-[Os(N-N)Cl₂] complexes (where (N-N) = poly(pyridyl ligand) were recorded in CH₃CN or DMSO solvent with 0.1 M tetraethylammonium perchlorate (TEAP) as supporting electrolyte. Phosphate buffer was made up using appropriate amounts of both Na₂HPO₄ and NaH₂PO₄ to give final pH value of 7.0 at room temperature.

Electrospray mass spectrometry (ESI-MS) was performed on a tandem quadrupole mass spectrometer in conjunction with tandem mass spectrometry. The complexes were delivered to the gas phase, using a Micromass, Qtof 1 spectrometer, by electrospraying 0.1 mM sample solutions of the complexes, dissolved in acetonitrile, at a flow rate of 30 μL min⁻¹. Spectra were recorded, in the positive ion mode over, typically, an average of 30 scans, using a cone voltage of 40 V, while keeping capillary constant at 3500 V.

C, H, and N elemental microanalyses were carried out by NUIG Microanalytical laboratory.

2.1.4 Synthesis of poly(N-vinylimidazole)

The monomer N–vinylimidazole, 99% (Aldrich) was purified by vacuum distillation, using a short path vacuum distillation Kugelrohr apparatus (Buch Gkr-5, Germany). This procedure removed the hydroquinone that inhibits polymerisation of the
monomer. The colourless monomer was then purged with nitrogen and bulk polymerisation was carried out using a free radical initiator 2,2'-azobisisobutyronitrile (AIBN) as follows, 10 ml (0.11 mol) of 1-vinylimidazole was dissolved in 30 ml of absolute ethanol, and then stirred with AIBN (362 mg, 0.0022 mmol) at 60 °C for 2 hours. This solution was then heated at reflux to achieve bulk polymerisation over 4 hrs. The product was filtered and the residue was then redissolved in ethanol. This polymer was precipitated by addition of the ethanolic solution to a large excess of diethyl ether and collected by filtration. The pale yellow polymer was dried under vacuum and then at 80 °C in a pre-heated oven. The product was ground (using a pestle and mortar) to form a fine off-white powder (2.31 g)

2.1.5 Synthesis of osmium polypyridyl complexes

Figure 2.3 Simplified reaction scheme for synthesis of Os polypyridyl complexes of general formula \textit{cis}-[Os(N-N)\textsubscript{2}Cl\textsubscript{2}].

General synthesis of \textit{cis}-Os(N-N)\textsubscript{2}Cl\textsubscript{2} complexes was carried out according to published procedures [21]. Generally, (NH\textsubscript{4})\textsubscript{2}OsCl\textsubscript{6} and a slight excess of twice the molar equivalent of the ligand (N-N) were dissolved in ethylene glycol and held at reflux for 45 minutes. After cooling to room temperature, an excess amount of aqueous sodium dithionite is added to reduce Os(III) to Os(II). The mixture was stirred in ice for 45 minutes before filtering. The precipitate is then washed with water followed by washing with large volumes of diethyl ether. The resulting complex is dried under vacuum. Yields in excess of 80% were consistently obtained. Complexes were characterised using CV, ESI-MS and CHN.
2.1.6 Synthesis of poly(N-vinylimidazole) polymers containing \( \text{cis-Os(N-N)Cl}_2 \) redox centres.

Poly(N-vinylimidazole) (PVI) polymers containing \( \text{cis-Os(N-N)Cl}_2 \) redox centers were synthesised according to literature procedures described by Forster and Vos [34]. The osmium complex, of general formula Os(N-N)Cl\(_2\), was allowed to reflux in ethanol for 20 minutes to ensure complete dissolution of the complex. A ten molar equivalent of PVI dissolved in ethanol was added slowly to the ethanol solution in 1 ml aliquots. The mixture was allowed to reflux for ~ 3 days and the reaction monitored at regular intervals using cyclic voltammetry. After the reaction reached completion the product was precipitated by adding the solution drop-wise to a solution of stirring diethyl ether. The product was then suction filtered and allowed to air-dry. It was then further dried at 50 °C in a pre-heated oven overnight.

![Diagram of reaction](image)

**Figure 2.4** Simplified reaction for synthesis of Os polymer complexes of general formula \( \text{cis-}[\text{(Os(N-N)Cl}_2\text{PVI})_{n+1}\text{Cl}]^+ \) (n = 9).

2.1.7 Characterisation of \( \text{cis-[Os(N-N)Cl}_2 \) complexes.

A range of \( \text{cis-osmium polypyridyl complexes} \) was synthesised following a general procedure described by Meyer and co-workers [21]. The solubility of each complex varied according to substitutions on the coordinating polypyridyl ligand. In general, solubility decreased with increasing electron-donating character of ligand substituent. For example, Os(dmobpy)\(_2\)Cl\(_2\) is less soluble than Os(4,4'-dimethyl-2,2'-bipyridine Os(dmbpy)\(_2\)Cl\(_2\) in acetonitrile, while the highest solubility was observed for Os(bpy)\(_2\)Cl\(_2\).
Table 2.1

Electrochemical and mass spec values evaluated for osmium complexes. (* recorded in CH₃CN solution with 0.1 M TEAP as supporting electrolyte, ** molecular ion detected by ESI-MS in CH₃CN solution).

<table>
<thead>
<tr>
<th>Complex</th>
<th>E°ʹ (vs Ag/AgCl)</th>
<th>m/z**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Os(bpy)₂Cl₂</td>
<td>0 mV</td>
<td>574</td>
</tr>
<tr>
<td>Os(dmbpy)₂Cl₂</td>
<td>−120 mV</td>
<td>630</td>
</tr>
<tr>
<td>Os(dmobpy)₂Cl₂</td>
<td>−270 mV</td>
<td>694</td>
</tr>
</tbody>
</table>

Characterisation of the osmium complexes was achieved using CV, ESI-MS and CHN microanalysis. Table 2.1 shows the redox potentials and molecular ion (m/z) evaluated for each of the Os(II) complexes. Formal redox potentials (E°ʹ) were calculated from the mean of the oxidation and reduction peak potentials, corresponding to the Os(II) and Os(III) redox couple, evaluated by CV.

It is evident that value of E°ʹ is highly sensitive to ligand substituent, varying between −270 and 0 mV (vs Ag/AgCl), depending on the nature of the substituent group. Also, the redox potentials observed for each of the different redox complexes comparable are comparable to results already determined by others [21, 34].
Figure 2.5 Proposed structure, CVs, and redox potential of (a) Os(bpy)$_2$Cl$_2$, (b) Os(dmbpy)$_2$Cl$_2$ and (c) Os(dmobpy)$_2$Cl$_2$. CVs recorded on films of redox complexes at GC electrodes in CH$_3$CN solution with 0.1 M TEAP as supporting electrolyte at room temperature at 100 mV s$^{-1}$ scan rate.

As a result of the complexity of NMR spectra of coordination compounds, and limited structural data produced by electrochemical and photochemical analysis, structural characterisation of cis-osmium compounds has heavily depended on microanalysis. The utilisation of ESI-MS and CHN microanalysis for the analysis of the above complexes has been employed in this thesis. The ESI-MS involves a gentle ionisation process, yielding multiply charged molecular ions with little or no fragmentation. During characterisation of osmium complexes, the highest mass ion observed consistently corresponded to the molecular ion ([Os(N-N)$_2$Cl$_2$]$^+$) of the complex under analysis. In addition, an isotopic distribution calculator (Sheffield
chemputer) was utilised to generate the theoretical isotopic distribution of the osmium complexes (Figure 2.6). Comparison between experimental and calculated isotopic distribution patterns show good correlation, see Figure 2.6.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Relative Intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>568</td>
<td>100</td>
</tr>
<tr>
<td>569</td>
<td>20</td>
</tr>
<tr>
<td>570</td>
<td>30</td>
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<tr>
<td>571</td>
<td>40</td>
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<td>50</td>
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<td>573</td>
<td>60</td>
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<td>574</td>
<td>70</td>
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<td>575</td>
<td>80</td>
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<td>90</td>
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<tr>
<td>580</td>
<td>40</td>
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</table>

<table>
<thead>
<tr>
<th>m/z</th>
<th>Relative Intensity (%)</th>
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<tbody>
<tr>
<td>624</td>
<td>100</td>
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<tr>
<td>625</td>
<td>20</td>
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<td>626</td>
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<td>636</td>
<td>40</td>
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</table>
Figure 2.6 Bar graph representing experimental (blue) and calculated (red) isotopic distribution pattern of (a) Os(bpy)$_2$Cl$_2$, (b) Os(dmbpy)$_2$Cl$_2$ and (c) Os(dmobpy)$_2$Cl$_2$, (cone voltage 40 V) in acetonitrile. Inset: structure of the Os complexes.

Further characterisation of the cis-Os(N-N)$_2$Cl$_2$ complexes was undertaken using elemental analysis (see Table 2.2).

Table 2.2 Elemental analysis for osmium complexes containing water of crystallisation showing theoretical (T) and observed (Obs) values

<table>
<thead>
<tr>
<th>Complex</th>
<th>% C</th>
<th>% H</th>
<th>% N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Os(dmobpy)$_2$Cl$_2$.2H$_2$O</td>
<td>40</td>
<td>33</td>
<td>7.7</td>
</tr>
<tr>
<td>Os(dmbpy)$_2$Cl$_2$.2H$_2$O</td>
<td>43</td>
<td>43</td>
<td>8.4</td>
</tr>
<tr>
<td>Os(bpy)$_2$Cl$_2$.2H$_2$O</td>
<td>39</td>
<td>39</td>
<td>9.2</td>
</tr>
</tbody>
</table>

It is important to note that the elemental analyses of osmium complexes are obtained using crude samples. The differences in theoretical and observed values are therefore as a result of impurities or solvent contained within these crude samples. Nonetheless, the crude complexes can be used to prepare redox polymers, as the required product is present in the crude sample, as evident from the MS and CV
results. The remaining impurities are removed through subsequent washing steps, after the PVI polymer substitution reaction as they do not co-ordinate to the polymer.

2.1.8 Synthesis and characterisation of poly(N-vinylimidazole) polymers containing cis-[Os(N-N)2Cl2] redox centres.

In the synthetic procedure, a Cl ligand from the previously synthesised Os complex is substituted with a pendant monomer of the PVI polymer, as ligand (1:10 ratio of Os and monomer unit added) to form polymer bound complexes. A summary of the proposed structure and the redox potentials of the polymer bound complexes determined using CV of films immobilised at the surface of GC electrodes is provided in Figure 2.5. These films were prepared by drop coat deposition of 3 μL of an 8 mg ml\(^{-1}\) aqueous solution of redox complex and 3 μL of a 15 mg ml\(^{-1}\) aqueous solution of epoxy crosslinker (polyethylene glycol-bis-diglycidyl ether, PEG). Slow scan voltammograms (1 mV s\(^{-1}\)) were recorded in 50 mM, phosphate buffer pH 7.4 at room temperature. The redox potentials observed for each of the different redox polymers are comparable to results already determined by others [14, 21, 34-38].
Figure 2.7 Proposed structure, CVs, and redox potential of (a) [Os(2,2'-bipyridine)$_2$(poly-vinylimidazole)$_{10}$Cl]$^+$ (OsbpyPVI), (b) [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$(PVI)$_{10}$Cl]$^+$ (OsdmmbpyPVI) and (c) [Os(4,4'-dimethoxy-2,2'-bipyridine)$_2$(PVI)$_{10}$Cl]$^+$ (OsdmobpyPVI). CVs recorded on films of redox polymer at GC electrodes in 50 mM, phosphate buffer pH 7.4 at room temperature at 1 mV s$^{-1}$ scan rate.

2.2 Immobilisation

Enzymatic fuel cells (EFCs) utilise biological catalysts in place of a metal catalyst for the conversion of chemical energy to electrical energy. EFCs allow miniaturisation through the removal of the proton exchange membrane as enzymes are substrate-specific at both the anode and the cathode, thus developing systems to power electronic device [39-41]. The incorporation of mediators in most EFC
electrodes allow for electron shuttling from the enzyme active site to electrodes as active sites are buried within a proteinaceous insulating envelope too distant from the electrode surface to permit rapid electron transfer to occur. Mediators possessing a redox potential suitable for rapid electron transfer between the enzyme active site and through the co-immobilisation of enzymes with electron-shuttling mediators on solid electrodes current capture can be improved [35, 42, 43]. Osmium-based mediators are selected due to the fact that they are stable in the reduced/oxidised Os(II/III) states [42] within biologically relevant potential ranges (−0.4 to 0.4 V vs Ag/AgCl). Also, the redox potential of the Os(II/III) metal centre transition can be tuned by selection of appropriate electron-donating/withdrawing coordinating ligands.

An important aspect to improving both EFC power output and operational stability is selection of an immobilisation strategy of enzyme and mediator. Methods such as: the covalent grafting of enzymes on derivatised electrode surfaces [5], the use of crosslinkers to bond the biocatalyst with the redox polymer chains [44] and/or employing layer-by-layer techniques [6, 45, 46] have been proposed to effectively trap the biocatalyst without affecting its activity. Co-immobilisation of enzymes within redox polymer hydrogels using a diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether (PEGDGE) is a well-established protocol for enzyme electrode production [47]. The use of glutaraldehyde (GA) solutions [7, 48, 49] or GA vapours [49, 50] to crosslink enzyme and redox polymer has been reported. In addition, recent reports on the use of GA vapours for enzyme co-immobilisation in redox polymer hydrogels show an increase in oxidation current and enzyme stability over non-crosslinked films [50]. The use of borohydride to reduce unstable Schiff bases, formed as a result of GA crosslinking to amines is also well established [51, 52]. Recently, GA vapours followed by a borohydride reduction (abbreviated as GA(R)), for preparation of stabilised redox hydrogel-based enzyme electrodes, with air-drying of the crosslinked films, yielded improvement in both glucose oxidation currents and stability [14, 36, 37].

Previous research shows that optimisation of component ratios of redox polymer and GOx enzyme improves glucose oxidation current signal and/or stability [37]. The addition of multi-walled carbon nanotubes (MWCNTs) into an enzyme/redox polymer film, crosslinked with glutaraldehyde vapours and reduced by sodium
borohydride solution was shown to offer improved stability of glucose oxidation current over other approaches investigated, retaining 77% of the initial current over 24 hours of continuous operation [36]. This report further demonstrated that the GA(R) crosslinking methodology used could be applied to immobilisation of redox polymers with low redox potential, more suited as fuel cell anodes [14, 37].

To investigate the immobilisation methodology for this thesis enzyme electrodes are prepared through co-immobilisation of enzyme, redox polymer and MWCNTs using the GA(R) crosslinking methodology. Glucose oxidase (GOx) is selected as the glucose oxidising enzyme for screening due to its commercial availability, high turnover rate and it has been used as the benchmark sugar oxidising enzyme [53]. An OsdmPbpyPVI redox polymer is selected due to its low redox potential (0.120 V vs Ag/AgCl) in comparison to Os(bpy)PVI (0.220 V vs Ag/AgCl) which has been used in previous research [14, 37]. The use of a lower redox potential can contribute to an overall increased difference in voltage between anode and cathode of an assembled EFC leading to improved power output of the fuel cell. To compare performance of enzyme electrodes in the presence of MWCNTs, amounts of GOx (0.048 mg) and redox polymer (0.048 mg) determined to provide highest current from previous studies [36] were retained for use in the preparation step, whilst the amount of MWCNTs was varied between 0 – 85 % w/w.

2.2.1 Enzyme electrode preparation
Electrodes were prepared from graphite rods, sourced from Graphite store, USA (3 mm diameter); they were cut to 9 cm length, shrouded with heat shrinking tubes and polished on fine grit paper to create a working surface area of 0.0707 cm². Immobilisation on films was achieved by depositing 9.6 µL of a 5 mg mL⁻¹ OsdmbpyPVI redox polymer, 4.8 µL of 10 mg mL⁻¹ solution of GOx and different ratios of a 46.25 mg mL⁻¹ dispersion of acid treated MWCNTs on the surface of 3 mm graphite working electrode and allowed to dry for 24 h. Glutaraldehyde crosslinking was achieved by exposing previously dried films to glutaraldehyde (GA) vapours in a sealed headspace for 30 minutes. Crosslinked electrodes were reduced by immersing in 1 M sodium borohydride solution (NaBH₄) for 5 seconds and then rinsed in Milli-Q water after which electrochemical testing commenced. For comparison PEGDGE crosslinking was achieved by adding 2 µL of 15 mg mL⁻¹ PEGDGE solution during immobilisation on the surface of the graphite working
electrode and allowing the subsequent mixture to cross-link and dry for 24 h prior to use.

2.2.2 GOx activity assays

GOx activity assays were conducted using an Agilent 8453 uv-visible spectroscopy system in a 3 ml cuvette (light path 10 mm) containing 0.1 M phosphate buffer mix (1 % o-Dianisidine, 0.4 mM) pH 7.0 (2.5 ml) saturated with oxygen for 10 minutes, 1 M glucose concentration (0.3 ml), horseradish peroxidase 200 µg/ml (HRP 0.1 ml). All measurements including the blank were recorded at 460 nm, the change of absorbance with time was measured and the slope extracted, from which the activity in U/ml was obtained. Solution phase measurements of the GOx enzyme activity were first conducted, from which an average enzymatic activity was obtained with example results shown in Figure 2.8.

Figure 2.8 An example of a slope obtained using solution phase kinetics.

