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Tailoring surfaces and supports for enzyme electrodes with application to biopower device development

Submitted by:
Peter Ó Conghaile B.Sc. (Hons)

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

Research was conducted in: School of Chemistry & Ryan Institute,
The National University of Ireland,
Galway

Month and Year of Submission: November 2013
Head of School: Professor Paul V Murphy
Supervisor of the Research: Professor Dónal Leech
Declaration

The contents of this thesis, except where otherwise stated, are based entirely on my own work and was carried out in the School of Chemistry & Ryan Institute, National University of Ireland Galway, Galway; Analytische Chemie-Electroanalytik & Sensorik, Ruhr-Universität Bochum, Bochum, Germany; Department of Analytical Chemistry/Biochemistry and Structural Biology, Lund University, Lund, Sweden and Biomedical Laboratory Science, Health and Society, Malmö University, Malmö, Sweden. I have not obtained a degree at NUI Galway or any other university on the basis of any of this work.

____________________
Peter Ó Conghaile
November 2013
To my mom and the loving memory of my dad.
I would like to express my gratitude to all who helped and assisted me throughout my postgraduate studies,

Firstly I would like to thank my supervisor, Professor Dónal Leech, for his advice, guidance and willingness to share his tremendous expertise. His boundless patience, encouragement and understanding are very much appreciated.

The School of Chemistry staff at NUIG, especially the technical staff, Marion, Gerry, Dermot and Seamas,

Professor Wolfgang Schuhmann, Dr. Sergey Shleev and Professor Lo Gorton for giving me the opportunity to work with them,

I thank my fellow labmates in the BERL group in NUIG (and in ELAN, Bochum and CMPS, Sweden) both past and present, for putting up with me, being a friend on the bad days and enjoying the good days together,

To my friends outside the office, for the much needed outlet that was needed.

My brothers, Paraíc, Máirtín, Gerald, Colin and Paul, and sisters. Margaret, Kathy and Eileen, for everything they’ve done for me and going out of their way to help me.

Finally, I would like to dedicate this thesis to my mother, Mary-Anne, and father, Máirtín, ar dheis Dé go raibh a anam dílis. Thank you for your never-ending support and giving me a life of opportunity.
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Abstract

The objective of this thesis was to investigate the integration of enzymes and redox mediators capable of transferring electrons between enzymes and electrodes. This was done with a view to developing a semi- or fully implantable, miniature, membrane-less enzymatic fuel cell (EFC) exploiting enzymatic oxidation of glucose coupled to the enzymatic reduction of dissolved dioxygen. Miniaturisation is possible if appropriate enzymes are selected as catalysts, instead of non-selective precious metal catalysts, by removal of ion-exchange membrane from assembled fuel cells.

Chapter two reports a novel method for the preparation of mediated biocatalytic enzyme electrodes for glucose oxidation, by cross-linking films of glucose oxidase, polymer supports, and a range of osmium complexes bearing functional groups on graphite electrodes. The redox potentials of the osmium complexes are manipulated by preparation of complexes using either 2,2′-bipyridine, or 2,2'-bipyridine with substitution of electron withdrawing or electron donating groups in the 4 and 4' positions, as ligands. Complexes with lower redox potential, desired for mediation of enzymatic glucose oxidation in fuel cell anodes, are based on use of 4,4'-dimethyl-2,2'-bipyridine or 4,4'-dimethoxy-2,2'-bipyridine as a ligand instead of 2,2'-bipyridine. Glucose oxidation current densities of 30 and 70 μA cm\(^{-2}\) at 0.2 and 0.35 V vs. Ag/AgCl applied potential were obtained for enzyme electrodes prepared using [osmium(4,4'-dimethoxy-2,2'-bipyridine)\(_2\)(4-aminomethylpyridine)Cl\(^+\)] complexes and [osmium(4,4'-dimethyl-2,2'-bipyridine)\(_2\)(4-aminomethylpyridine)Cl\(^+\)] compared to 120 μA cm\(^{-2}\) at 0.45 V vs. Ag/AgCl for the enzyme electrode using [osmium(2,2'-bipyridine)\(_2\)(4-aminomethylpyridine)Cl\(^+\)], under pseudo-physiological conditions in 5 mM glucose, however stability of signals proved insufficient for long-term operation. The availability of redox mediators, polymer supports and cross-linkers offers wide scope for investigation of anchoring and cross-linking methodology that may improve current generation and stability to provide enzyme electrodes capable for application to longer-term glucose biosensors and anodes in enzymatic fuel cells.

Chapter three focuses on an investigation of enzyme electrodes for oxygen reduction at high potentials, for application to EFC cathodes, based on co-
immobilisation of multi-copper oxidases, such as bilirubin oxidase or a *Streptomyces coelicolor* laccase (SLac), with osmium redox complexes possessing an amine-functional group, in the presence of multi-walled carbon nanotubes (MWCNTs) at graphite electrodes. Oxygen reduction current densities of 0.8 mA cm$^{-2}$ under pseudo-physiological conditions at 0 V vs. Ag/AgCl are obtained by co-immobilisation of SLac, polyallylamine, MWCNTs and an [osmium(2,2′-bipyridine)$_2$(4-aminomethylpyridine)Cl]$^+$ complex. Enzyme electrodes prepared by incorporation of added MWCNT as a support in oxygen saturated, 150 mM NaCl, 50 mM phosphate buffer solution at 37°C, demonstrate a 3-fold increase in oxygen reduction current densities over those prepared without MWCNT. EFCs were assembled by combining the SLac-based enzyme electrode as a cathode, with glucose-oxidising anodes, based on either a pyrroloquinoline quinone or FAD-dependent glucose dehydrogenases and selected osmium redox complexes. The EFC assembled based on the PQQ-dependent glucose dehydrogenase enzyme electrode as the anode provides a maximum power density of 66 μW cm$^{-2}$ in 5 mM glucose, 150 mM NaCl, phosphate buffer solution at 37°C. On operation in human serum, although the EFC power dropped to 37 μW cm$^{-2}$, it still represents the highest reported power density to date for an enzymatic fuel cell operating in serum.

Chapter four reports studies on enzyme electrodes for glucose oxidation prepared using films of novel osmium complex-modified redox polymers as mediators for application to biosensors or biofuel cells. These novel redox polymers were developed by coupling osmium complexes containing amine functional groups to synthetic epoxy-functionalised polymers, providing the possibility of tuning both redox polymer potential, by variation in Os complex ligand, and redox polymer physicochemical properties, by variation in monomer selection and ratio. The capability of the redox polymers to function as mediators for glucose oxidation was tested by co-immobilisation onto graphite with glucose oxidase or an FAD-dependent glucose dehydrogenase using a range of crosslinkers and in the presence and absence of MWCNT. Glucose oxidation current densities as high as 560 μA cm$^{-2}$ were obtained in 100 mM glucose, 150 mM NaCl, phosphate buffer solution at 0.45 V vs. Ag/AgCl. Films prepared by crosslinking polymer bound-[Os(4,4′-dimethoxy-2,2′-bipyridine)$_2$(4-aminomethylpyridine)Cl]$^+$, an FAD-
dependent glucose dehydrogenase, and carbon nanotubes provided current densities of 215 μA cm\(^{-2}\) in 5mM glucose at the lower potential of 0.2 V vs. Ag/AgCl, showing some promise for application to glucose oxidising EFCs.