To calculate the activity in U/ml, the following formula was used [54].

\[ \text{Slope}/60 \times \text{VA} \times \text{DF} \]

\[ \text{U/ml} = \frac{\text{Slope}/60 \times \text{VA} \times \text{DF}}{11.3 \times \text{VE}} \]

VA = Total volume in cuvette in ml
DF = Dilution Factor (ml)
11.3 = Extinction Coefficient of o-Dianisidine
VE = Volume of Enzyme ml
By using different concentrations of GOx an average enzymatic activity of is 234.3 ± 82 U ml\(^{-1}\) is obtained which correlates well with the enzymatic activity provided by Sigma-Aldrich (100 – 250 U/ml).

### 2.2.3 Enzyme Electrode Electrochemical Characterisation

Cyclic voltammetry (CV) is initially used to verify electrochemical response for enzyme electrodes, recorded in the presence and absence of substrate in PBS at 37 °C. The formal redox potential (E\(^\circ\)) for the Os(II/III) transition of the OsdmbpyPVI redox polymer, estimated from the midpoint between the oxidation and reduction peak potentials by CV recorded at 1 mV s\(^{-1}\) scan rate in the absence of substrate, is confirmed as 120 ± 10 mV vs Ag/AgCl. This agrees well with the value reported for other enzyme electrodes prepared using this redox polymer [14, 21, 34-38]. Where there are no lateral interactions between surface confined redox centers and a rapid equilibrium is established with the electrode (ideal reversible system), a zero value for peak-to-peak splitting (\(\Delta E_p\)) and a full width at half-maximum (FWHM) of 90.6/n mV are expected for a one-electron transfer [55]. The full width half maximum (FWHM) for oxidation of the enzyme electrode redox polymer under the same conditions is 0.11 ± 0.01 V vs Ag/AgCl which is close to the predicted response of 0.09 V for an ideal surface-confined one electron redox system [55]. Furthermore, the \(\Delta E_p\) is 0.022 ± 0.004 V, which is less than the 0.059 V separation expected for a one electron redox process under semi-infinite diffusion control [55] although deviating slightly from the predicted response of 0 V for an ideal surface-confined one electron [55]. This deviation from the expected theoretical value of a surface confined one electron reversible redox couple, suggests the influence of semi-infinite diffusion, and interaction within the film, on the overall electrochemical response. [56, 57]. At slow-scan (<20 mV s\(^{-1}\)) cyclic voltammograms measured in the absence of glucose display peak currents that vary linearly with scan rate. This linear dependence is as a result of the fact that the surface bound osmium redox centres are not free to diffuse to the electrode surface unlike in a solution phase electrochemistry, but are confined to it. Over long experimental time-scales (slow-scan), the polymer film is exhaustively oxidised or reduced and, under these conditions, the depletion layer extends all the way to the film/solution interface. As a result, the voltammetric wave is modified, compared to a wave for a solution species, producing symmetrical oxidation and reduction waves.
around peak potential ($E_p$), and peak currents ($i_p$) that show a linear dependence on scan rate [55]. At higher scan rates, peak currents vary linearly with the square root of scan rate as only a fraction of the total amount of the electroactive species immobilised at the electrode surface is electrolysed and the depletion zone remains well within the film. Under these conditions, semi-infinite linear diffusion dominates and, resembling a solution phase response [55, 58]. An estimate of redox-active osmium surface coverage ($\Gamma_{Os}$) on the electrode is obtained by integrating the charge passed where the film is comprehensively electrolysed under the slow-scan-rate CV peak. The use of equation 1.13, with $Q$ determined by integration of the anodic peak in the cyclic voltammogram, yielded $\Gamma_{Os}$ values of $67.5 \pm 1.74$ nmol cm$^{-2}$ and $135 \pm 4.56$ nmol cm$^{-2}$, for the absence and presence of MWCNTs, respectively. These values are characteristic of multilayer formation and are comparable to those reported by others from co-immobilisation of Os(dm bpy)PVI with GOx and MWCNTs on graphite electrodes [36, 37].

**Figure 2.9** Slow-scan (1 mV s$^{-1}$) CV in PBS at 37 °C of enzyme electrodes prepared using GA(R) crosslinking of Os(dm bpy)PVI and GOx on electrodes in either the presence (--) or absence (—) of 79% MWCNTs.

Addition of MWCNTs is shown to increase the surface coverage of osmium on enzyme electrodes in comparison to electrodes prepared without addition of MWCNTs (Figure 2.9 and 2.10). This increase in surface coverage provides improved enzyme retention and/or addressing the redox polymer on the electrode
surface [59], subsequently leading to improved glucose oxidation current density. Amounts higher than approximately 70% w/w MWCNTs result in a decrease in the surface coverage (Figure 2.12). This decrease in the surface coverage could be as a result of parts of the films detaching from the electrode surface as a result of the effervescence from hydrogen gas production using borohydride as a reducing agent (Figure 2.10 and 2.11).

**Figure 2.10** Image showing effervescence occurring in sodium borohydride solution, resulting in detachment of films from electrodes.

**Figure 2.11** Image showing film detachment from electrode surface using scanning electron microscopy.
The retention of enzyme activity at the electrodes is estimated and compared with the retention of redox active polymer. A series of electrodes was prepared and the retained enzyme activity spectrophotometrically assayed using established procedures [54] (Figure 2.12). An increase in retained enzyme activity is observed as a function of MWCNT, up to amounts of approximately 79% w/w MWCNT, above which the retained enzyme activity decreases. The retained enzyme activity correlates well with observed glucose oxidation amperometric response (Figure 2.14). A similar decrease with higher amounts of carbon nanotubes as components in enzyme electrodes has been previously reported by Joshi et al. [59] for studies using single-walled carbon nanotubes and they suggest that electron transfer from enzyme to redox centre of the polymer might be limiting as the amount of carbon nanotubes on enzyme electrodes is increased.

![Figure 2.12 Plot of activity (blue dash) and $\Gamma_{Os}$ (black dot) as a function of % w/w MWCNTs added for enzyme electrodes prepared by co-immobilisation of MWCNT, OsdmbpyPVI and GOx (n=4).](image)

Slow scan CV and fixed-potential amperometry is used to measure glucose oxidation currents for enzyme electrodes. The addition of high concentrations (100 mM) of glucose to electrochemical cells resulted in sigmoidal slow-scan cyclic voltammograms for all enzymatic electrodes (Figure 2.13), as expected for an electrocatalytic (EC') process [55].
Enzyme electrodes prepared with MWCNTs display higher current densities compared to those prepared without MWCNTs (Figure 2.14). The highest current densities were obtained for electrodes with 79% w/w MWCNTs prepared with GOx. The decrease in current density for MWCNT content greater than 79% correlates with the decrease in retained enzyme activity within the enzymatic electrode films, when assayed spectrophotometrically suggesting that retention of enzyme activity is the key parameter that controls the value of the saturated current density for these electrodes.

**Figure 2.13** CVs at 1 mV s⁻¹ in quiescent 50 mM phosphate buffer solution, 150 mM NaCl at 37 °C, of films of Os(dmbpy)PVI co-immobilised with GOx in 5 mM glucose (—) and with 78.7% w/w MWCNTs in 100 mM glucose (−−). See Figure 2.10 for CV in absence of glucose.
Glucose oxidation current densities of 5 mM glucose (red) and enzymatic activities (blue). Films were prepared with GOx (0.048 mg, 12 U activity), redox polymer (0.048 mg) and a range of MWCNTs % w/w.

Further comparison across the range of glucose concentrations and MWCNT amounts was undertaken by adding substrate to the electrochemical cell with enzyme electrodes polarised at 0.35 V vs Ag/AgCl, to ensure sufficient overpotential is applied, and using gentle stirring of solutions at 150 rpm to minimise substrate depletion, to generate maximum glucose catalytic currents. The resulting amperometric current densities showed higher currents in comparison to that observed using cyclic voltammetry at 100 mM glucose using 78.7% w/w MWCNTs (see Figure 2.13 and Table 2.4); this is as a result of introducing convection using 150 rpm stirring. For example, a current density of 5 mA cm$^{-2}$ can be estimated at 0.35 V from the CV in Figure 2.13 for oxidation of 100 mM glucose by films of Os(dmbpy)PVI co-immobilised with GOx, whereas a current density of 6 mA cm$^{-2}$ is obtained at the same applied potential during amperometry.
Glucose oxidation current densities estimated from steady state amperometry at an applied potential of 0.35 V with stirring at 150 rpm, as a function of glucose concentration, for films using Os(dmbpy)PVI co-immobilised with GOx and 82.2% w/w MWCNTs (red square) and Michaelis-Menten steady state approximation (blue solid) for the same enzyme electrodes.

For all enzyme electrodes, the increase in glucose oxidation current density as a function of glucose concentration is apparent (see Figure 2.15), with substrate saturation at concentrations greater than 50 mM. This follows the trend expected for the steady-state approximation of Michaelis–Menten enzyme kinetics, with an initial linear increase in current density that subsequently approaches a maximum at high glucose concentrations. Nonlinear least-squares fitting of the experimental points to the Michaelis–Menten equation was used to provide apparent Michaelis constants ($K_{M^{app}}$) and maximum saturated current densities ($j_{max}$) for enzyme electrodes as shown in Table 2.3.
Table 2.3 $K_M^{\text{app}}$ and $j_{\text{max}}$ estimated from amperometric response using Michaelis-Menten enzyme kinetics for films containing Os(dmbpy)PVI, GOx and MWCNTs on graphite electrodes. n=4

<table>
<thead>
<tr>
<th>% w/w MWCNTS</th>
<th>$j_{\text{max}}$ (mA cm$^{-2}$)</th>
<th>$K_M$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.4 ± 0.2</td>
<td>17.0 ± 1.4</td>
</tr>
<tr>
<td>48</td>
<td>2.8 ± 2.1</td>
<td>15.5 ± 0.1</td>
</tr>
<tr>
<td>65</td>
<td>3.0 ± 0.3</td>
<td>16.3 ± 0.5</td>
</tr>
<tr>
<td>74</td>
<td>3.4 ± 0.4</td>
<td>11.3 ± 4.0</td>
</tr>
<tr>
<td>79</td>
<td>7.5 ± 0.5</td>
<td>14.3 ± 1.4</td>
</tr>
<tr>
<td>82</td>
<td>2.5 ± 0.3</td>
<td>7.0 ± 0</td>
</tr>
<tr>
<td>85</td>
<td>2.4 ± 0.2</td>
<td>7.0 ± 1.4</td>
</tr>
</tbody>
</table>

The $j_{\text{max}}$ observed from amperometric response of the curve-fitting Michaelis-Menten model also showed significant increase in glucose oxidation currents as a function of MWCNTs amount and then a sharp decrease with MWCNTs addition of masses greater than 79% w/w (see Table 2.4). The $K_M^{\text{app}}$ value at 82% w/w MWCNTs correlates with that observed in previous reports [36, 37, 60-62]. This value is lower than the one reported for the native GOx from *Aspergillus niger* in solution (27 mM) [63, 60].

The operational stability is a key factor essential for further development of continuous-use enzyme electrodes for application to biosensing and biocatalytic fuel cells. A 20 h test for stability of glucose oxidation current was carried out in order to extract the half-life of the films in order to measure the operational stability of enzyme electrodes.
Figure 2.16 Decrease in glucose oxidation current (ln) over time for films prepared with 0% MWCNT (light blue), 48.1% MWCNTs (red), 64.9% MWCNTs (green), 73.5% MWCNTs (purple), 78.7% MWCNTs (black), 82.2% MWCNTs (blue) and 84.7% MWCNTs (orange). Data points sampled from 0.35 V vs Ag/AgCl amperometry in 50 mM pH 7.4 phosphate buffer solution at 37 °C containing 150 mM NaCl and 100 mM glucose, stirred at 150 rpm.

The stability of the response for glucose oxidation was estimated using amperometry and a plot of natural logarithm as a function of time over 20 h was used to estimate enzyme electrode half-life (t½) (see Figure 2.16). Assuming pseudo-first order process, the integrated first order rate law with respect to a reactant A is

$$\ln j = -kt + \ln j_0$$  \hspace{1cm} Equation 2.2

Where j is the current density, k is rate constant, t is time and j₀ is initial concentration of reactant. A plot of ln j vs t gives a straight line with a slope of −k. The half-life (t½) of a first order reaction is independent of the initial concentration and given by:

$$t_{1/2} = \frac{\ln(2)}{k}$$  \hspace{1cm} Equation 2.3

Assumption of such a simple model provides linear plots for all of the approaches, with the reported half-life for these films extracted only from the linear portion of the plot over the 8–20 h time period.
Table 2.4 Initial and 20 h glucose oxidation current density values obtained from amperometry at an applied potential of 0.35 V vs Ag/AgCl in slowly stirred (150 rpm) in 50 mM buffer solution containing 150 mM NaCl at 37 °C and 100 mM glucose (n=4).

<table>
<thead>
<tr>
<th>% w/w MWCNTs</th>
<th>Initial glucose current density (mA cm⁻²)</th>
<th>% Glucose current remaining at 20 h</th>
<th>Calculated t₁/₂ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.5 ± 0.6</td>
<td>34 ± 8</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>48</td>
<td>3.1 ± 0.3</td>
<td>36 ± 6</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>65</td>
<td>3.2 ± 0.1</td>
<td>92 ± 2</td>
<td>153 ± 49</td>
</tr>
<tr>
<td>74</td>
<td>3.9 ± 0.8</td>
<td>68 ± 8</td>
<td>37 ± 7</td>
</tr>
<tr>
<td>79</td>
<td>6.6 ± 0.3</td>
<td>46 ± 6</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>82</td>
<td>2.3 ± 0.3</td>
<td>81 ± 3</td>
<td>48 ± 5</td>
</tr>
<tr>
<td>85</td>
<td>1.9 ± 0.2</td>
<td>68 ± 8</td>
<td>33 ± 9</td>
</tr>
</tbody>
</table>

From the results obtained an improved stability of films containing MWCNTs in comparison to the absence of MWCNTs for glucose oxidation current is observed. This is to be expected as others have reported improved retention of glucose oxidation currents as a result of added MWCNTs [36, 37]. The estimated half-life shows an increase only for enzyme electrodes prepared with 65% w/w MWCNTs (Table 2.4). This increase is similar to that previously reported by MacAodha et al. [36] for enzyme electrodes using the same crosslinking methodology, but for addition of 82% MWCNTs for their electrodes. However, the half-life observed for 82% MWCNTs of the same co-immobilised enzyme electrodes as MacAodha et al. only yielded 47.5 h. This notable difference in the half-life could be as a result of film detachment during the crosslinking process which could in turn affect the operation of enzyme electrodes. On the other hand, when the crosslinking process is successful (no film detachment on electrode surface), there is a significant improvement in both glucose oxidation currents and stability (see Figure 2.16 and Table 2.4). Electrodes prepared using GA(R) crosslinking were further compared with electrodes prepared utilising PEGDGE crosslinking see Table 2.5.
Table 2.5 Response of films prepared using PEGDGE and GA(R) crosslinking methodologies. Current density values obtained from amperometry at an applied potential of 0.35 V vs Ag/AgCl in slowly stirred (150 rpm) in 50 mM buffer solution containing 150 mM NaCl at 37 °C and 100 mM glucose (n=4).

<table>
<thead>
<tr>
<th>% w/w MWCNTs</th>
<th>PEGDGE</th>
<th>GA(R)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Gamma_{Os}$ nmols/cm²</td>
<td>Current density (mA cm⁻²)</td>
</tr>
<tr>
<td>0</td>
<td>71 ± 4</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>48</td>
<td>107 ± 8</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>65</td>
<td>116 ± 5</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>74</td>
<td>117 ± 4</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>79</td>
<td>127 ± 8</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>82</td>
<td>126 ± 4</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>85</td>
<td>129 ± 2</td>
<td>2.1 ± 0.2</td>
</tr>
</tbody>
</table>

In Table 2.5 $\Gamma_{Os}$ obtained for enzyme electrodes prepared using PEDGDE shows a continuous increase with increasing MWCNTs which is expected as reported previously by Joshi et al. [59]. In contrast electrodes prepared using GA(R) show a decrease in $\Gamma_{Os}$ for MWCNT amounts greater than 74%. Whilst higher current densities are achieved for GA(R) electrodes in comparison to the PEGDGE electrodes, the $t_{1/2}$ for PEGDGE electrodes show increased stability with added MWCNTs whereas the $t_{1/2}$ for GA(R) electrodes varied widely. This suggests that the utilisation of PEGDGE crosslinking provides for more control in enzyme electrode preparation in comparison to GA(R).

2.3 Conclusions

The synthesis and characterisation of a range of osmium polypyridyl complexes is described. Subsequent to the production of cis-[Os(N-N)_2Cl_2], one of the Cl ligands is substituted with a pendant monomer of a poly(N-vinylimidazole) (PVI) polymer, as ligand (1:10 ratio of Os complex and monomer unit added) to form polymer bound complexes suitable for adsorption to solid electrode surfaces. Furthermore, we
compared the performances of enzymatic electrodes for glucose oxidation by varying the amount of MWCNTs added to films prepared with and GOx co-immobilised with an osmium-based redox polymer on graphite electrodes. The addition of increasing amounts of MWCNTs to enzyme electrodes increased the amount of redox polymer that was co-immobilised and electronically coupled within the enzyme films. In addition, glucose-oxidation activity was measured spectrophotometrically within the enzymatic electrode films and reached a maximum value for electrodes prepared with 79% w/w MWCNTs, and decreased in the films with higher amounts of MWCNTs, thereby indicating that the retention of enzyme activity within films is the key parameter influencing current density for these electrodes. The enzyme electrodes prepared using the optimised amount of MWCNTs provided up to 6.1 mA cm\(^{-2}\) glucose-oxidation current density at the relatively low applied anode potential of 0.12 V vs Ag/AgCl for the GOx-based electrodes in PBS at 37 °C. Operation at pseudo-physiological conditions of 5 mM glucose in PBS at 37 °C resulted in current densities of 1.4 mA cm\(^{-2}\). Electrodes prepared using GA(R) crosslinking were further compared with electrodes prepared using PEGDGE crosslinking. The results imply that utilisation of PEGDGE crosslinking provides for more control in enzyme electrode preparation in comparison to GA(R). Although the results obtained in the GA(R) study show improvement in current densities achieved, the use of the crosslinking methodology may affect the performance of enzyme electrodes due to less control in the borohydride reduction step, which could result in detachment of films on electrode surface. Due to difficulties in the control during enzyme electrode preparation using GA(R) crosslinking, PEGDGE, as the more widely accepted methodology, is utilised as a crosslinker in chapter 3, 4 and 5 of this thesis.
2.4 References


Chapter 3

Published as:

Effect of Multi-Walled Carbon Nanotubes on Glucose Oxidation by Glucose Oxidase or a Flavin-Dependent Glucose Dehydrogenase in Redox-Polymer-Mediated Enzymatic Fuel Cell Anodes


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Co-author contributions:

I synthesised the osmium redox systems, and performed the laboratory work, the analysis, and wrote the first draft of the publication.