Chapter five reports on use of a fragmented form of a deglycosylated pyranose dehydrogenase (fdgPDH), produced when the deglycosylated enzyme (dgPDH) loses a C-terminal fragment when stored in buffer solution at 4 °C, as a glucose-oxidising catalyst for EFC anodes. A comparison of the capability of three forms of PDH, the native glycosylated enzyme (gPDH), the dgPDH and the fdgPDH, to function as catalysts for glucose oxidation when co-immobilised with osmium redox polymers on graphite electrodes, using flow injection amperometry and cyclic voltammetry, is reported. Higher glucose oxidation current densities are observed for using the fdgPDH when osmium redox polymers with low redox potentials, \([\text{Os(4,4'-dimethoxy-2,2'-bipyridine)2(poly-vinylimidazole)10Cl}]^+\) and \([\text{Os(4,4'-dimethyl-2,2'-bipyridine)2(poly-vinylimidazole)10Cl}]^+\), are selected for the comparison. Under pseudo-physiological conditions, glucose oxidation current densities of ~0.3 mA cm\(^{-2}\) are obtained from films containing \([\text{Os(4,4'-dimethyl-2,2'-bipyridine)2(poly-vinylimidazole)10Cl}]^+\) and fdgPDH at 0 V vs Ag/AgCl in 5 mM glucose, 150 mM NaCl, phosphate buffer solution. Improved access of the substrate to the active site and improved communication between enzyme and mediator within the film, possibly due to higher local concentration of redox complex, are suggested as two main reasons for the improved current generation of enzyme electrodes prepared using the fdgPDH when compared with those prepared using the gPDH and dgPDH. Operation of an assembled, membrane-less EFC in physiological solutions, human saliva and blood is demonstrated to provide power to an electronic device to enable wireless transmission of sensing data. The EFC is prepared using the fdgPDH co-immobilised on graphite with \([\text{Os(4,4'-dimethyl-2,2'-bipyridine)2(poly-vinylimidazole)10Cl}]^+\) and MWCNT as anode, coupled to an oxygen-reducing cathode based on adsorption of a bilirubin oxidase on gold nanoparticles. Maximum power densities of up to 325 μW cm\(^{-2}\) were obtained in 5 mM glucose, 150 mM NaCl, phosphate buffer solution. When tested in whole human blood a power density of 80 μW cm\(^{-2}\) was achieved, the highest power density reported to date for EFCs operating in human blood.
Finally, chapter 6 summarises my research attempts to address some of the problems associated with the integration of biocatalysts and mediators, and looking at a range of techniques to improve the current output and stability of these modified enzyme-based electrodes through the incorporation of nanoparticles and/or different immobilising techniques. Chapter 6 also provides some opinion on the direction such research may take in the future. My PhD also included the synthesis of a range of redox polymers, which were also distributed to a range of collaborating partners resulting in a number of publications which continues to grow. Although these results are not discussed in this thesis, an appendix included providing a list of my co-authored publications along with oral and poster presentations and research visits made over the course of my PhD studies.
Chapter 1: Introduction

1.1 Introduction

In the field of medical device technology there has been an increased interest in deployment of implantable and semi-implantable devices such as pacemakers, insulin pumps and defibrillators, to name but a few. There is a growing trend towards the miniaturisation and integration of these devices for controlling and monitoring of various medical conditions [1, 2]. In the mid-1960s, the idea of being able to use cell-free enzyme systems to provide sufficient power to sustain pacemakers or a permanently implantable artificial heart was explored [2, 3]. However, it soon became apparent that they could not meet the power and operational lifetime required for such devices with this research being largely abandoned until its re-emergence in the late 1990s, due in part to these energy demanding devices becoming more sophisticated in design, and microelectronics requiring smaller and lower energy consuming power sources that are able to sustain operation over long periods of time. At present, these implantable devices are powered by highly reactive lithium or alkaline electrolyte batteries, providing electricity from internal chemical reactions. The battery contains components that are corrosive and highly toxic to humans, and therefore they require protective casings, seals and membranes to ensure isolation from the surrounding environment and from each other, avoiding unwanted chemical side reactions. Development of implanted devices that take advantage of fuels present in the bloodstream to generate power and thus continue to generate power throughout the host’s lifespan would represent a step-change in medical device technology. Enzymatic fuel cells (EFC) can potentially do this by electrolysing ambient body fuels and oxidant such as glucose and oxygen and converting them into benign by-products, a conversion process through which electrical energy can be generated from chemical energy [1, 2, 4–6]. Although there is promise in EFC systems, there are still a number of challenges to be addressed, such as obtaining improved stability and power density. There is also the issue that implanted devices, when exposed to interstitial fluids or blood, can develop a protein layer later forming a three dimensional biofilm which may reduce the efficacy of a potentially implanted EFC device [7–9].
1.2 Fuel cells

Generation of electricity via the oxidation of a fuel at the anode and the reduction of an oxidant at the cathode is achievable by an electrochemical energy conversion device known as a fuel cell. In 1838, German physicist Christian Friedrich Schönbein [10] discovered the principle of a fuel cell which was then demonstrated the following year by Sir William Robert Grove [11]. The power output of a fuel cell is a function of the rate of transfer of electrons through an external circuit and the cell voltage. The cell voltage is the potential difference between the anode (Ea) and the cathode (Ec), and the cell voltage may be affected by irreversible losses in the voltage (η) due to kinetic limitations of the electron transfer process at the electrode interfaces, ohmic resistances and concentration gradients, equation 1.1 [6].

\[ V_{\text{CELL}} = (E_c - E_a) - \eta \quad (1.1) \]

The operating principle of a fuel cell is similar to that of a battery as they both use a chemical reaction to provide electricity. A battery stores the chemical reactants, usually metal compounds like lithium, zinc or manganese, for energy generation, while a fuel cell is an open system that creates energy from the reactants (fuel and oxidant) that are supplied externally to the device. Theoretically a fuel cell is able to function indefinitely as long as fuel and oxidant are in constant supply [2]. Figure 1.1 illustrates an example of one such fuel cell, the hydrogen-oxygen proton-exchange membrane fuel cell (PEMFC). The anodic and cathodic half reactions taking place within the PEMFC are illustrated in table 1.1.
Figure 1.1: Operational principle of a PEMFC [12]

Table 1.1: Anodic and cathodic half reactions, along with their standard potentials, taking place within the PEMFC

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<th>Half-reaction</th>
<th>( E^0 ) (V)</th>
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<tr>
<td>Anode</td>
<td>( \text{H}_2 \rightarrow 2, \text{e}^- + 2, \text{H}^+ )</td>
<td>0</td>
</tr>
<tr>
<td>Cathode</td>
<td>( \text{O}_2 + 4, \text{e}^- + 4, \text{H}^+ \rightarrow 2, \text{H}_2\text{O} )</td>
<td>1.23</td>
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At the anode, protons and electrons are produced when hydrogen gas is oxidised via a metal catalyst. The protons migrate towards the cathode through the proton exchange membrane that separates the two electrodes and the electrons pass through an external circuit creating the electron flow. At the cathode oxygen is catalytically combined with the protons and electrons to form water. The standard potential difference (\( \Delta E^0 \)) between the anode and cathode of a PEMFC fuel cell is, from table 1.1, 1.23 V [13]. The catalyst usually used is platinum metal, a highly effective but expensive catalyst. Platinum, as a catalyst, however, is non-selective, necessitating the use of a membrane and a protective casing to prevent migration of the fuel and oxidant to, and reaction at, opposing electrodes.
1.2.1 Biofuel cells

Based on the same principle as fuel cells, biocatalytic fuel cells (biofuel cells) are fuel cells which rely upon biocatalytic reactions at the electrodes to convert chemical fuels and oxidants into electrical power. Biofuel cells can be categorised into two types: microbial and enzymatic [14]. Microbial fuel cells (MFCs) use living microbes to convert a fuel and oxidant to electrical power, whereas enzymatic fuel cells use catalysts extracted from organisms for the conversion process. In both instances the active component (i.e. microbe or enzyme) can either be free in solution or confined to the electrode surface. Enzymatic biofuel cells operate in general using either mediated or mediatorless approaches. The work carried out within this thesis focuses on fundamental research aimed at enhancing power and stability of enzymatic biofuel cells [1-5, 7-10], so the concept of microbial fuel cells will only be briefly presented.

1.2.1.1 Microbial fuel cells

Microbial fuel cells (MFCs) are fuel cells that employ whole living organisms, such as algae or bacteria, to generate power from organic substrates (waste) as a fuel [15–18]. A typical MFC consists of anode and cathode compartments connected by a load and separated by an ion exchange membrane. The anode contains mixed or pure cultures of microbial organisms that are used to catalyse the decomposition of the organic matter into electrons and protons. Electrons are transferred to the cathode compartment through an external circuit, and cations migrate to the cathode compartment through the membrane. Electrons and protons are consumed in the cathode compartment, combining with oxygen to form water. A metal is usually used to catalyse oxygen reduction, although it has recently been shown that microorganisms can be used for this purpose as well [19].

1.2.1.2 Enzymatic fuel cells

An enzymatic biofuel cell is a specific type of fuel cell which utilises enzymes as a (bio)catalyst, instead of metal catalysts, to convert chemical energy to electrical energy using biochemical pathways [1, 2, 4–6]. The first glucose/oxygen fuel cell
was reported by Yahiro et al. in 1964 [20], demonstrating current production using a redox reaction of a flavoprotein enzyme for glucose oxidation. It was not until the early 1990s that there was renewed interest in the study of biocatalytic fuel cells and an upsurge in research was driven by the advances in biosensor design and enzyme electrochemistry [1, 2, 4–6].