Dónal Leech, as the project supervisor, contributed through guidance and advice throughout and wrote the final draft of the publication.
Effect of Multi-Walled Carbon Nanotubes on Glucose Oxidation by Glucose Oxidase or a Flavin-Dependent Glucose Dehydrogenase in Redox-Polymer-Mediated Enzymatic Fuel Cell Anodes

3.1 Abstract
The addition of multi-walled carbon nanotubes (MWCNTs) to enzymatic electrodes based on either glucose oxidase (GOx), or an oxygen-insensitive flavin adenine dinucleotide-dependent glucose dehydrogenase (FADGDH), increases the amount of [Os(4,4’-dimethyl-2,2’-bipyridine)2(polyvinylimidazole)10Cl]Cl redox polymer at the electrode surface, indicating that MWCNTs provide a surface for the immobilisation of film components. Glucose oxidation is highest for films with 68% w/w MWCNTs, and a decrease is observed with larger amounts; this decrease is related to a decrease in retained enzyme activity. Enzymatic electrodes provide 4.2 mA cm⁻² current density at 0.12 V vs Ag/AgCl, for GOx-based electrodes, compared to 2.7 mA cm⁻² for FADGDH-based electrodes in 50 mM phosphate-buffered saline containing 150 mM NaCl at 37 °C. Current densities of 0.52 and 1.1 mA cm⁻² are obtained for FADGDH and GOx-based electrodes, respectively, operating at physiologically relevant 5 mM glucose concentrations. These enzymatic electrodes, thus, show promise for application as anodes in enzymatic fuel cells for in vivo or ex vivo power generation.
3.2 Introduction

Enzymatic fuel cells (EFCs), in which the enzyme catalyst replaces traditional metal catalysts, operate by converting chemical energy into electrical energy [1]. A potential advantage of using enzyme catalysts, apart from moderate operating conditions and their sustainability, is the specificity they can exhibit for the reactions they catalyse, compared to metal catalysts. This specificity, in fuel cell anodes and cathodes of EFCs, eliminates the need for casings and ion-exchange membranes, and led to renewed interest in recent years that focused on the development of potentially implantable or portable, miniaturised, membrane-less EFCs operating on carbohydrates as fuel [2–4]. There remain, however, significant issues with the proposed technology, such as low power/current outputs, instability of enzymes and incomplete oxidation of fuels, which have thus instigated a range of studies to improve the performance of EFCs [5–7]. Over the past decades, advancements have been made in maximising current capture, as a result of enzymatic redox reactions in enzymatic-electrode technology, through the co-immobilisation of enzymes with electron-shuttling mediators on solid electrodes [4, 8, 9]. Synthetic polymers, such as poly(N-vinylimidazole) and poly(4-vinylpyridine), containing osmium pendant moieties have been the focus of much of this research [9–14], owing to the ability to modulate the mediator redox potential of the central Os metal by using coordinating ligands, the relative stability of the resulting complexes in the reduced/oxidised states Os(II/III), and because the hydrogel characteristics of enzymatic electrodes based on redox-polymer films permit rapid mass and charge transport, thus generating substantial current signals [9, 15, 16].

Matching the enzyme with mediator and immobilisation approach is important to provide the highest current signal, at an appropriate redox potential, to permit application of enzymatic electrodes as biosensors, or as electrodes in EFCs. Recent methods for improved integration of components of enzymatic electrodes to provide for increased current as a function of underlying geometric electrode area have focused on the addition of conductive particles to enzymatic electrode films. For example, it has been reported that enzymatic electrodes for glucose oxidation containing carbon micro-, meso- and nanostructured materials show improved performance over those prepared without addition of such materials [15, 17–20]. Carbon nanotubes (CNTs) [21] exhibit properties that make them attractive to use as
components in electrochemical devices [22, 23]. Recent studies have shown that CNTs can promote electron transfer from proteins to electrode surfaces [24], which is attributed to their high electrical conductivity. Multi-walled CNTs (MWCNTs) consist of multiple rolled layers of concentric tubes of graphene, whereas single-walled CNTs (SWCNTs) consist of a single tube of graphene [3]. The reported faster electron-transfer rate for different redox reactions, and better electrical conductivity of MWCNTs, compared to SWCNTs [5], make MWCNTs more attractive for use in enzymatic electrode applications. For example, improved glucose-oxidation current density, and stability of current signals, is observed upon addition of MWCNTs to films for enzymatic electrodes composed of cross-linked \([\text{Os}(2,2'\text{-bipyridine})_2(\text{polyvinylimidazole})_{10}\text{Cl}]\text{Cl}\) redox polymer and the enzyme glucose oxidase (GOx) [15]. This approach was subsequently adopted to provide a comparison of assembled EFC operation from anodes of glucose-oxidising enzymes, and cathodes of oxygen-reducing multicopper oxygenases, with no attempt to study the effect of varying the amount of MWCNTs on enzymatic-electrode or EFC performance [15, 25]. Herein, we report on the effect of varying the amount of MWCNTs on the response of enzymatic electrodes to glucose oxidation. These enzymatic electrodes were prepared by co-immobilisation on graphite of an \([\text{Os}(4,4'\text{-dimethyl-2,2'\text{-bipyridine}})_2(\text{polyvinylimidazole})_{10}\text{Cl}]\text{Cl}\) redox polymer \([\text{Os-(dmnbpy)}_2\text{PVI}], and either GOx, or a flavin adenine dinucleotide dependent glucose dehydrogenase (FADGDH). \text{Os(dmbpy)}_2\text{PVI} was selected instead of the \([\text{Os}(2,2'\text{-bipyridine})_2(\text{polyvinylimidazole})_{10}\text{Cl}]\text{Cl}\) redox polymer, because of its lower redox potential (0.12 V vs Ag/AgCl) [25], thus providing the possibility of a higher cell voltage for an EFC assembled with it. GOX has been used as a benchmark for the performance of electrodes based on glucose-oxidising enzymes, owing to its high glucose turnover rate [9] and commercial availability, however, its main disadvantage is its sensitivity to oxygen [16, 26], which can affect the power output of an EFC [26, 27]. Thus, we also compared the performances of enzymatic electrodes based on GOx and the oxygen-insensitive enzyme FADGDH [26].
3.3 Experimental Section

3.3.1 Materials
The Os(dmbpy)PVI polymer was synthesised according to literature procedures [29–30]. All chemicals were purchased from Sigma–Aldrich, unless otherwise stated. All aqueous solutions were prepared in Milli-Q water (18 MΩ cm), unless otherwise stated. GOx from Aspergillus niger (Sigma–Aldrich, EC 1.1.3.4.) and an FADGDH from Aspergillus sp. (Sekisui, Cambridge, MA; GLDE-70-1192, EC 1.1.99.10) were used as received. MWCNTs (product 659258; Sigma–Aldrich) were treated by stirring in nitric acid at reflux for 6 h, and were isolated by filtration.

3.3.2 Methods
GOx activity was determined using the o-Dianisidine and horseradish peroxidase-coupled spectrophotometric assay by monitoring absorbance changes at 460 nm on an Agilent 8453 UV/Vis spectrophotometer [35]. The average activity obtained was 234 ± 82 U mg⁻¹, which correlated well with the activity reported by Sigma–Aldrich (100 – 250 U mg⁻¹). The FADGDH activity was determined using an NTB–PMS-coupled spectrophotometric assay by monitoring absorbance changes at 570 nm [36], with an average activity obtained of 266 ± 58 U mg⁻¹.

Working electrodes were prepared from graphite rods (3 mm diameter, NC001295; GraphiteStore.com, Inc., Buffalo Grove, IL) cut to 9 cm length, shrouded with heat-shrinking tubing and polished on fine grit paper to create a working geometric surface area of 0.0707 cm². Enzymatic electrodes were prepared by depositing aqueous solutions of redox polymer (5 mg mL⁻¹, 9.6 µL), enzyme (10 mg mL⁻¹, 4.8 µL, ≈12 U), PEGDGE cross-linker (15 mg mL⁻¹, 2 µL), and different volumes of a dispersion of acid-treated MWCNTs (46 mg mL⁻¹) on the surface of the graphite working electrode and allowing the subsequent mixture to cross-link and dry for 24 h prior to use.

Electrochemical tests were conducted using a CH Instruments 1030a multichannel potentiostat (IJ Cambria, Llanelli, UK) on a three-electrode cell containing PBS [prepared using a mixture of disodium hydrogen phosphate and sodium dihydrogen phosphate salts (50 mM), NaCl (150 mM), pH 7.4], at 37 °C, using a custom built
Ag/AgCl, the enzymatic electrode as the working electrode, and a platinum mesh counter electrode (Goodfellow, Huntingdon, UK).

3.4 Results and Discussion

Enzymatic electrodes for glucose oxidation were prepared initially by co-immobilisation, using a polyethylene glycol diglycidyl ether (PEGDGE) diepoxide cross-linker [2–4, 28] of Os(dmbpy)PVI and either GOx or FADGDH with a range of amounts of MWCNTs. To test the effect of adding different amounts of MWCNTs to the enzymatic electrodes, the ratio of Os(dmbpy)PVI to enzyme was kept constant (1:1, w/w), as this was previously reported to provide maximum glucose-oxidation current for similar systems [15], and the amount of MWCNTs was varied. The enzymatic electrodes were characterised using cyclic voltammetry (CV) and amperometry in the presence and absence of the substrate glucose. In the absence of glucose, cyclic voltammograms recorded for all enzymatic electrodes (examples are shown in Figure 1) display peaks for oxidation and reduction centred at 0.14 ± 0.02 V vs Ag/AgCl, which can be assigned to the Os(II/III) transition of the redox polymer, as this value is similar to the redox potentials observed for Os(dmbpy)PVI in phosphate-buffered saline (PBS) solution (not shown), Os(dmbpy)PVI films on electrodes [29, 30], and for enzymatic electrodes based on immobilisation of this redox polymer [4, 15, 18, 25, 31].

The slow scan (< 20 mV s⁻¹), cyclic voltammograms measured in the absence of glucose display peak currents that vary linearly with scan rate, which is characteristic of a surface-confined redox layer response [32]. An estimate of redox-active osmium surface coverage ($\Gamma_{\text{Os}}$) on the electrode is therefore obtained by integrating the area under slow scan rate CV peak. $\Gamma_{\text{Os}}$ values of 85 ± 10 and 103 ± 5 nmol cm⁻², which are indicative of multilayer formation, were obtained for enzymatic electrodes containing FADGDH and GOx, respectively, and these are comparable to values reported by others for enzymatic electrodes based on co-immobilisation of enzymes and osmium-containing redox polymers [15, 25, 33]. The peak currents scale linearly with the square root of the scan rate at higher scan rates, which indicates semi-infinite diffusion within the films, as expected for multi-layered redox-active films on electrodes [32]. CV of enzymatic electrodes containing MWCNTs and either FADGDH or GOx at slow scan rates yielded peak-to-peak potential differences of 22 ± 4 and 18 ± 9 mV, respectively; these values are less than the 59 mV expected for a
one-electron redox process under semi-infinite diffusion control [32], but greater than the value expected for a surface-confined species, perhaps because of non-idealities as a result of interactions within the films [33] and/or dispersion in the redox kinetics [34].

Figure 1 Slow-scan (2 mV s⁻¹) CV in PBS at 37 °C of films of FADGDH (A) and GOx (B) co-immobilised with Os(dmbpy)PVI (—) and 68% (→) and 81% (→→) w/w MWCNTs.
An increase in the Os surface coverage with an increase in the amount of MWCNTs was observed for all enzymatic electrodes (Figure 2) with \( \Gamma_{\text{Os}} \) values of up to 150 nmol cm\(^{-2}\) obtained for enzymatic electrodes prepared by the deposition of solutions containing 81% w/w MWCNTs. This suggests that although the same amount of redox polymer was deposited on all enzymatic electrodes, not all the Os redox centres were either retained or electrically communicating with the electrode for films prepared with lower amounts of MWCNTs. The addition of MWCNTs provides an increased surface area, as evident from CV experiments (Figure 1), in which the capacitive current flowing increases as a function of the amount of MWCNT added, thus allowing for improved retention and/or addressing of the deposited Os redox polymer [6].

To estimate the retention of enzyme activity at the enzymatic electrodes and compare this with the retention of redox active polymer, a series of electrodes was prepared and the retained enzyme activity spectrophotometrically assayed using established procedures [35, 36] (Figure 2). An increase in retained enzyme activity was observed for GOx- and FADGDH-based enzymatic electrodes as a function of MWCNT, up to amounts of approximately 70% w/w MWCNT, above which the retained enzyme activity decreases. Others have reported a similar response of redox-polymer-based enzymatic electrodes by using SWCNTs [6], and suggest that as more CNTs are added there is an eventual decrease when electron transfer from enzyme to redox centre of the polymer becomes limiting. Although enzymatic electrodes prepared using FADGDH or GOx display similar Os surface coverage, it is interesting to note that electrodes prepared with FADGDH show considerably lower retained enzyme activity compared to those prepared with GOx. The maximum retained enzyme activity of 12.2 ± 3.4 U for GOx based electrodes at a MWCNT loading of 68% w/w is similar to the enzyme activity of the deposition solution (≈12 U), confirming that practically all of the GOx within the films is accessible to glucose and O\(_2\) as an electron acceptor, under the conditions of the assay. The maximum retained enzyme activity of 3.5 ± 0.4 U for FADGDH-based electrodes, also at a MWCNT loading of 68% w/w, indicates that only approximately 30% of the enzyme activity in the deposition solution (≈12 U) is retained, possibly owing to the relative inaccessibility of the FADGDH to the nitrotetrazolium blue (NTB) electron acceptor when co-
immobilised within the films, compared to that of \( \text{O}_2 \) for GOx, under the conditions of the assay.

**Figure 2** Plot of glucose-oxidation activity measured using spectrophotometry ( ), and \( \Gamma_{\text{Os}} \) from slow-scan CV ( and ), as a function of MWNCT amount for enzymatic electrodes prepared using A) FADGDH and B) GOx.
3.4.1 Glucose Oxidation Current

A better way to compare the activity of glucose oxidation within the enzymatic electrodes is to use the steady-state oxidation current signal in the presence of glucose. Thus, glucose oxidation currents as a function of MWCNT content were measured using slow-scan CV and fixed-potential amperometry. The addition of high concentrations (100 mM) of glucose to electrochemical cells resulted in sigmoidal slow-scan cyclic voltammograms for all enzymatic electrodes (Figure 3), as expected for an electrocatalytic (EC') process [32]. The onset of glucose oxidation was at approximately −0.1 V vs Ag/AgCl, half-wave potentials were close to the redox potential of the redox polymer, and steady-state maximum catalytic currents were achieved at potentials above 0.25 V for all enzymatic electrodes. The cyclic voltammograms for enzymatic electrodes containing 68% MWCNTs show fluctuations in the steady-state glucose-oxidation current densities, which suggests diffusion limitations, possibly owing to substrate diffusion within the film, given the independence of the current density on glucose concentration at this high glucose concentration (see below) [37]. For an initial comparison, steady-state catalytic current densities, extracted at 0.25 V from the cyclic voltammograms, of 2.83 mA cm\(^{-2}\) and 3.71 mA cm\(^{-2}\) were achieved for enzymatic electrodes containing FADGDH and GOx, respectively, using 68% w/w MWCNT at 100 mM glucose. Further comparison across the range of glucose concentrations and MWCNT amounts was undertaken by adding substrate to the electrochemical cell with enzymatic electrodes polarised at 0.25 V for all systems, to permit extraction of catalytic current densities. The resulting amperometric current densities (Figure 4) were similar in magnitude to the catalytic plateau current densities observed with slow-scan CV (Figure 3) for all systems, which confirmed the steady state.
Figure 3 Slow-scan (2 mV s$^{-1}$) CV in PBS containing glucose (100 mM) at 37 °C of films of FADGDH (A) and GOx (B) co-immobilised with Os(dmbpy)PVI (-----) and 68% (—) and 81% (-----) w/w MWCNTs.