The most commonly described enzymatic fuel cell (EFC) prototype in the literature consists of coupling the oxidation of glucose at the anode to an O$_2$ reducing cathode [21–29] although other systems based on H$_2$/O$_2$ [30], glucose/H$_2$O$_2$ [31], fructose/O$_2$ [32], NADH/H$_2$O$_2$ [33], methanol/O$_2$ [34], and alcohol/O$_2$ [35] have also been reported. The electrochemical reactions of a glucose/O$_2$ EFC consist of two separate reactions: an oxidation half-reaction occurring at the anode and a reduction half-reaction occurring at the cathode (Figure 1.2). At the anode, β-$d$-glucose passes over the anode and is catalytically split into β-$d$-gluconolactone producing hydrogen ions, which travel through the electrolyte to the cathode, and electrons that travel through the circuit to the cathode (scheme 1.1). A reduction half reaction occurs at the cathode where oxygen is catalytically reduced by an enzyme and combines with protons to form water (scheme 1.2). The power output of the cell is the product of the cell voltage (ΔE) and the cell current (I).

**Figure 1.2:** Operating principle of a fully enzymatic glucose/oxygen biofuel cell, with potentials quoted vs. Ag/AgCl at pH 7.4.
Scheme 1.1 (anode): Oxidation of β-d-glucose to β-d-gluconolactone catalysed by glucose oxidase (GOx)

\[
O_2 + 4 H^+ + 4 e^- \rightarrow 2H_2O
\]

Scheme 1.2 (cathode): Reduction of dioxygen to water catalysed by multicopper oxidase (MCO).

In order for an EFC to be able to rival the lithium ion battery, an operational lifetime of greater than 5 years may be required. Although these non-rechargeable batteries have been successfully employed in implantable medical devices for over 4 decades, their lifespan is usually much shorter than the desired period of implantation and depletion of the battery has resulted in the replacement 20% of the 200,000 pacemaker implants each year in the United States alone [36, 37]. An alternate approach may be use of implanted or semi-implanted enzyme electrodes to self-power (from glucose oxidation and oxygen reduction) short-lived continuous monitoring systems, that may be replaced regularly, whilst responding to analyte concentration (i.e. glucose) in body fluids, by arranging it so that one of the EFC electrodes limits current flow [38]. If implantation of a glucose/O_2 EFC is to be considered a realistic possibility, it is critical that it operate effectively under physiological conditions (i.e. pH 7.4, 37 °C, 5 mM glucose, 150 mM NaCl).

1.3 Enzymatic catalysts

Enzymes are naturally occurring molecules which possess many attractive features for applications as biological catalysts in organic synthesis [39], biosensors [40] and biofuel cells [4, 14]. In bioelectrocatalysis, enzymes are attractive for use due to their high substrate specificity, efficient conversion of substrates to products, moderate operating temperatures, and activity under mild
conditions, such as physiological pH. Enzymes are generally substrate specific and larger than their respective substrates. Only a specific part of the enzyme takes part in an enzymatic reaction. The active site is where substrate conversion to product occurs, usually close to the substrate binding pocket. Selection of enzyme(s) as redox catalysts provides an opportunity for oxidation of a wider variety of fuels compared to inorganic catalysts, whilst also being renewable and relatively inexpensive to produce [4]. As a result, there has been extensive research into engineering and optimising enzymatic catalysts as a means of increasing efficiency of an enzyme or to widen the substrate specificity to a larger group of fuels.

1.3.1 Enzymes for application to substrate oxidation

There are a wide range of fuels available, such as ethanol, methanol, formic acid and glucose to name but a few, based on availability of redox enzymes suitable for oxidation of these fuel in a biocatalytic anode [41]. Some of the biological catalysts employed for biofuel cell anodes are presented, along with their optimum pH and substrate in table 1.2. The focus of the studies performed within this thesis, especially in the context of enzymatic fuel cells with in-vivo application, has centred upon utilising glucose as the fuel, due to its relatively high concentration of ca. 5-8 mM present in blood [42, 43].

1.3.1.1 Glucose oxidase

Glucose oxidase (GOx) (EC 1.1.3.4) is a dimeric protein capable of catalysing the oxidation of β-D-glucose into D-gluconolactone which subsequently hydrolyses to gluconic acid. GOx requires a cofactor, flavin adenine dinucleotide (FAD), to be able to function as a biocatalyst. Figure 1.3 shows a representation of the crystal structure of one of the GOx monomers, and Figure 1.4 displays the ligand environment of the FAD active site deeply buried (~1.5 nm) within the crystal structure of the GOx [44]. The FAD functions as initial electron acceptor and is reduced to FADH₂, which is then subsequently oxidised by the final electron acceptor, oxygen, the natural acceptor, as in the equations 1.2 and 1.3, or an artificial acceptor (redox mediator) which replaces oxygen in the electron transfer
mechanism. The natural reaction of oxygen with GOx competes with the electrode for electrons lowering the performance of the anode, producing hydrogen peroxide and depleting $O_2$ concentration available as oxidant to the cathode in a membrane-less EFC setup, equation 1.3.

Figure 1.3: Crystal structure of the *Aspergillus niger* glucose oxidase (PDB ID: 3QVP, Kommoju et al.[44])
Figure 1.4: Ligand environment of the FAD deeply buried (~1.5 nm) within the structure of GOx.

\[
\text{GOx (FAD) + 2 e}^- + 2 \text{H}^+ \rightleftharpoons \text{GOx (FADH}_2\text{)} 
\]

\[
\text{GOx (FADH}_2\text{)} + \text{O}_2 \rightarrow \text{GOx (FAD) + H}_2\text{O}_2
\]

Although the FAD is deeply buried within the enzyme there have been numerous studies purporting to show direct electron transfer (DET) from GOx to solid electrode substrates. The most persuasive report of DET to a fully active GOx has been presented by Mano and co-workers [45] who observe a redox couple in cyclic voltammograms at $-0.49$ V vs. Ag/AgCl, attributed to the FAD/FADH$_2$ redox process within a deglycosylated GOx adsorbed on a glassy carbon electrode. Glucose oxidising current density of 23 $\mu$A cm$^{-2}$ at $-0.2$ V vs. Ag/AgCl was observed indicating that catalytic activity of the enzyme is retained. Many others have reported DET from GOx, usually on nanocomposite electrode materials [46]. However, recent reports dispute DET as a mechanism for catalytic current generation in GOx-based electrodes deposited along with carbon nanotubes [47]. Despite the apparent simplicity of DET, catalytic currents may be hindered due to the large distance (negligible beyond 2 nm) of closest approach of the FAD active site to electrode material, further hampered by incorrect orientation of enzymes on the electrode surface. One way of overcoming these
limitations is to immobilise the enzyme within a three dimensional mediating matrix. Employing such an approach enables addressing by the electrode of multilayers of enzyme and easier access of the mediator to the enzyme active site if the matrix is sufficiently solvated and swollen (vide infra).

1.3.1.2 Dehydrogenases

The dehydrogenases are a class of enzyme that may prove advantageous over GOx for use in EFCs, due to their ability to oxidise fuels without competition from oxygen as electron acceptor, and thus without producing hydrogen peroxide, as in equations 1.2 and 1.3 for GOx. They are capable of oxidising a substrate by transferring one or more protons to an acceptor such as nicotinamide adenine dinucleotide (NAD\(^+\)), a flavin co-factor such as FAD or a pyrrolo-quinoline quinone (PQQ) co-factor. The NAD\(^+\)/NADH cofactor, as illustrated in figure 1.5, is not directly bound to the enzyme although its presence is necessary in the bioelectrocatalytic function of the enzyme. The NADH co-factor itself acts as a carrier of two electrons and one proton. However it is not attractive for redox signalling in biocatalysis due to the high overpotential for, [48] and the electrochemically irreversible nature of, the NAD\(^+\)/NADH redox process.

![Figure 1.5: Structure and reaction of the NAD\(^+\)/NADH co-factor involving a 2 electron hydride transfer.](image)

Glucose dehydrogenase (GDH) is a class of enzyme that utilises either a NAD [49], PQQ [50], or an FAD [51] as a cofactor for oxidation of glucose. The PQQ co-factor has a thermodynamic redox potential of approximately \(-0.16\) V vs. Ag/AgCl at pH 7.2 [52], however, application of the PQQ-dependent GDH to
biocatalytic fuel cells may be limited due to its relative instability compared to GOx [53]. Nonetheless, the application of electrodes modified with the PQQ-dependent GDH as biocatalytic anodes has been investigated owing to its insensitivity to oxygen, the fact that the co-factor is bound to the enzyme, unlike NAD$^+$-dependent GDH, and the high catalytic efficiency of this PQQ-dependent GDH [53, 54].