For all enzymatic electrodes, the increase in glucose-oxidation current density as a function of glucose concentration followed the trend expected for the steady-state approximation of Michaelis–Menten enzyme kinetics, with an initial linear increase in current density that subsequently approached a maximum at high glucose
concentrations. Nonlinear least-squares fitting of the experimental points to the Michaelis–Menten equation was used to provide apparent Michaelis constants ($K_{M^{app}}$) and maximum saturated current densities ($j_{max}$) for each enzymatic electrode; the overall results are compiled in Table 1. The $K_{M^{app}}$ values obtained for all enzymatic electrodes are similar, although values for the GOx-based electrodes are slightly lower, at 8.7 ± 1.8 mM, compared to 13.6 ± 2.0 mM obtained for FADGDH-based electrodes, which agrees with the published solution-phase values [15, 25, 38] and suggests that GOx has a slightly better affinity for glucose compared to FADGDH.
Figure 4 Glucose oxidation current densities as a function of glucose concentration, measured in PBS at 37 °C with stirring at 150 rpm at an applied potential of 0.25 V, for enzymatic electrodes using FADGDH (A) and GOx (B) co-immobilised with Os(dmbpy)PVI (–) and 68% (▲) and 81% (■) w/w MWCNTs.

Enzymatic electrodes prepared with MWCNTs display higher saturated current densities compared to those prepared without MWCNTs (Figure 5 and Table 1). The
highest saturated current densities were obtained for electrodes with 68% w/w MWCNTs prepared with either FADGDH or GOx. The decrease in $j_{\text{max}}$ if MWCNT content is higher than 68% correlates with the decrease in retained enzyme activity within the enzymatic electrode films, when assayed spectrophotometrically (Figure 2), suggesting that retention of enzyme activity is the key parameter that controls the value of the saturated current density for these electrodes. Notably, although the retained spectrophotometrically assayed activity for the FADGDH enzymatic electrodes was at most only 30% of the activity of the deposition solutions compared to almost 100% for the best GOx-based enzymatic electrodes. $j_{\text{max}}$ values for all FADGDH based enzymatic electrodes were of similar magnitude to that obtained with GOx-based enzymatic electrodes (Table 1), achieving on average 85% of the GOx-based enzymatic electrode current density. This finding, again, indicates that access of the NTB electron acceptor, or the phenazine methosulfate (PMS) mediator, to the FADGDH within the films of the enzymatic electrodes might contribute to the low retained activity assayed for these enzymatic electrodes.

Figure 5 Plot of current density as a function of MWCNT amount for enzymatic electrodes based on FADGDH (●) and GOx (■) co-immobilised with Os(dmbpy)PVI in PBS containing glucose (100 mM) at 37 °C with stirring at 150 rpm at an applied potential of 0.25 V.
## Table 1. Apparent Michaelis-Menten constants and stability parameters for glucose-oxidising enzyme electrodes

<table>
<thead>
<tr>
<th>% w/w MWCNTs</th>
<th>( j_{\text{max}} ) (mA cm(^{-2}))</th>
<th>( K_{M}^{\text{app}} ) (mM)</th>
<th>( j_{0}^{[a]} ) in 100 mM glucose (mA cm(^{-2}))</th>
<th>( % j_{0}^{[b]} ) in 100 mM glucose after 20 h</th>
<th>( t_{\frac{1}{2}} ) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FADGDH enzyme electrodes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.7 ± 0.3</td>
<td>12 ± 3.8</td>
<td>0.7 ± 0.2</td>
<td>30 ± 11</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>41</td>
<td>1.7 ± 0.7</td>
<td>16 ± 6.7</td>
<td>1.6 ± 0.6</td>
<td>29 ± 6</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>59</td>
<td>2.2 ± 0.6</td>
<td>13 ± 1.7</td>
<td>2.2 ± 0.6</td>
<td>27 ± 5</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>68</td>
<td>2.7 ± 0.6</td>
<td>12 ± 1.0</td>
<td>2.6 ± 0.6</td>
<td>25 ± 8</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>74</td>
<td>2.0 ± 0.6</td>
<td>14 ± 2.6</td>
<td>2.1 ± 0.7</td>
<td>38 ± 19</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>78</td>
<td>2.2 ± 0.6</td>
<td>17 ± 4.8</td>
<td>2.1 ± 0.6</td>
<td>25 ± 12</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>81</td>
<td>2.1 ± 0.5</td>
<td>12 ± 4.4</td>
<td>2.0 ± 0.5</td>
<td>24 ± 12</td>
<td>13 ± 5</td>
</tr>
<tr>
<td><strong>GOx enzyme electrodes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.1 ± 0.2</td>
<td>5.5 ± 0.9</td>
<td>0.9 ± 0.2</td>
<td>39 ± 9</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>41</td>
<td>2.1 ± 0.2</td>
<td>8.8 ± 0.3</td>
<td>1.9 ± 0.2</td>
<td>55 ± 3</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>59</td>
<td>2.3 ± 0.3</td>
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<td>2.2 ± 0.3</td>
<td>59 ± 3</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>68</td>
<td>4.2 ± 0.4</td>
<td>9.5 ± 0.5</td>
<td>3.7 ± 0.4</td>
<td>52 ± 6</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>74</td>
<td>2.4 ± 0.3</td>
<td>8.3 ± 0.5</td>
<td>2.2 ± 0.3</td>
<td>58 ± 7</td>
<td>28 ± 7</td>
</tr>
<tr>
<td>78</td>
<td>2.1 ± 0.3</td>
<td>9.3 ± 1.0</td>
<td>1.9 ± 0.3</td>
<td>55 ± 8</td>
<td>27 ± 8</td>
</tr>
<tr>
<td>81</td>
<td>2.1 ± 0.2</td>
<td>11.5 ± 1.0</td>
<td>1.9 ± 0.1</td>
<td>55 ± 9</td>
<td>25 ± 9</td>
</tr>
</tbody>
</table>

[a] In 100 mM glucose. [b] In 100 mM glucose after 20 h.

Testing for stability of glucose-oxidation current generation under operational conditions is important for the application of such enzymatic electrodes to continuous glucose monitoring, or electricity generating, devices. The results of stability tests carried out in 100 mM glucose solutions, using an applied potential of 0.25 V over 20 h in PBS at 37 °C, with stirring at 150 rpm, are shown in Table 1.
The glucose-oxidation current measured was fitted to a simple exponential decay, assuming first-order kinetics for the decrease in current, to permit extraction of the enzymatic electrode half-life ($t_{1/2}$; Table 1). The results obtained for the FADGDH-based electrodes showed no significant difference in either the percentage of initial glucose oxidation current density after 20 h ($<40\%$), or the calculated $t_{1/2}$ for the current decay profile (all $\approx11$ h), as a function of the amount of added MWCNTs. This agrees with results reported by others for similar enzymatic electrodes but without added MWCNTs [15, 25, 38]. The GOx-based electrodes showed similar stability parameters to those based on FADGDH if no MWCNTs were added. However, if MWCNTs were added in the enzymatic electrode preparation step, significant increases in the percentage of initial glucose-oxidation current density after 20 h (all $\approx55\%$) and the calculated $t_{1/2}$ for the current decay profile (all $\approx26$ h) were observed. A similar increase in operational stability of the glucose-oxidation current has been reported for enzymatic electrodes prepared using GOx, crosslinked to an Os-containing redox polymer by glutaraldehyde, with added MWCNTs [15, 25].

The lower stability of the FADGDH-based electrodes in the presence of MWCNTs compared to those of GOx might be the result of differences in lysine and histidine content that is available for cross-linking by the diepoxide; the primary sequence of FADGDH contains 26 lysine and seven histidine residues [39], whereas GOx has 14 lysine and 20 histidine residues [40]. However, the difference in stability might instead arise from differences in the thermal stability of enzyme activity. This is supported by the fact that redox activity is retained to similar extents for these enzymatic electrodes, as the percentage of GOs remaining after 20 h is 46% (FADGDH) and 52% (GOx) for films without MWCNTs, and 72% (FADGDH) and 77% (GOx) for 68% w/w MWCNTs, respectively.
3.5 Conclusions

Increases in enzymatic electrode current density and stability of current density output through the addition of nanomaterials have been reported previously [6, 15, 31]; thus, we compared the performances of enzymatic electrodes for glucose oxidation by varying the amount of MWCNTs added to films prepared with two glucose-oxidising enzymes (FADGDH and GOx) co-immobilised with an osmium-based redox polymer on graphite electrodes. The addition of increasing amounts of MWCNTs to enzymatic electrodes increased the amount of redox polymer that was co-immobilised and electronically coupled within the enzyme films. However, the glucose-oxidation activity, assayed spectrophotometrically or by measuring the glucose-oxidation current generated within the enzymatic electrode films, reached a maximum value for electrodes prepared with 68% w/w MWCNTs, and decreased in the films with larger amounts of MWCNTs, thus indicating that the retention of enzyme activity within films is the key parameter influencing current density for these electrodes. Interestingly, there is no evidence of a threshold amount of MWCNTs required to permit percolation of charge through the enzymatic electrode films; MWCNTs simply increase surface area for the retention of redox polymer and enzyme in the film. The enzymatic electrodes prepared using the optimised amount of MWCNTs provided up to 4.2 mA cm\(^{-2}\) glucose-oxidation current density at the relatively low applied anode potential of 0.12 V vs Ag/AgCl for the GOx-based electrodes in PBS at 37 °C, compared to 2.7 mA cm\(^{-2}\) for the FADGDH-based electrodes. Operation in pseudo-physiological conditions of 5 mM glucose in PBS at 37 °C resulted in current densities of 0.52 and 1.1 mA cm\(^{-2}\) for the FADGDH and GOx-based enzymatic electrodes, respectively. These electrodes, thus, show promise for application as anodes in EFCs for \textit{in vivo} or \textit{ex vivo} power generation.

3.6 Acknowledgements

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3.7 References


Chapter 4

Published as

Glucose oxidation by osmium redox polymer mediated enzyme electrodes operating at low potential and in oxygen, for application to enzymatic fuel cells

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Co-author contributions:

I synthesised the osmium redox systems, and performed the laboratory work, the analysis, and wrote the first draft of the publication.

Peter Ó Conghaile and Paul Kavanagh contributed advice during the laboratory work.

Dónal Leech, as the project supervisor, contributed through guidance and advice throughout and wrote the final draft of the publication.
Glucose oxidation by osmium redox polymer mediated enzyme electrodes operating at low potential and in oxygen, for application to enzymatic fuel cells

4.1 Abstract

Graphite electrodes modified with a redox polymer, \([\text{Os}(4,4'-\text{dimethoxy-2,2'}'-\text{bipyridine})_2(\text{polyvinyl imidazole})_{10}\text{Cl}]\text{Cl} \ (E^\circ = -0.02 \text{ V vs Ag/AgCl})\), crosslinked with glucose oxidising enzymes and various amounts of multi-walled carbon nanotubes are investigated for current generation in the presence of glucose in physiological buffer solutions. Enzyme electrodes based on glucose oxidase and FAD-dependent glucose dehydrogenase are compared in the presence and absence of oxygen. The highest glucose oxidation currents are produced from enzyme electrodes containing 68 \% w/w multi-walled carbon nanotubes in the deposition matrix. The FAD-dependent glucose dehydrogenase and glucose oxidase enzyme electrodes provide similar current density of ~0.8 mA cm\(^{-2}\) in de-oxygenated 50 mM phosphate-buffered saline at 37 °C containing 5 mM glucose concentration. Current densities under the same conditions, but in the presence of oxygen are 0.50 mA cm\(^{-2}\) and 0.27 mA cm\(^{-2}\), for glucose dehydrogenase and glucose oxidase enzyme electrodes, respectively, with decreased currents a result of oxygen reduction by the redox polymer in both cases, and oxygen acting as a co-substrate for the glucose oxidase-based electrodes. Application of the anodes in membrane-less enzymatic fuel cells is demonstrated by connection to cathodes prepared by co-immobilisation of \([\text{Os}(2,2'-\text{bipyridine})_2(\text{polyvinyl imidazole})_{10}\text{Cl}]\text{Cl}\) redox polymer, \(\text{Myrothecium verrucaria}\) bilirubin oxidase and multi-walled carbon nanotubes on graphite electrodes. Power densities of up to 270 µW cm\(^{-2}\) are achieved, showing promise for \textit{in vivo} or \textit{ex vivo} power generation under these conditions.
4.2 Introduction

Enzymatic biofuel cells (EFCs) generate electrical energy through enzymatic reactions oxidising fuel at the anode and reducing oxidant at the cathode [1, 2]. The oxidoreductase enzymes used in EFCs provide specificity at fuel cell electrodes that open the possibility for miniaturisation of the system by eliminating the need for casings and separating membranes [3-5]. In addition, enzyme catalysts can oxidise complex fuels, for example glucose at the anode, under mild conditions (20 - 40 °C, neutral pH) compared to metal-based fuel cell catalysts. Research on EFCs is of increasing interest as such miniaturised membrane-less EFCs could generate power from sugar and oxygen present in physiological fluids or easily transportable packs, thus providing potential for application to power portable electronic devices, biosensors and implantable devices [6-8].

In most EFC electrodes mediators are incorporated to allow for electron shuttling from the enzyme active site to electrodes as active sites are buried within a proteinaceous insulating envelope too distant from the electrode surface to permit rapid electron transfer to occur. Mediators possessing a redox potential suitable for rapid electron transfer between the enzyme active site and the electrode surface can improve current capture at an electrode surface. In redox mediation, to have an effective electron exchange, the thermodynamic redox potentials of the enzyme and the mediator should be tuned. The tuning of these potentials is a compromise between the need to have a high cell voltage and a high mediated current. Mediators can be sub-classified into organic or metal-based mediators [9], with the focus here on metal based osmium complexes as mediators. The osmium complexes are selected due to the fact that they are stable in the reduced/oxidised Os(II/III) states [10] within biologically relevant potential ranges (−0.4 to 0.4 V vs Ag/AgCl). Also, the redox potential of the Os(II/III) metal centre transition can be tuned by selection of appropriate electron-donating/withdrawing coordinating ligands.

Integration of nanomaterials as components of enzyme electrodes provides increased current capture as demonstrated by inclusion of gold nanoparticles, platinum nanoparticles, carbon nanoparticles, etc. [11-15]. The use of multi-walled carbon nanotubes (MWCNTs) has been shown to improve glucose oxidation currents for enzyme electrodes prepared by crosslinking the nanomaterial with enzymes and osmium-based redox polymers [16-19], attributed to improved retention of enzyme.
activity [20]. We previously reported that enzyme electrodes prepared by crosslinking glucose-oxidising enzymes and an \([\text{Os}(4,4\text{'-dimethyl-2,2\text{'-bipyridine})_2(\text{polyvinyl imidazole})_{10}\text{Cl}]\text{Cl} \ (\text{Os(dmbpy})\text{PVI})\) redox polymer with increasing amounts of MWCNTs maximum current density is obtained for enzyme electrodes containing 68% w/w MWCNTs, beyond which the retained enzyme activity decreases [20]. Glucose oxidase, GOx, is a widely used glucose oxidising enzyme due to its commercial availability and high turnover rate, thus becoming a benchmark for comparison of glucose oxidising enzyme electrodes. The main disadvantage however is its sensitivity to oxygen [21, 22], which can reduce the power output of EFCs [22, 23] through oxygen competition for electrons [10]. As a result, oxygen-insensitive glucose oxidising enzymes, such as the glucose dehydrogenases, may be utilised to alleviate this issue.

Here we report on the use of an \([\text{Os}(4,4\text{'-dimethoxy-2,2\text{'-bipyridine})_2(\text{polyvinyl imidazole})_{10}\text{Cl}]\text{Cl} \ (\text{Os(dmobpy})\text{PVI})\) co-immobilised with either GOx, or FADGDH on graphite electrodes with varying amounts of MWCNTs, in an attempt to improve the current and cell voltage of glucose|oxygen EFCs. The Os(dmobpy)PVI is as it has a lower reported redox potential, \(-0.02 \text{ V vs Ag/AgCl}\), compared to Os(dmbpy)PVI previously used, \(0.12 \text{ V vs Ag/AgCl}\) [21, 24, 25]. This lower anode mediator redox potential can contribute to an overall increased difference in potential between anode and cathode of an assembled EFC leading to improved power output of the fuel cell. As before [20] MWCNT addition to Os(dmobpy)PVI enzyme electrodes is optimised for current output. The electrodes are then used as anodes in membrane-less EFCs by combining with a cathode consisting of bilirubin oxidase (BOd) crosslinked with MWCNTs and \([\text{Os}(2,2\text{'-bipyridine})_2(\text{polyvinyl imidazole})_{10}\text{Cl}]\text{Cl} \ (\text{Os(bpy})\text{PVI})\) redox polymer on graphite electrodes [26].

4.3 Experimental

4.3.1 Materials
All chemicals and biochemicals were purchased from Sigma-Aldrich, unless otherwise stated. The glucose oxidase is from \textit{Aspergillus niger} (GOx EC 1.1.3.4.) and the FAD-dependent glucose dehydrogenase is from \textit{Aspergillus sp.} (FADGDH 1.1.99.10, Sekisui, Cambridge, USA; product GLDE-70-1192). The \textit{Myrothecium verrucaria} bilirubin oxidase (MvBOd) is provided by Amano Enzyme Inc. (Nagoya,
Japan). The MWCNTs (product 659258; Sigma-Aldrich) were pre-treated under reflux in concentrated nitric acid for 6 h and isolated by filtration. Polyethylene glycol diglycidyl ether (PEGDGE) was purchased from Sigma-Aldrich (average $M_n \sim 526$). All aqueous solutions unless otherwise stated were prepared in Milli-Q water (18 MΩ cm). Redox polymers Os(dmobpy)PVI, Os(dmbpy)PVI and Os(bpy)PVI were synthesised according to literature procedures [27, 28].

4.3.2 Anode enzyme electrode preparation

Electrodes were prepared from graphite rods (Graphite store, USA, 3.0 mm diameter, NC001295) insulated with heat shrink tubing and the exposed disk polished on fine grit paper to create a geometric working surface area of 0.0707 cm$^2$. Enzyme electrodes assembly was achieved by depositing 9.6 µL of a 5 mg mL$^{-1}$ redox polymer aqueous solution, 4.8 µL of a 10 mg mL$^{-1}$ enzyme aqueous solution, 2 µL of a 15 mg mL$^{-1}$ PEGDGE aqueous solution and different volumes of a 46 mg mL$^{-1}$ aqueous dispersion of acid-treated MWCNTs on the surface of the graphite working electrode and allowing the deposition to dry for 24 h.

4.3.3 Electrochemical measurements

Electrochemical tests were conducted using a CH Instrument 1030 multichannel potentiostat in a three electrode cell containing 50 mM phosphate buffer saline (PBS, 150 mM NaCl) pH 7.4, at 37 °C as electrolyte and a Ag/AgCl reference electrode, a graphite working electrode and a platinum mesh counter electrode (Goodfellow).

4.3.4 Enzyme activity assays

The GOx activity was determined using an o-Dianisidine, horseradish peroxidase coupled spectrophotometric assay by monitoring absorbance change (Agilent 8453 UV-visible spectrophotometer) at 460 nm [29]. The average activity obtained was 234 ± 82 U mg$^{-1}$ which correlates well with the reported activity from Sigma-Aldrich (100 – 250 U mg$^{-1}$).

The FADGDH activity was determined using a nitrotetrazolium blue (NTB) and phenazine methosulfate (PMS) coupled spectrophotometric assay by monitoring absorbance change at 570 nm [30]. The average activity obtained was 266 ± 58 U mg$^{-1}$ which is lower than that reported from Sekisui (1180 U mg$^{-1}$). These assays were also utilised to estimate the activity of the enzyme when immobilised on the
electrode surface, assuming that the enzyme activity remains the same at the surface as it was for the solution-phase assay.

4.3.5 Fuel cell assembly and testing
The EFCs were constructed by combining anode enzyme electrodes with a previously described enzymatic cathode [26]. These cathode enzyme electrodes were prepared as described for anode enzyme electrodes except using MvBOd as enzyme, Os(bpy)PVI as redox polymer and a volume of the 46 mg mL\(^{-1}\) aqueous dispersion of acid-treated MWCNTs to provide 78 % w/w MWCNTs in the coating procedure. The EFC current and power densities were estimated from linear sweep voltammetry (LSV) obtained at 1 mV s\(^{-1}\) and normalised to the geometric area of the current-limiting electrode. All electrodes were operated in phosphate buffered saline (PBS, 50 mM phosphate buffer containing 150 mM NaCl, pH 7.4) solutions at 37 °C unless otherwise stated.

4.4 Results and discussion
A substantial increase in current density due to added MWCNTs was previously reported for enzyme electrodes prepared using co-immobilisation of Os(dmbpy)PVI redox polymer and glucose-oxidising enzymes on graphite electrodes, thereby showing promise for generation of higher current and application as EFC anodes [20]. The voltage, and hence power output, of an assembled EFC can be increased if an anode can provide current density at lower operating redox potentials. This study thus targets research on using enzyme electrodes prepared by co-immobilisation in a similar matrix to that previously reported [20] but replacing the Os(dmbpy)PVI redox polymer with a redox polymer, Os(dmobpy)PVI, that has a lower redox potential, −0.02 V vs Ag/AgCl [21, 24, 25]. Verification of the electrochemical response of Os(dmobpy)PVI was undertaken using cyclic voltammetry (CV) in the presence and absence of substrate in PBS at 37 °C. The formal redox potential (\(E^\circ\)) for the Os(II/III) transition of the redox polymer, estimated from the midpoint between the oxidation and reduction peak potentials by CV recorded at 1 mV s\(^{-1}\) scan rate in the absence of substrate, is confirmed as −0.02 V ± 0.01 vs Ag/AgCl, which agrees well with the value reported for other enzyme electrodes prepared using this redox polymer [21, 24, 25]. Under the same conditions, the full width half maximum (FWHM) for oxidation of the enzyme electrode redox polymer is 0.110 ± 0.005 V vs Ag/AgCl, which is close to the predicted response of 0.09 V for an ideal
surface-confined one electron redox system [31]. In addition, the difference in oxidation and reduction peak potentials ($\Delta E_p$) is $0.022 \pm 0.002$ V, which is less than the 0.059 V separation expected for a one electron redox process under semi-infinite diffusion control (although deviating slightly from the predicted response of 0 V for an ideal surface-confined one electron redox system [31], presumably due to non-idealities within the film [32], and/or dispersion in redox kinetics [33]). The CV at slow scan rates ($< 20$ mV s$^{-1}$) in the absence of substrate display peak currents that vary linearly with scan rate, which again is indicative of surface-confined response [31]. At higher scan rates ($> 20$ mV s$^{-1}$), the peak currents scale linearly with the square root of the scan rate, indicating semi-infinite diffusional charge transport limitations within the films, as expected for multi-layered films [31]. The area under the peak for CV recorded at slow scan rates in the absence of substrate can be integrated to provide an estimate of osmium surface coverage, $\Gamma_{Os}$ of $68 \pm 7$ nmol cm$^{-2}$ and $33 \pm 4$ nmol cm$^{-2}$ in the presence and absence of MWCNTs, respectively. These values are characteristic of multilayer formation and are comparable to those reported by others from co-immobilisation of Os(dmobpy)PVI with GOx and MWCNTs on graphite electrodes [24, 34]. It is interesting to note that the $\Gamma_{Os}$ observed for the Os(dmobpy)PVI systems shows a 60% decrease in comparison to that observed for the Os(dmbpy)PVI systems [20] both in the presence and in the absence of MWCNT. Experiments conducted using solution phase UV-Visible spectrophotometry show a 20% decrease in absorbance, at 480 nm characteristic of the MLCT band for octahedral complexes of osmium [35], for solutions of Os(dmobpy)PVI in comparison to that for Os(dmbpy)PVI. The $\Gamma_{Os}$ difference may therefore be attributed to a lower amount of osmium complex co-ordinatively bound to PVI for the Os(dmobpy)PVI in comparison to that for Os(dmbpy)PVI.
Figure 1 Slow scan (1 mV s\(^{-1}\)) CV for enzyme electrodes prepared by co-immobilisation of FADGDH and Os(dmobpy)PVI without (solid line) or with (dashed line) addition of MWCNTs (80% w/w).

For the Os(dmobpy)PVI enzyme electrodes, an increase in the osmium surface coverage with an increase in the amount of MWCNTs added is apparent for electrodes prepared using both FADGDH and GOx as enzymes (Figure 2), as previously observed for enzyme electrodes based on Os(dmobpy)PVI [20]. This finding suggests that the addition of MWCNTs provides for increased surface area and allows for improved retention of osmium on the electrode surface [20].

Spectrophotometric assays at both FADGDH and GOx enzyme electrodes were carried out in order to estimate enzymatic activity retention as a function of MWCNT amounts on electrode surfaces. As observed in previous reports [15, 20], an increase in retained enzymatic activity is observed for all enzyme electrodes as a function of MWCNTs, up to amounts of approximately 68% (w/w) MWCNTs, above which the retained enzymatic activity decreases. This decrease suggests that electron transfer from enzyme to electron acceptor co-substrate becomes limiting with higher amounts of carbon nanotube added [15]. It is important to note that although the enzymatic activity deposited on electrodes is similar for FADGDH and GOx electrodes, 14 ± 4 and 11 ± 4 U, respectively, the FADGDH electrodes show lower retained spectrophotometrically assessed enzyme activity compared to GOx...
electrodes (Figure 2). We previously postulated that the PMS/NTB co-substrate (electron acceptors) used in the assay of FADGH enzyme activity might not access enzyme active sites within redox polymer enzyme electrodes to the same extent as the oxygen co-substrate (electron acceptor) used for the GOx activity assay [20]. The GOx enzyme electrodes prepared using Os(dmobpy)PVI show slightly lower retained enzyme activity in comparison to the previously reported Os(dmbpy)PVI systems [20] possibly because of the lower osmium surface coverage observed.

**Figure 2** Plot of % retained glucose oxidation activity (♦) and $\Gamma_{\text{Os}}$ (■) as a function of % w/w MWCNTs added for enzyme electrodes prepared by co-immobilisation of MWCNT, Os(dmobpy)PVI and either FADGDH (A) or GOx (B).
4.4.1 Glucose oxidation

Slow scan CV and fixed-potential amperometry is used to measure glucose oxidation currents for enzyme electrodes in the presence and absence of oxygen. A sigmoidal-shaped CV is obtained upon the addition of glucose to the electrochemical cells (Figure 3), as expected for an electrocatalytic (EC) process [31]. The onset potential for glucose oxidation is approximately $\approx -0.15$ V vs Ag/AgCl and the observed half-wave potentials are close to the redox potential of the redox polymer for all enzyme electrodes studied. Maximum catalytic currents are achieved at applied potentials above 0.15 V vs Ag/AgCl for all Os(dmobpy)PVI enzyme electrodes.

Figure 3 Slow scan (1 mV s$^{-1}$) CV recorded under 5 mM glucose (solid line) and 100 mM glucose (dotted line) concentrations for enzyme electrodes prepared by co-immobilisation of FADGDH and Os(dmobpy)PVI

Further screening of Os(dmobpy)PVI enzyme electrodes is thus undertaken using amperometry with an applied potential of 0.15 V to ensure sufficient overpotential is applied, and using gentle stirring of solutions at 150 rpm to minimise substrate depletion, to generate maximum catalytic currents. An increase in amperometric glucose oxidation current with increased amounts of MWCNTs is observed up to amounts equivalent to $\approx 70\%$ w/w MWCNT (Figure 4). Maximum glucose oxidation current densities in the presence of 100 mM glucose of $2.30 \pm 0.31$ mA cm$^{-2}$ and
1.96 ± 0.07 mA cm$^{-2}$ are obtained for 68% w/w MWCNTs for FADGDH and GOx enzyme electrodes, respectively (Figure 4). Amounts of MWCNTs deposited above ~70% w/w result in lower currents. As noted previously, retained enzyme activity decreases for MWCNT amounts above approximately 70% w/w indicating that this parameter may control the glucose oxidation current density output for these enzyme electrodes.

![Plot of glucose oxidation current density as a function of % MWCNT added in the preparation step for enzyme electrodes prepared by co-immobilisation of MWCNT, Os(dmobpy)PVI and either FADGDH (♦) or GOx (■). Currents recorded by amperometry in PBS solutions containing glucose (100 mM) at 37 °C with stirring at 150 rpm at an applied potential of 0.15 V.](image)

**Figure 4** Plot of glucose oxidation current density as a function of % MWCNT added in the preparation step for enzyme electrodes prepared by co-immobilisation of MWCNT, Os(dmobpy)PVI and either FADGDH (♦) or GOx (■). Currents recorded by amperometry in PBS solutions containing glucose (100 mM) at 37 °C with stirring at 150 rpm at an applied potential of 0.15 V.

### 4.4.2 Effect of oxygen

Experiments were conducted using the amount of MWCNTs to produce the maximum glucose current (68% w/w) in order to evaluate the role and effect of oxygen on these enzyme electrodes. A decrease in glucose oxidation current density for all systems in the presence of oxygen is apparent (Figure 5 and 6). In oxygen saturated electrolyte the FADGDH-based enzyme electrodes show a decrease in current density of 37% in solutions of both 5 mM and 30 mM glucose, for example. The GOx-based enzyme electrodes show a 64% decrease in current density in oxygen saturated electrolyte for solutions containing 5 mM glucose and a 43%
decrease in glucose solutions of 30 mM (Figure 5 and 6). Currents observed from controls carried out in the absence of glucose and presence of oxygen (not shown) suggest that reduction of molecular oxygen by the osmium redox polymer, which in turn reduces the amount of osmium redox centres that can be accessed by the electrons from glucose substrate, is the reason for a decrease in current density for all enzyme electrodes in the presence of oxygen, including those based on FADGH. Reduction currents evident in the CVs (Figure 5) in the presence of oxygen support this hypothesis. This has been previously reported on by Prévoteau et al. [21, 36] who demonstrated that molecular oxygen could be reduced by osmium based redox polymers with a formal redox potential ≤ +0.07 V vs Ag/AgCl [21, 36]. The decrease in current density in 5 mM glucose concentrations for GOx-based enzyme electrodes over and above the decrease observed for the FADGDH-based enzyme electrodes is as a result of the competition for electrons from the oxygen co-substrate for GOx [29, 36-38], forming H₂O₂ in the process. Under higher glucose concentrations (e.g. 30 mM) less current is proportionally “lost” to oxygen.
Figure 5 Slow scan (1 mV s\(^{-1}\)) CV of enzyme electrodes prepared by co-immobilisation of Os(dmobpy)PVI, 68% w/w MWCNT and either FADGDH (A) or GOx (B) in the presence (solid line) or absence (dotted line) of oxygen. Other conditions as in Figure 4.

The amperometric glucose oxidation current densities are similar to the current densities measured at the same applied potential (+0.15 V) in the slow scan CVs.
(Figure 5 and 6). An estimate of apparent Michaelis constant, $K_{M_{\text{app}}}$, and maximum saturated currents $j_{\text{max}}$ is obtained by non-linear least-squares fitting of the data, recorded in the absence of oxygen in Figure 6, to a Michaelis-Menten model and using the Lineweaver-Burke plots of the transformed data to verify the fit. The $K_{M_{\text{app}}}$ values observed for each of the systems without MWCNTs for GOx and FADGDH are $6.3 \pm 0.6$ mM and $11 \pm 3.5$ mM respectively, which agree well with reported values [19, 20, 39], and suggest that GOx displays higher affinity for glucose compared to FADGDH under these conditions. Reports have suggested that mass transport can be a limiting factor for these enzyme electrodes [40, 41], and the $K_{M_{\text{app}}}$ may therefore reflect instead a difference in mass transport and not affinity, particularly as deviations from the simple Michaelis-Menten model are observed at higher glucose concentrations. In the presence of oxygen, a sigmoidal shape is observed for current response as a function of glucose concentration for enzyme electrodes containing GOx, with low current up to 4 mM glucose and a sharp increase in current up to 20 mM glucose followed by a gradual levelling off of the current, due to the reduction of oxygen by the GOx and the osmium redox polymer as reported on by others [21, 42, 43].
Figure 6 Glucose oxidation current density as a function of glucose concentration recorded for enzyme electrodes prepared by co-immobilisation of Os(dmobpy)PVI, 68% w/w MWCNT and either FADGDH (A) or GOx (B) in the absence (●) or presence (▲) of oxygen. Other conditions as in Figure 4.

4.4.3 Stability
Operational stability tests carried out in 100 mM glucose solutions in PBS using amperometry at 0.15 V, show 30% retained glucose oxidation current after 20 hours
for FADGDH-based enzyme electrodes compared to 70% retained glucose oxidation current after 20 hrs for GOx-based enzymes electrodes, both in the absence of oxygen. This agrees well with results reported by others using similar enzyme electrodes with and without added MWCNTs [19, 20, 24, 37]. The improved stability observed for GOx-based electrodes is suggested to arise from differences in the thermal stability of enzymes [20]. For oxygen saturated systems, a significant decrease in stability is observed with the FADGDH-based enzyme electrodes retaining only ~3% of current after 20 hours compared to retentions of ~ 15% for the GOx-based enzyme electrodes. This loss in current may be as a result of peroxide production under oxygen saturated conditions for both enzyme electrodes that damages the enzymes. Previous reports have demonstrated that H₂O₂ is produced as a result of the reduction of molecular oxygen by osmium complexes [37] in the presence of oxygen.

4.4.4 Enzymatic Fuel Cells

A potential application for EFCs is to supply energy to medical implants relying on the electrochemical reactions of glucose and oxygen as fuel and oxidant which are present in vivo. We have assembled for testing under pseudo-physiological conditions EFCs using anodes prepared by co-immobilisation of Os(dmbpy)PVI or Os(dmobpy)PVI redox polymers with either the FADGDH or GOx enzymes and 68% w/w MWCNTs. Enzyme electrodes chosen as cathodes are based on previous reports [26] of Os(bpy)PVI redox polymer co-immobilised with MvBOd and 78% MWCNTs.

Assembled EFCs based on anodes of Os(dmobpy)PVI co-immobilised with FADGDH and 68% MWCNTs yield highest power output of 270 µW cm⁻² (Figure 7). This lower power output observed for EFCs with GOx-based anodes is as a result of the competition between mediator and oxygen as co-substrates (electron acceptors) of GOx under the low (5 mM) glucose concentrations, as evident from data in Figure 5 and 6. The higher power output observed for EFCs with anodes based on Os(dmobpy)PVI co-immobilised with FADGDH, in comparison to anodes based on Os(dmbpy)PVI is as a result of the higher cell voltage (lower anode operating potential) (Figure 7).
Figure 7 Power curves recorded for fully membrane-less enzymatic fuel cells in PBS containing 5 mM glucose, saturated O$_2$ at 37 °C. Anode enzyme electrodes prepared by co-immobilisation of 68% w/w MWCNT and either FADGDH (A) or GOx (B) with Os(dmobpy)PVI (solid line) or Os(dmbpy)PVI (dotted line). Cathode enzyme electrodes prepared by co-immobilisation of Os(bpy)PVI, 78% w/w MWCNT and MvBod. Power densities normalised to the geometric area of the current-limiting electrode.

In an attempt to evaluate the factors limiting the power output for the EFCs, polarisation curves were computed from linear sweep voltammetry at 1 mV s$^{-1}$ slow
scan for the anode and cathode enzyme electrodes for each EFC. The polarisation curves in Figure 8 indicate that the current at the cathode (as cathode and anode areas are the same) limits power produced at the assembled fuel cell for the EFC assembled using anodes based on Os(dmobpy)PVI co-immobilised with FADGDH. For all other EFCs assembled the current density at the anode was observed to limit the power produced (not shown).

![Figure 8](image_url) Polarisation curves recorded in PBS containing 5 mM glucose and saturated O$_2$ at 37 °C for enzyme electrodes prepared by co-immobilisation of 68 % w/w MWCNT with either FADGDH and Os(dmobpy)PVI (dotted line) or $Mv$BOd and Os(bpy)PVI (dashed line) reported vs Ag/AgCl, and for the enzyme electrodes assembled as a membrane-less fuel cell (solid line). Current densities normalised to electrode geometric area. Other conditions as in Figure 7.

The maximum power density for the EFC assembled using anodes based on Os(dmobpy)PVI co-immobilised with FADGDH is observed at 0.35 V. Previously Kim et al. reported on a membrane-less EFC operating at a power density of 50 µW cm$^{-2}$ at a 0.5 V cell voltage under physiological conditions (air saturated pH 7.4, 140 mM NaCl, 37.5 °C in 15 mM glucose concentration) [44], with the increased voltage due to the differences in redox potential of the osmium redox polymers selected. Comparison with other EFC results has proven difficult due to differences in operating conditions, such as pH, glucose concentrations and also electrode preparation methodologies. For example, Soukharev et al. [45] report an EFC using
GOx and fungal laccase, co-immobilised with osmium redox polymers on 7 µm diameter, 2 cm long, carbon fibres, produces a power density of 350 µW cm$^{-2}$ in 15 mM glucose solutions. Under similar conditions, except in 5mM glucose solutions and using GOx sourced from *Penicillium pinophilum*, an EFC was reported to produce a power density of 280 µW cm$^{-2}$ [46], which is the highest operating EFC in 5mM glucose to our knowledge. Our EFCs produce power densities of similar magnitude at macro-electrodes (compared to the microelectrodes used by others).

4.5 Conclusions

We compare the performances of glucose oxidising enzyme electrodes prepared using FADGDH and GOx with varying amounts of MWCNTs, co-immobilised with a low-potential osmium-based redox polymer on graphite electrodes. The maximum current is from enzyme electrodes prepared with 68 % w/w MWCNTs, as previously observed [20]. A marked decrease in current is observed for GOx-based electrodes when operated in the presence of oxygen and at low glucose concentrations, in comparison to the FADGDH-based electrodes. In addition, oxygen reduction by the mediator is apparent for all enzyme electrodes, including FADGDH-based electrodes, in oxygen saturated conditions. EFCs assembled from anodes based on Os(dmobpy)PVI or Os(dmbpy)PVI with FADGDH or GOx and a cathode containing MvBOd co-immobilised with Os(bpy)PVI and MWCNTs were tested for power generation under pseudo-physiological conditions. A maximum power output of 270 µW cm$^{-2}$ is achieved for EFCs using anodes based on Os(dmobpy)PVI co-immobilised with FADGDH and 68 % MWCNTs in oxygen-saturated, 5 mM glucose solutions.

4.6 Acknowledgments

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4.7 References


Chapter 5

Using design of experiment approach to optimise amounts of polymer, enzymes and multiwalled carbon nanotubes for use as anodes in an enzymatic fuel cell

5.1 Abstract
To improve the performance of glucose oxidising bioanodes, a design of experiment methodology is investigated. Graphite electrodes are modified with a redox polymer, [Os(4,4'-dimethoxy-2,2'-bipyridine)$_2$(polyvinylimidazole)$_{10}$Cl]Cl ($E^{\circ'} = -0.02$ V vs Ag/AgCl), crosslinked with a flavin adenine dinucleotide glucose dehydrogenase and multi-walled carbon nanotubes for electrocatalytic oxidation of glucose. Optimised amount of enzyme electrode components display maximum current densities of $1 \pm 0.1$ mA cm$^{-2}$ and $3.5 \pm 0.2$ mA cm$^{-2}$ at 0.15 V vs Ag/AgCl 50 mM phosphate-buffered saline at 37 °C containing 5 mM and 100 mM glucose concentrations respectively, largely consistent with the predicted values. Application of the anodes in membrane-less enzymatic fuel cells is demonstrated by connection to cathodes prepared by co-immobilisation of [Os(2,2'-bipyridine)$_2$(polyvinylimidazole)$_{10}$Cl]Cl redox polymer, Myrothecium verrucaria bilirubin oxidase and multi-walled carbon nanotubes on graphite electrodes. Power densities of up to 285 µW cm$^{-2}$, 146 µW cm$^{-2}$ and 60 µW cm$^{-2}$ are achieved in pseudo physiological buffer, artificial plasma and human plasma respectively, showing promise for in vivo or ex vivo power generation under these conditions.
5.2 Introduction

Enzymatic fuel cells (EFCs) are electrochemical devices that utilise biocatalyst for the conversion of chemical energy to electrical energy [1-3]. In EFCs, enzymes are used as biocatalysts as a replacement for the conventional metal catalyst, due to their specificity towards reactions they catalyse [1, 4, 5]. This specificity at fuel cell electrodes allows for the possibility of developing miniaturised, potentially implantable or portable membrane-less EFCs through the elimination of the need for casings and separating membranes [2-4, 6]. Another potential advantage of the use of enzyme catalyst over metal catalyst is its ability to operate under relatively mild conditions (20-40 °C, neutral pH), this thereby makes them attractive for power generation in vivo via the utilisation of fuels and oxidants such as glucose and oxygen present in the bloodstream [7-9].

The addition of redox mediators improves shuttling of electrons to enzyme-based electrodes between the active site and electrode surface, as active sites are buried within a proteinaceous insulating envelope too distant from the electrode surface to permit rapid electron transfer to occur. Thereby making electron transfer independent of orientation or proximity of the enzyme active site to the electrode surface, in comparison to that for direct electron-transfer mechanisms between enzyme and electrode [5, 10]. Current output from enzyme electrodes depends on selection of a mediator with appropriate structure (enzyme affinity) and suitable redox potential (driving force) for rapid electron transfer between the enzyme active site and the electrode surface [11, 12]. Osmium based mediators have been widely used [2, 13-15], owing to the ability to modulate the mediator redox potential of the central Os metal by using coordinating ligands, the relative stability of the resulting complexes in the reduced/oxidised states (OsII/OsIII), and because the hydrogel characteristics of enzymatic electrodes based on redox-polymer films permit rapid mass and charge transport, thus generating substantial current signals [2, 16, 17]. The inclusion of multi-walled carbon nanotubes (MWCNTs) has been shown to improve glucose oxidation currents for enzyme electrodes prepared by crosslinking the nanomaterial with enzymes and osmium-based redox mediators [8, 18-20], attributed to improved retention of enzyme activity [21, 22].

To enhance the current output of an enzymatic biofuel cell (EFC), optimisation of relative components (osmium redox polymer, FADGDH and MWCNTs) is very
crucial. The application of experimental design is necessary in an optimization strategy that involves various independent variables affecting the responding factors unlike the classical method of varying on factor at a time. Kumar et al. [23] used a response surface design of experiment methodology to optimise components used to construct enzyme electrodes. They report on a 32% increase in glucose oxidation current in comparison to that observed for enzyme electrodes optimised by varying of one factor at a time [23, 24]. The aim of this experiment is to apply experimental design for the optimisation of relative components for use in either biosensor or EFC applications. Previous research on the optimisation of component ratios of redox polymers, MWCNTs and GOx/FADGDH enzyme was achieved by varying one factor at a time [21]; this proved effective however this approach will allow valid statistical analysis to be carried out in an organized manner. The [Os(4,4'-dimethoxy-2,2'-bipyridine)$_2$(poly-vinylimidazole)$_{10}$Cl]$^+$ (Os(dmobpy)PVI) polymer was selected for screening due to its lower redox potential −0.02 V vs Ag/AgCl compared to [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$(poly-vinylimidazole)$_{10}$Cl]$^+$ (Os(dmbpy)PVI) 0.12 V vs Ag/AgCl which should possibly provide increased power output due to greater potential difference between the anode and the cathode, also reports have shown Os(dmobpy)PVI to have better binding affinities for enzyme as compared to Os(dmbpy)PVI [25], which should possibly contribute to improved current densities. FADGDH enzyme was selected for screening due to its oxygen-insensitive advantage in comparison with the oxygen-sensitive GOx which can affect the power output of EFCs.
5.3 Experimental

5.3.1 Materials
All chemicals and biochemicals were purchased from Sigma-Aldrich, unless otherwise stated. The flavin dependent glucose dehydrogenase is from *Aspergillus* sp. (FADGDH 1.1.99.10, Sekisui, Cambridge, USA; product GLDE-70-1192). The *Myrothecium verrucaria* bilirubin oxidase (*Mv*BOd) is provided by Amano Enzyme Inc. (Nagoya, Japan), Plasma from human (product 9523; Sigma-Aldrich). The MWCNTs (product 659258; Sigma-Aldrich) were pre-treated under reflux in concentrated nitric acid for 6 h and isolated by filtration. Polyethylene glycol diglycidyl ether (PEGDGE) was purchased from Sigma-Aldrich (average $M_n \approx 526$). All aqueous solutions unless otherwise stated were prepared in Milli-Q water (18 MΩ cm). Os(dmbpy)PVI redox polymer was synthesised according to literature procedures [26, 27].

5.3.2 Anode enzyme electrode preparation
Electrodes were prepared from graphite rods (Graphite store, USA, 3.0 mm diameter, NC001295) insulated with heat shrink tubing and the exposed disk polished on fine grit paper to create a geometric working surface area of 0.0707 cm$^2$. Enzyme electrodes assembly was achieved by depositing solutions of a 5 mg mL$^{-1}$ redox polymer aqueous solution, 10 mg mL$^{-1}$ FADGDH aqueous solution, 2 µL of a 15 mg mL$^{-1}$ PEGDGE aqueous solution and 46 mg mL$^{-1}$ aqueous dispersion of acid-treated MWCNTs on the surface of the graphite working electrode and allowing the deposition to dry for 24 h. The amount of different components of MWCNTs, Os(dmobpy)PVI and FADGDH added in the enzyme electrode preparation step is determined by the Design Expert Software (version 9, STAT-EASE Inc., Minneapolis, USA).
Table 5.1 The main factors determining the performance of the bioanode in the enzyme electrode preparation step and their levels.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Low level (−1)</th>
<th>Central point (0)</th>
<th>High level (+1)</th>
</tr>
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<tbody>
<tr>
<td>MWCNTs (µg)</td>
<td>0 (0 µL)</td>
<td>300 (6.5 µL)</td>
<td>600 (13 µL)</td>
</tr>
<tr>
<td>Os(dmobpy)PVI (µg)</td>
<td>10 (2 µL)</td>
<td>55 (11 µL)</td>
<td>100 (20 µL)</td>
</tr>
<tr>
<td>FADGDH</td>
<td>20 (2 µL)</td>
<td>60 (6 µL)</td>
<td>100 (10 µL)</td>
</tr>
</tbody>
</table>

5.3.3 Electrochemical measurements

Electrochemical tests were conducted using a CH Instrument 1030 multichannel potentiostat in a three electrode cell containing 50 mM phosphate buffer saline (PBS, 150 mM NaCl) pH 7.4, at 37 °C as electrolyte and a Ag/AgCl reference electrode, a graphite working electrode and a platinum mesh counter electrode (Goodfellow).

5.3.4 Fuel cell assembly and testing

The EFCs were constructed by combining the optimised anode enzyme electrodes with a previously described enzymatic cathode [28]. These cathode enzyme electrodes were prepared as described for anode enzyme electrodes except using MvBOd as enzyme, Os(bpy)PVI as redox polymer and a volume of the 46 mg mL\(^{-1}\) aqueous dispersion of acid-treated MWCNTs to provide 78 % w/w MWCNTs in the coating procedure. The EFC current and power densities were estimated from linear sweep voltammetry (LSV) obtained at 1 mV s\(^{-1}\) and normalised to the geometric area of the current-limiting electrode.

All tests were conducted, unless otherwise stated, in 50 mM phosphate buffer solution, pH 7.4, containing 150 mM NaCl (PBS), at 37 °C by using a CH Instruments (IICambria) 1030A multi-channel potentiostat. Platinum mesh and Ag/AgCl were used as counter and reference electrode, respectively, for all voltammetry. Oxygen saturation was estimated, by using a dissolved oxygen electrode and meter (EUTech Instruments), to occur at approximately 0.22 mM O\(_2\), achieved by bubbling oxygen into the solution. The artificial plasma contained uric acid (68.5 mg L\(^{-1}\)), ascorbic acid (9.5 mg L\(^{-1}\)), fructose (36 mg L\(^{-1}\)), lactose (5.5 mg L\(^{-1}\)), urea (267 mg L\(^{-1}\)), glucose (916.5 mg L\(^{-1}\)), cysteine (18 mg L\(^{-1}\)), sodium
chloride (6.75 g L\(^{-1}\)), sodium bicarbonate (2.138 g L\(^{-1}\)), calcium sulfate (23.8 mg L\(^{-1}\)), magnesium sulfate (104.5 mg L\(^{-1}\)) and bovine serum albumin (7 g L\(^{-1}\)) [29].

5.4 Results and discussion

Enzyme electrodes were initially modified through the co-immobilisation of Os(dmobpy)PVI redox polymer, FADGDH and MWCNTs, using a PEGDGE di-epoxide cross-linker on graphite electrode. Cyclic voltammetry (CV) is used to characterise the Os(II/III) transition for the Os(dmobpy)PVI in the enzyme electrode for the presence and absence of substrate. CVs recorded at 1 mV s\(^{-1}\) scan in the absence of glucose displayed oxidation and reduction peaks centred at −0.02 V vs Ag/AgCl, which is similar to the redox potential previously reported for the osmium polymer in solution and on electrode surface [16, 19, 25]. In the absence of substrate, peak currents vary linearly with scan rate at slow scan rates (< 20 mV s\(^{-1}\)), thereby indicating a surface-confined response [30]. At high scan rates (> 20 mV s\(^{-1}\)), CVs display peak currents that scale linearly to the square roots of scan rates, which indicates semi-infinite diffusion control as expected for the formation of multilayered films on electrode surface [30]. The osmium surface coverage (\(\Gamma_{Os}\)) for the redox polymer is calculated by integrating the area under the peak for CVs recorded at slow scan rates, the \(\Gamma_{Os}\) is comparable to previously reported values in the presence of MWCNTs [19]. The addition of glucose to the electrochemical cells was measured at slow scan rates resulted in a sigmoidal-shaped CV for enzyme electrodes (Figure 5.1), which is characteristic of an electrocatalytic (EC') process [30]. A notable difference in shift ~ 100 mV for the half-wave potential (\(E_{1/2}\)) of the sigmoidal-shaped catalytic CV for enzyme electrodes in the presence of glucose in comparison to the absence of glucose is observed. Others have reported that a shift in the \(E_{1/2}\) of sigmoidal-shaped catalytic CV for enzyme electrodes indicate glucose substrate transport limitation and occurs when a mediated-limited case and a substrate-limited case is crossed [31, 32].
Catalytic current densities for enzyme electrodes are extracted from steady state amperometry at an applied potential of 0.15 V, in which glucose oxidation current density is measured as a function of glucose concentrations (Figure 5.1). Amperometric current densities obtained are similar in magnitude to the catalytic plateau current densities observed with the slow-scan CV, thereby confirming steady state. An increase in glucose oxidation current density as a function of glucose concentration is observed, with substrate saturation at concentrations greater than 50 mM glucose for all enzyme electrodes. Apparent Michaelis-Menten constants, $K_M^{app}$, and maximum current densities $j_{max}$ can be estimated using non-linear least squares to fit the amperometric plots to the Michaelis-Menten equation (Figure 5.2). The $K_M^{app}$ of $16 \pm 1$ mM is obtained for enzyme electrodes co-immobilised with Os(dmobpy)PVI, MWCNTs and FADGDH and is similar to values obtained from previous report [19, 21].

**Figure 5.1** 1 mV s$^{-1}$ CVs of Os(dmobpy)PVI (80 µg) films co-immobilised with FADGDH (64 µg) and MWCNTs (540 µg) in PBS containing no glucose ( ), 5 mM glucose ( ) and 100 mM glucose ( ) concentrations.
Figure 5.2 Glucose oxidation current densities as a function of glucose concentration, measured in PBS at 37 °C with stirring at 150 rpm at an applied potential of 0.15 V, for enzymatic electrodes using Os(dmobpy)PVI (80 µg) co-immobilised with FADGDH (64 µg) and MWCNTs (540 µg).

5.4.1 Design of experiments

For the optimisation of bioanodes, a DoE based on response surface factorial Box–Behnken Design (BBD) with a three level factorial design is used to evaluate the main effect and interaction between the MWCNTs, osmium redox polymer and FADGDH components required to prepare glucose-oxidising enzyme electrodes. The Box–Behnken design requires an experiment number according to $N = 2k (k − 1) + C_0$ [33], where $k$ is the number of factors, and $C_0$ is the number of central points. The 16 run experimental design is used to demonstrate the relative significance of the bioanode components and enhance its current density in pseudo-physiological conditions. The range and level of components investigated are given in Tables 5.1 and 5.2.
Table 5.2 Design layout showing the amount of relative components and their responses

<table>
<thead>
<tr>
<th>Run</th>
<th>Os(dmobpy)PVI</th>
<th>MWCNTs</th>
<th>FADGDH</th>
<th>Response Current density/ µA cm(^{-2}) (5 mM)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1</td>
<td>0</td>
<td>1</td>
<td>0.36</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.75</td>
<td>0.13</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0.77</td>
<td>0.08</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>1</td>
<td>-1</td>
<td>0.58</td>
<td>0.04</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.85</td>
<td>0.10</td>
</tr>
<tr>
<td>6</td>
<td>-1</td>
<td>-1</td>
<td>0</td>
<td>0.23</td>
<td>0.08</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>-1</td>
<td>1</td>
<td>0.58</td>
<td>0.12</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>0</td>
<td>-1</td>
<td>0.63</td>
<td>0.14</td>
</tr>
<tr>
<td>9</td>
<td>-1</td>
<td>0</td>
<td>-1</td>
<td>0.21</td>
<td>0.07</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.70</td>
<td>0.10</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>-1</td>
<td>-1</td>
<td>0.39</td>
<td>0.05</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.79</td>
<td>0.11</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>-1</td>
<td>0</td>
<td>0.49</td>
<td>0.05</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.83</td>
<td>0.15</td>
</tr>
<tr>
<td>15</td>
<td>-1</td>
<td>1</td>
<td>0</td>
<td>0.14</td>
<td>0.05</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0.87</td>
<td>0.24</td>
</tr>
</tbody>
</table>

The low levels of FADGDH and Os(dmobpy)PVI in this design are selected to be 20 µg and 10 µg respectively as a minimum level requirement for the production of glucose oxidation current density based on previous reports [2, 10, 14, 23, 34]. The high levels selected for each component are as a result of the difficulties that may be accompanied in co-immobilisation of higher amounts on electrode surface. For example, if higher amounts of the components are added, it would be difficult to control the drop-coat on the electrode surface. In this response model, analysis of variance (ANOVA) is used for statistical testing and the data extracted demonstrates whether or not the model is statistically significant [35]. The coefficient of variance
(R²), Fisher’s test (F-test) and probability (p-value) are used to evaluate the significance of the deviation from a null hypothesis [35]. For example, the low F-value (19.43) and p-value (0.0009) evaluated (Table 3) suggests that the model is statistically significant, therefore, the higher the values, the more likely the rejection of the null hypothesis that the data show no variation. Furthermore, the coefficient of determination (R²) between predicted and observed responses was evaluated to be 0.97, when the amount of variation in the model was adjusted, the measure of the adjusted R² (Adj R²) value is 0.92, thereby suggesting significant correlation. These results are similar to those observed previously by Kumar et al. [23] on enzyme electrodes co-immobilised using MWCNTs, GOx, osmium redox complex and carboxymethylated dextran in physiological conditions. There were no irregularities observed in the DoE model from the residual diagnostics, as the predicted current densities of enzyme electrodes correlated well with the observed current densities (see Figure 5.3).

**Table 5.3** Analysis of variance (ANOVA) for response surface quadratic model of the enzyme electrode response

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degree of freedom</th>
<th>Mean square</th>
<th>F-value</th>
<th>P-value</th>
<th>Prob &gt; F</th>
<th>R²</th>
<th>Adj R²</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>0.85</td>
<td>9</td>
<td>0.095</td>
<td>19.43</td>
<td>0.0009</td>
<td>0.9</td>
<td>0.92</td>
<td>Significant</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>0.029</td>
<td>6</td>
<td>4.9 × 10⁻³</td>
<td>0.90</td>
<td>0.5336</td>
<td>7</td>
<td></td>
<td>Not significant</td>
<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td>0.014</td>
<td>3</td>
<td>4.6 × 10⁻³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure error</td>
<td>0.015</td>
<td>3</td>
<td>5.1 × 10⁻³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.88</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.3 Observed amperometric glucose oxidation current densities (mA cm\(^{-2}\)) vs the predicted current densities (mA cm\(^{-2}\)) at 0.15 V vs Ag/AgCl in 5 mM glucose solution.

5.4.1.1 Validation of model

In order for a system to be optimised, it is imperative to demonstrate that the model is a reasonable representation of the actual system and that it reproduces system behaviour with enough fidelity to satisfy analysis objectives. Model validation was tested based on values randomised by the model together with their predicted results (Table 5.4) for pseudo physiological conditions (5 mM glucose, 50 mM phosphate buffered saline, PBS, pH 7.4, 37 °C). Enzyme electrodes were prepared through the co-immobilisation of the different components using the randomised amounts; experiments were carried out under pseudo physiological conditions and the results achieved are as shown in Table 5.4.
Table 5.4 Model validation showing predicted and experimental values of the enzyme electrode response at pseudo physiological conditions (n = 4).

<table>
<thead>
<tr>
<th>Os(dmobpy) PVI (µg)</th>
<th>MWCNTs (µg)</th>
<th>FADGDH (µg)</th>
<th>Predicted Current density mA cm⁻²</th>
<th>Actual Current density mA cm⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>56 (11 µL)</td>
<td>316 (6.8 µL)</td>
<td>63 (6.3 µL)</td>
<td>0.80 ± 0.07</td>
<td>0.81 ± 0.05</td>
</tr>
<tr>
<td>100 (20 µL)</td>
<td>316 (6.8 µL)</td>
<td>63 (6.3 µL)</td>
<td>0.78 ± 0.07</td>
<td>0.89 ± 0.04</td>
</tr>
<tr>
<td>100 (20 µL)</td>
<td>600 (13 µL)</td>
<td>63 (6.3 µL)</td>
<td>0.81 ± 0.07</td>
<td>0.88 ± 0.07</td>
</tr>
<tr>
<td>100 (20 µL)</td>
<td>600 (13 µL)</td>
<td>97 (9.7 µL)</td>
<td>0.88 ± 0.07</td>
<td>1.07 ± 0.13</td>
</tr>
<tr>
<td>100 (20 µL)</td>
<td>600 (13 µL)</td>
<td>26 (2.6 µL)</td>
<td>0.67 ± 0.07</td>
<td>0.96 ± 0.09</td>
</tr>
<tr>
<td>100 (20 µL)</td>
<td>291 (6.3 µL)</td>
<td>26 (2.6 µL)</td>
<td>0.64 ± 0.07</td>
<td>0.78 ± 0.15</td>
</tr>
<tr>
<td>54 (11 µL)</td>
<td>600 (13 µL)</td>
<td>26 (2.6 µL)</td>
<td>0.59 ± 0.07</td>
<td>0.60 ± 0.09</td>
</tr>
<tr>
<td>100 (20 µL)</td>
<td>600 (13 µL)</td>
<td>100 (10 µL)</td>
<td>0.89 ± 0.07</td>
<td>1.06 ± 0.27</td>
</tr>
</tbody>
</table>

A plot of predicted current density vs observed current density to show the linear relationship is constructed by the model using the above results (Table 5.5 and Figure 5.4). A correlation (R²) of 0.8897 is achieved suggesting that the model is valid. Minor deviations on certain points in the model might be as a result of human error during experiments.
Table 5.5 Box-Behnken Model validation of variables and measured amperometric current density response at 0.2 V vs Ag/AgCl for enzyme electrodes under pseudo-physiological conditions

<table>
<thead>
<tr>
<th>Run</th>
<th>Predicted values (mA cm(^{-2}))</th>
<th>Observed values (mA cm(^{-2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>0.23</td>
</tr>
<tr>
<td>2</td>
<td>0.18</td>
<td>0.14</td>
</tr>
<tr>
<td>3</td>
<td>0.78</td>
<td>0.75</td>
</tr>
<tr>
<td>4</td>
<td>0.82</td>
<td>0.87</td>
</tr>
<tr>
<td>5</td>
<td>0.78</td>
<td>0.85</td>
</tr>
<tr>
<td>6</td>
<td>0.45</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>0.18</td>
<td>0.21</td>
</tr>
<tr>
<td>8</td>
<td>0.81</td>
<td>0.78</td>
</tr>
<tr>
<td>9</td>
<td>0.82</td>
<td>0.8</td>
</tr>
<tr>
<td>10</td>
<td>0.62</td>
<td>0.63</td>
</tr>
<tr>
<td>11</td>
<td>0.78</td>
<td>0.7</td>
</tr>
<tr>
<td>12</td>
<td>0.6</td>
<td>0.58</td>
</tr>
<tr>
<td>13</td>
<td>0.78</td>
<td>0.84</td>
</tr>
<tr>
<td>14</td>
<td>0.38</td>
<td>0.37</td>
</tr>
<tr>
<td>15</td>
<td>0.44</td>
<td>0.39</td>
</tr>
<tr>
<td>16</td>
<td>0.57</td>
<td>0.59</td>
</tr>
<tr>
<td>17</td>
<td>0.8</td>
<td>0.81</td>
</tr>
<tr>
<td>18</td>
<td>0.78</td>
<td>0.89</td>
</tr>
<tr>
<td>19</td>
<td>0.81</td>
<td>0.88</td>
</tr>
<tr>
<td>20</td>
<td>0.88</td>
<td>1.07</td>
</tr>
<tr>
<td>21</td>
<td>0.67</td>
<td>0.96</td>
</tr>
<tr>
<td>22</td>
<td>0.64</td>
<td>0.78</td>
</tr>
<tr>
<td>23</td>
<td>0.59</td>
<td>0.6</td>
</tr>
<tr>
<td>24</td>
<td>0.89</td>
<td>1.06</td>
</tr>
</tbody>
</table>
Figure 5.4 A plot of predicted current density vs observed current density for enzyme electrodes prepared according to Table 5.4.

5.4.1.2 Optimisation of bioanode

Following the response achieved from enzyme electrodes prepared using this model, MWCNTs and Os(dmobpy)PVI were shown to be the main factors contributing to the enhanced current densities (Figure 5.5). This is not surprising as previous reports have shown that the addition of MWCNTs on the enzyme electrode increased the amount of redox polymer that is co-immobilised and electronically coupled within the enzyme films [21]. In order to evaluate the experimental parameters for enzyme electrode preparation to achieve the highest current density under these conditions, an optimisation study was carried out from the validated DoE model. Table 5.6 shows the optimum of these components based on the combination of the response surface model and contours (Figure 5.5), together with the predicted and experimentally determined enzymatic response.
**Figure 5.5** Contour plot for MWCNTs vs Os(dmobpy)PVI showing glucose oxidation current density values attributed to the contours in mA cm$^{-2}$.

**Table 5.6** Optimisation showing predicted and experimental values of the enzyme electrode response at pseudo physiological conditions ($n = 5$).

<table>
<thead>
<tr>
<th>Os(dmobpy)PVI (µg)</th>
<th>MWCNTs (µg)</th>
<th>FADGDH (µg)</th>
<th>Predicted Current density mA cm$^{-2}$</th>
<th>Actual Current density mA cm$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 (16 µL)</td>
<td>540 (12 µL)</td>
<td>64 (6.4 µL)</td>
<td>1.00 ± 0.07</td>
<td>1.00 ± 0.10</td>
</tr>
</tbody>
</table>

Enhanced current densities of $1 ± 0.1$ mA cm$^{-2}$ are obtained for the DoE optimised enzyme electrodes in physiological relevant glucose solutions, which is 30 % higher than previously obtained ($0.8$ mA cm$^{-2}$) for enzyme electrodes under similar conditions but using the one factor at a time (OFAT) approach [22]. Figure 5.1 and
5.2 shows the electrochemical response for the optimised enzyme electrodes with a maximum current density $j_{\text{max}}$ of $4.32 \pm 0.05 \text{ mA cm}^{-2}$ in comparison to previously reported $j_{\text{max}}$ of $2.30 \pm 0.31 \text{ mA cm}^{-2}$ for similar system except using the OFAT approach.

### 5.4.1.3 Optimised bioanodes in oxygen

Optimised bioanodes were further tested in the presence of oxygen in order to evaluate the effect of oxygen on these enzyme electrodes as their eventual application would be operation in EFCs. A decrease in glucose oxidation current density is apparent for enzyme electrodes in the presence of oxygen (Figure 5.6). A 17\% decrease in 5 mM glucose concentration is observed in the presence of oxygen. Previous reports have shown a 37\% decrease utilising the same components and methodology [22]. This difference could be as a result of the total w/w of MWCNTs added, as 79\% MWCNTs was added in the optimised bioanodes while 68\% MWCNTs was used in previous report [22]. Rajavel et al. [36] recently reported on the adsorption of oxygen molecules to MWCNTs. According to the report, oxygen interaction on the MWCNT depends on the available number of carboxyl functional sites at the nanotube surface, the lattice defects and quality of nanotube structures [36]. They further report that defect induced CNTs have a maximum adsorption energy of $-3.381 \text{ eV}$, which shows that oxygen molecules are chemically bound with defect sites in the nanotubes while pure CNTs show lower adsorption energy ($-0.753 \text{ eV}$) than defect nanotubes because of the physisorption of oxygen molecules [36]. Although we are unsure whether or not there are defects in the MWCNTs used throughout this study, it is possible that the higher amounts of MWCNTs on the electrode surface allows for more oxygen molecules to be adsorbed onto the MWCNTs thereby reducing the amount of oxygen detected on the enzyme electrode. The decrease in glucose currents in the presence of oxygen suggests that molecular oxygen is reduced by the osmium redox polymer, which in turn decreases the amount of osmium redox centres that can be accessed by the electrons from glucose substrate, thus the decrease in current density for all enzyme electrodes in the presence of oxygen [22]. This is demonstrated by Prévotaeu et al. and Osadebe et al. that molecular oxygen could be reduced by osmium based redox polymers with a formal redox potential $<-0.07 \text{ V vs Ag/AgCl}$ [16, 22, 37].
Figure 5.6 Glucose oxidation current density observed as a function of glucose concentration for optimised enzyme electrodes (Os(dmobpy)PVI (80 µg) co-immobilised with FADGDH (64 µg) and MWCNTs (540 µg) measured in PBS at 37 °C with stirring at 150 rpm at an applied potential of 0.15 V, for enzymatic electrodes in the presence (●) and absence of oxygen (●).

5.4.2 Enzymatic fuel cells

An enzymatic fuel cell is assembled based on the utilisation of glucose as fuel and oxygen as oxidant for testing under pseudo-physiological conditions using anodes prepared by co-immobilisation of or Os(dmobpy)PVI redox polymers with FADGDH and MWCNTs using amounts optimised from the DoE approach. Enzyme electrodes chosen as cathodes are based on previous reports [22, 28] of Os(bpy)PVI redox polymer co-immobilised with MvBOd and 78% w/w MWCNTs.

5.4.2.1 EFCs in pseudo physiological buffer

A potential application for EFC system is to power implantable medical devices via the oxidation of glucose as fuel and the reduction of oxygen as oxidant available in the bloodstream. Assembled EFC is first tested in pseudo physiological buffer conditions in 0.2 mM O₂ in an attempt to mimic in vivo human conditions. An average power density of 270 ± 15 µW cm⁻² is achieved (Figure 5.7) with a maximum power output of 285 µW cm⁻². The polarisation curves (Figure 5.8) for enzyme electrodes and the assembled EFC indicate that the cathode current density limits power produced at the assembled fuel cell. The maximum power density for
the EFC assembled is observed at ~0.3 V and is similar to that obtained previously [22]. Others have reported on a membraneless EFC operating at higher cell voltages, for example Kim et al. [38] on an EFC producing a power density of 50 µW cm$^{-2}$ at a 0.5 V cell voltage under physiological conditions (air saturated pH 7.4, 140 mM NaCl, 37.5 °C in 15 mM glucose concentration) [38] with the increased voltage due to the differences in redox potential of the osmium redox polymers selected. Due to differences in operating conditions, such as pH, glucose concentrations and also electrode preparation methodologies, comparison with other EFC results has proven difficult. For example, Soukharev et al. [39] report an EFC using GOx and fungal laccase, co-immobilised with osmium redox polymers on 7 mm diameter, 2 cm long, and carbon fibres, produces a power density of 350 µW cm$^{-2}$ in 15 mM glucose solutions. However, when the same EFC is tested for operation in 5 mM glucose solutions and using GOx sourced from *Penicillium pinophilum*, a power density of 280 µW cm$^{-2}$ is achieved [40] and it is similar to the maximum power density obtained in Figure 5.7. MacAodha et al. [28] has previously reported on an EFC operations in pseudo physiological conditions using similar enzyme electrodes but with Os(dmbpy)PVI redox polymer at the anode while *Myceliophthora thermophila* laccase enzyme was used at the biocathode produced a power density in buffer of 145 µW cm$^{-2}$ [28]. More recently, Osadebe et al. [22] reports on an EFC producing a maximum power output of 270 µW cm$^{-2}$ operating in pseudo physiological conditions, which is similar to those reported in Figure 5.7 with anodes based on Os(dmobpy) PVI co-immobilised with FADGDH and 68% w/w MWCNTs [22].

### 5.4.2.2 EFCs in artificial plasma

Assembled EFCs operating in artificial plasma, prepared according to Burtis et al. [29] allows for the reproduction of physiological conditions present in vivo without the associated risks of whole blood samples. An average power density of 109 ± 37 µW cm$^{-2}$ (Figure 5.7) with a maximum power output of 146 µW cm$^{-2}$ is achieved for EFC in artificial plasma. Maximum power output produced in artificial plasma is approximately half of that observed for the same EFC operating in PBS; a similar difference has been reported by MacAodha et al. [28]. This difference in power output observed between artificial plasma and PBS is probably due to the presence of antioxidants and enzyme-inhibiting compounds in the adopted artificial plasma solutions [41-43]. Furthermore, another contributing factor to the low power output
observed for EFC in artificial plasma relative to that in buffer is the oxygen concentration. In the artificial plasma, the oxygen concentration was determined to be lower than that achieved in PBS, with a concentration maintained at approximately 0.06 mM by means of oxygen bubbling through the solution compared to 0.125 mM in PBS. Previous reports on EFC operations using similar enzyme electrodes but with Os(dmbpy)PVI redox polymer at the anode while *Myceliophthora thermophila* laccase enzyme was used at the biocathode produced a power density in artificial plasma of 60 µW cm\(^{-2}\) [28]. To evaluate the factors limiting the power output for the EFCs, polarisation curves at the anode and cathode enzyme electrodes from the 1 mV s\(^{-1}\) slow scan CVs are used to compute cell polarisation curves for each EFC (see Figure 5.8). The polarisation curves indicate that the current at the cathode (as cathode and anode areas are the same) limits power produced at the assembled fuel cell for the EFC.

### 5.4.2.3 EFCs in human plasma

Further experiments were conducted to test for EFC operations in human plasma in an attempt to evaluate the effect of the deployment in real physiological solutions. The human plasma sample is used as purchased; it is originally extracted from whole human blood using anticoagulants to remove red blood cells. An average power density of 53 ± 9 µW cm\(^{-2}\) (Figure 5.7) with a maximum power output of 60 µW cm\(^{-2}\) is achieved for EFC in human plasma. The maximum power output observed for human plasma is approximately half of that observed in artificial plasma. This difference is possibly due to the fact that blood plasma contains additional components such as blood clotting factors, lipids, hormones, enzymes, antibodies, and other proteins/components not present in artificial plasma, some of which are enzyme-inhibiting [44]. Although the oxygen concentration measured in human plasma is 0.1 mM and similar to previous reported value [44, 45], the power density achieved is lower in comparison to artificial plasma which is probably due to the additional factors such as hormones, proteins etc. present in human blood but not in the artificial plasma [45]. The polarisation curves for the EFC shown in Figure 5.8 indicates that the cathode still limits the power produced in the EFC.

Others have reported on an EFC based on direct electron transfer using cellulobiose dehydrogenase enzyme at the anode and *MvBOd* enzyme at the cathode, in human serum [42, 44], human plasma and human blood samples [44], where no significant
change in power output between PBS and real physiological solutions were observed but a maximum power density of 4 µW is observed. A recent study on EFC utilising FADGDH with a ferrocene (FcMe2–C3–LPEI) redox hydrogel as bioanode and direct electron transfer at the biocathode using MvBOd immobilised onto multi-walled carbon nanotubes modified with anthracene moieties was investigated in human serum and citrate/phosphate buffer. Power densities of ~58 µW cm⁻² and ~45 µW cm⁻² are achieved in human serum and citrate/phosphate buffer respectively at 37 °C [7], which is the highest recorded power density in human serum to my knowledge. Ó Conghaile et al. [46] recently reports on an EFC constructed using enzyme electrodes of Os(dmbpy)PVI, MWCNT, and deglycosylated Pyranose dehydrogenase at anodes and Bod on AuNP substrate as cathode. Average power densities of up to 275 ± 50 µW cm⁻² were achieved in pseudo physiological conditions, and 73 ± 7 µW cm⁻² when tested in whole human blood which is the highest reported power generation in human blood to date [46].
Figure 5.7 Power curves recorded for fully membrane-less enzymatic fuel cells by 1 mV s$^{-1}$ linear sweep voltammetry at 37 °C in 50 mM PBS containing 5 mM glucose (blue dots), artificial plasma (red dash) and human plasma (green solid) for optimised bioanodes prepared by co-immobilisation Os(dmobpy)PVI 80 µg co-immobilised with FADGDH 64 µg and MWCNTs 540 µg. Cathode enzyme electrodes prepared by co-immobilisation of Os(bpy)PVI, MWCNT and MvBod. Power densities normalised to the geometric area of the current-limiting electrode.
Figure 5.8 Polarisation curves computed from 1 mV s\(^{-1}\) linear sweep voltammetry recorded in 5 mM glucose and O\(_2\) at 37 °C for optimised enzyme electrodes prepared by co-immobilisation Os(dmobpy)PVI 80 µg co-immobilised with FADGDH 64 µg and MWCNTs 540 µg (red dotted line). Cathode enzyme electrode prepared by co-immobilisation of MvBOd and Os(bpy)PVI (blue dashed line) reported vs Ag/AgCl, and for the enzyme electrodes assembled as a membrane-less fuel cell (black solid line) in PBS (A), artificial plasma (B) and human plasma (C). Current densities normalised to electrode geometric area.
5.5 Conclusions
Enzyme electrodes components were optimised and verified using response surface methodology to produce a current density of ~1.1 mA cm$^{-2}$ in physiological relevant glucose solutions 30% higher than previously observed for enzyme electrodes optimised by varying one electrode at a time. EFCs were assembled and tested for power generation to compare performances in PBS, artificial plasma and human plasma using the optimised bioanodes and a cathode containing $Mv$BOd co-immobilised with Os(bpy)PVI and MWCNTs. EFCs produced highest power densities of 285 µW cm$^{-2}$, 146 µW cm$^{-2}$ and 60 µW cm$^{-2}$ in PBS, artificial plasma and human plasma respectively which is the highest reported power densities in these solutions to date.
5.6 References


Chapter 6: Conclusions and future directions

6.1 Conclusions

The main aim of this thesis is to investigate different strategies for the optimisation of biocatalytic anodes for use in an enzymatic fuel cell capable of operating under in vivo conditions, with the aim of producing improved current densities over other methodologies.

Chapter 2 described the synthesis and characterisation of a range of osmium polypyridyl complexes. The complexes were characterised electrochemically and using a range of analytical techniques, including CHN microanalysis and mass spectrometry. The resulting osmium polypyridyl complexes are further used in the synthesis and electrochemical characterisation of redox polymers. The electrochemical characteristics of films of the redox polymers on electrodes were determined using CV. OsdmbpyPVI redox polymer previously synthesised is further co-immobilised with GOx on graphite electrodes in the presence of MWCNTs. The enzyme electrodes were co-immobilised using glutaraldehyde vapour with a NaBH₄ reduction crosslinking methodology. Although the use of the glutaraldehyde vapour with a NaBH₄ reduction crosslinking strategy show improved current signals and/or stability, the use of the crosslinking methodology may affect the performance of enzyme electrodes due to less control in the borohydride reduction step, which could result in detachment of films on electrode surface. Due to difficulties in the control during enzyme electrode preparation using GA(R) crosslinking, PEGDGE, as the more widely accepted methodology, is utilised as a crosslinker in chapters 3, 4 and 5 of this thesis.

Chapter 3 focused on comparing the performance of enzyme electrodes for glucose oxidation as a function of amount of added MWCNT to films prepared based on FADGDH or GOx as glucose-oxidising enzyme co-immobilised with an Os(dmbpy)PVI redox polymer on graphite electrodes. The addition of increasing amounts of MWCNTs to enzymatic electrodes increased the amount of redox polymer that was co-immobilised and electronically coupled within the enzyme films. However, the glucose-oxidation activity, assayed spectrophotometrically or by measuring the glucose-oxidation current generated within the enzymatic electrode films, reached a maximum value for electrodes prepared with 68% w/w MWCNTs,
and decreased in the films with larger amounts of MWCNTs, thereby indicating that the retention of enzyme activity within films is the key parameter influencing current density for these electrodes. The enzymatic electrodes prepared using the optimised amount of MWCNTs provided up to 4.2 mA cm$^{-2}$ glucose-oxidation current density at the relatively low applied anode potential of 0.12 V vs Ag/AgCl for the GOx-based electrodes in PBS at 37 °C, compared to 2.7 mA cm$^{-2}$ for the FADGDH-based electrodes. Operation in pseudo-physiological conditions of 5 mM glucose in PBS at 37 °C resulted in current densities of 0.52 and 1.1 mA cm$^{-2}$ for the FADGDH and GOx-based enzymatic electrodes, respectively. These electrodes, thus, show promise for application as anodes in EFCs for in vivo or ex vivo power generation.

Chapter 4 focused on the performances of glucose oxidising enzyme electrodes prepared using FADGDH and GOx with varying amounts of MWCNTs, co-immobilised with a low potential osmium-based redox polymer on graphite electrodes. The maximum current is from enzyme electrodes prepared with 68% w/w MWCNTs, as previously observed in chapter 3. A marked decrease in current is observed for GOx-based electrodes when operated in the presence of oxygen and at low glucose concentrations, in comparison to the FADGDH-based electrodes. In addition, oxygen reduction by the mediator is apparent for all enzyme electrodes, including FADGDH-based electrodes, in oxygen saturated conditions. EFCs assembled from anodes based on Os(dmobpy)PVI or Os(dmbpy)PVI with FADGDH or GOx and a cathode containing $\text{Mv} \text{BOd}$ co-immobilised with Os(bpy)PVI and MWCNTs were tested for power generation under pseudo-physiological conditions. A maximum power output of 270 μW cm$^{-2}$ is achieved for EFCs using anodes based on Os(dmobpy)PVI co-immobilised with FADGDH and 68% MWCNTs in oxygen-saturated, 5 mM glucose solutions.

Chapter 5 focused on the optimisation of individual components such as MWCNTs, FADGDH and Os(dmobpy)PVI using a response surface methodology in order to maximise current capture on enzyme electrode surface. The DoE model is developed and validated for enzyme electrode performance in pseudo-physiological conditions (5 mM glucose, 50 mM phosphate buffered saline, pH 7.4, 37 °C). The components were co-immobilised using PEGDGE on graphite electrode surface as previously reported in chapter 3 and 4 with an optimised value of 1 mA cm$^{-2}$ under pseudo physiological conditions. The optimised amounts were further used as bioanodes and
a cathode containing $Mv$BOd co-immobilised with Os(bpy)PVI and MWCNTs for EFC application. The EFCs produced highest power densities of 285 $\mu$W cm$^{-2}$, 146 $\mu$W cm$^{-2}$ and 60 $\mu$W cm$^{-2}$ in PBS, artificial plasma and human plasma respectively which is the highest reported power densities in these solutions to date.

### 6.2 Future directions

Future work could focus on the optimisation of combinations of redox polymer, nanostructured materials and oxygen reducing enzymes with the aim of enhancing current density for use as cathodes in biofuel cells or biosensors. This thesis is focused on the optimisation of anodes for utilisation in EFC using different methodologies (detailed in chapter 2, 3, 4 and 5). However, an optimisation study for the utilisation of an osmium based redox polymer with *Myrothecium verrucaria* bilirubin oxidase ($Mv$BOd) in the presence of MWCNTs as in these EFC systems has not been fully investigated. A design of experiment methodology as illustrated in chapter 5 can be utilised to optimise relative components used in cathode thereby improving current density obtained and subsequently power output produced in an EFC.

Another future direction is the development of a compact paper-based microfluidic EFC for use as *in vivo* applications. As the eventual application of EFCs would be utilisation as power supply for implantable and semi-implantable devices, miniaturisation of EFC at microscale would be required due to medical devices becoming more sophisticated. The use of microfabrication techniques provides an alternate route for miniaturisation in an EFC. Microfluidics is the science of fluid flow and transport phenomena in microstructures with at least one characteristic dimension in the range of 1–1000 $\mu$m [1]. A microfluidic fuel cell comprises of a fluid delivery and removal, reaction sites and electrode structures all confined to a microfluidic channel. Microfluidic fuel cells are laminar flow-based fuel cells and describe a group of fuel cells capable of operation within the framework of a microfluidic chip [2]. In a microfluidic cell design, the anolyte and catholyte flowing solutions are effectively separated as their mixing is restricted to a narrow interdiffusion region between two streams. In most common configurations, microfluidic fuel cells utilise the laminar flow characteristic, this flow regime is characterised by low Reynolds’ numbers:
\[ R_e = \frac{\rho U D_h}{\mu} \quad \text{Equation 6.1} \]

Where \( \rho \) is the fluid density (kg m\(^{-3}\)), \( U \) is the average velocity (m s\(^{-1}\)), \( D_h \) is the hydraulic diameter (m), and \( \mu \) is the dynamic viscosity (kg m\(^{-1}\) s\(^{-1}\)). In the construction of microfluidics EFC devices, channel structure are fabricated using polymeric materials such as polydimethylsiloxane (PDMS) is used. PDMS generally has benign properties for fuel cell applications; it is relatively inert and compatible with most solvents and electrolytes [3]. The obtained channel structure is sealed to a glass or PDMS substrate, either reversibly as is, or irreversibly following plasma-treating of both parts [4]. The use of plasma-treating also renders the channel walls hydrophilic, which is useful for microfluidic fuel cells to promote wetting and reduce pressure drop in the channel [4]. Also, channel structures can be fabricated by casting the PDMS on photolithographically patterned photoresist [5, 6]. The most common architecture for microfluidic fuel cells is the Y-shaped configuration, where two streams are horizontally combined in a microchannel, flowing over a reaction region where the fuel cell electrodes are placed (see Figure 6.1). The fuel is contained in one laminar stream while the second laminar stream contains the oxidant. Reports have shown that glass or plastic based microfluidic fuel cell utilising external pressure sources such as pumps to maintain the reactants in motion could limit their miniaturisation and portability [7]. The use of paper instead of glass or plastic allows for passive liquid transport through capillary action and could thereby eliminate the need for external pumping devices required for glass or plastic based microfluidic fuel cells [8, 9]. In addition, these paper based biodevices are simple to operate, fast, relatively inexpensive in comparison to glass or plastic [10, 11] and can prove advantageous for use in EFCs.
6.3 Three dimensional micro-channelled CNT scaffolds

To maximise the extent by which EFC can be miniaturised, research in the utilisation of three dimensional micro-channelled electrodes has been of great interest. For example, Katuri et al. [13] utilised MWCNT/chitosan (MWCNT/CHI) microchanneled structures in flow-through configuration as 3D anodes for application to microbial fuel cells with which a volumetric power density of 2 kW m\(^{-3}\) was obtained. The MWCNTS/CHI electrodes are constructed entirely from the components of the biocatalytic films usually deposited on graphite or glassy carbon electrodes based on previously published approach using ice segregation induced self-assembly (ISISA). ISISA is a technique that is simple to deploy, consisting of harnessing unidirectional freezing of any diluted aqueous suspension, by means of controlled immersion of the suspension into liquid nitrogen bath [14-21]. As the ice forms, the matter is segregated to the external boundaries of the ice, as the growth of ice continues the matter is compacted, forming the channel walls. The resulting frozen suspension is freeze-dried, forming cryogels containing macroporous structures characterized by “walls” of matter enclosing empty areas where ice crystals originally resided thereby resulting in a micro-channelled structure. ISISA scaffolds using dispersed suspension of CNT and chitosan to favour MWCNT dispersion and ensure homogeneity of the suspension have been previously reported,
for example, as supports for anodes in fuel cells [13, 22, 23]. Preliminary research is being directed at the formation of scaffolds, for use as biocathodes in EFCs. ISISA comprises of MWCNT suspensions dispersed using chitosan, for subsequent coupling of enzyme. Oxygen reducing enzyme $Mv$BOd is selected as cathode catalyst owing to its ability to undergo direct electron transfer [24] as previously discussed in the introduction chapter. An added advantage to $Mv$BOd also is that it works best at physiological conditions [25, 26], thereby making it attractive for use in EFCs. A cyclic voltammogram of the current density provided by the chitosan coupled $Mv$BOd/MWCNT scaffold with PBS in oxygen saturated conditions is shown in Figure 6.1. In addition, enzyme amount added to the scaffold was increased to test its effect on current density (Figure 6.2).

![Image](image.png)

**Figure 6.2** Slow scan CV (1 mV s$^{-1}$) of scaffolds prepared with MWCNT (75 mg mL$^{-1}$/chitosan, with 10 mgs (—), 20 mgs (—) and 30 mgs (—) of $Mv$BOd, in 50 mM PBS, pH 7.4, 150mM NaCl, in oxygen saturated conditions at 37 °C.

Increased current is observed with increased amount of enzyme with a current density of up to 0.4 mA cm$^{-2}$ achieved with the addition of 30 mg of $Mv$BOd (2.5 U mg$^{-1}$ activity). Although there has been report showing direct electron transfer using
gold microelectrodes and/or modified with gold nanoparticles [27] with current
densities of up to $-0.4 \text{ mA cm}^{-2}$ for fuel cell cathodes, there has been little or
nothing reported using the ISISA technique for the formation of 3D microchanneled
CNT scaffolds for use as biocathodes in EFC. Future studies should be directed
toward the optimisation of these scaffolds, in an effort to enhance direct electron
transfer and thereby resulting in high-current producing electrodes.

6.4 References


Appendix
Publications and presentations
Publications


• **I. Osadebe**, R. Kumar, P. Ó Conghaile, D. Leech, “Using design of experiment approach to optimise amounts of polymer, enzymes and multiwalled carbon nanotubes for use as anodes in an enzymatic fuel cell, in preparation

Selected Presentations
Oral presentations

• **Isioma Osadebe**, Rakesh Kumar, Peter Ó Conghaile, Domhnall Mac Aodha, Paul Kavanagh, Dónal Leech, “Combining enzymes, mediators and carbon nanotube supports for preparation of high current density glucose-oxidising biofuel cell anodes” in the 65th annual ISE meeting Lausanne, Switzerland September 2014.

Poster presentations

• **Isioma Osadebe**, Rakesh Kumar, Peter Ó Conghaile, Paul Kavanagh and Dónal Leech, “High current glucose anodes for application to enzymatic fuel cells” in Analytical Research Forum in UK, July 2015.
• **I. Osadebe**, Conan Mercer, Rakesh Kumar, Peter Ó Conghaile, Paul Kavanagh, D. Leech, “Electron transfer using osmium based mediators in biosensors and biofuel cells” in NUI Galway and University of Limerick Alliance Research day, Galway, Ireland, April 2015.

• **Isioma Osadebe**, Rakesh Kumar, Peter Ó Conghaile, Domhnall MacAodha, Paul Kavanagh, Dónal Leech, “Combining enzymes, mediators and conductive supports for anodes in biofuel cells” in CASi 2013: Cork.