Another dehydrogenase enzyme that has attracted considerable attention over recent years is pyranose dehydrogenase (PDH). PDH does not utilise oxygen as an electron acceptor, compared to, for example, pyranose oxidase. It also possesses a wider range of substrate specificity and regio-selectively which can be attributed to the unique structure of the region surrounding the flavin pocket [55, 56].

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Acronym</th>
<th>Substrate</th>
<th>Optimal pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol dehydrogenase (NAD)</td>
<td>ADH</td>
<td>Ethanol</td>
<td>8.6-9.0</td>
</tr>
<tr>
<td>Methanol dehydrogenase (PQQ)</td>
<td>MDH</td>
<td>Methanol</td>
<td>8.5</td>
</tr>
<tr>
<td>Formate dehydrogenase (PQQ)</td>
<td>FDH</td>
<td>Formic acid</td>
<td>7.5</td>
</tr>
<tr>
<td>Glucose oxidase (FAD)</td>
<td>GOx</td>
<td>Glucose</td>
<td>4.5-7.5</td>
</tr>
<tr>
<td>Glucose dehydrogenase (FAD)</td>
<td>FADGDH</td>
<td>Glucose</td>
<td>7-8</td>
</tr>
<tr>
<td>Glucose dehydrogenase (PQQ)</td>
<td>PQQGDH</td>
<td>Glucose</td>
<td>6.5-9</td>
</tr>
<tr>
<td>Pyranose dehydrogenase</td>
<td>PDH</td>
<td>various pentoses, hexoses, mono- and oligosaccharides, as well as various glycosides</td>
<td>8.5</td>
</tr>
<tr>
<td>Pyranose oxidase</td>
<td>POx</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Cellobiose dehydrogenase</td>
<td>CDH</td>
<td>large variety of carbohydrate substrates</td>
<td>4-7</td>
</tr>
</tbody>
</table>

Table 1.2: Biocatalysts of interest for use as part of the anodic constituent of a biofuel cell.
Naturally occurring enzymes, known as multi-copper oxidases (MCOs), provide an alternative to non-specific metal catalysts, such as platinum, and have shown promise as efficient oxygen reduction reaction (ORR) catalysts for the 4 electron reduction of oxygen to water when immobilised at electrode surfaces [57–59]. A laccase sourced from *Corsiolum hirsutus*, when co-immobilised with an osmium redox polymer at a carbon fibre electrode, and operated in a 0.1 M pH 5 citrate buffer is a superior ORR catalyst to platinum, operating in its optimal electrolyte of 0.5 M H₂SO₄[60]. In addition to increased electrode stability, the ORR reaction proceeded at just −0.07 V below the O₂ / H₂O half-cell reversible potential in the same electrolyte, ca. 0.4 V more positive than the ORR catalysed by platinum under the same conditions [60]. Enzymatic biocatalysts also offer additional substrate selectivity, compared to Pt, in favour of the oxidant over the fuel, eliminating the requirement for compartmentalisation of the anode and cathode, thus allowing miniaturisation.

There are several enzymes that could be employed as biocatalysts for reduction of oxidants, table 1.3. These include peroxidases which utilise hydrogen peroxide as the oxidant. However, peroxidases are not an option for use in EFC for *in vivo* applications due to the associated toxicity of peroxide and that it can be oxidised at the anode. Cathodes based on MCO for ORR can be classified into two groups: those based on DET and those based on mediated electron transfer (MET). Biocathodes displaying mediatorless direct electron transfer are often produced using electrode substrates modified with conductive nanocomposites, such as gold or carbon nanoparticles, to increase the electrode surface area and enable enhanced heterogeneous electron transfer. Mediated systems require the enzyme to be co-immobilised with a mediator, facilitating the shuttling of electrons between the protein and the conductive surface. Osmium-based redox complexes and polymers are among the most common mediators for the ORR catalysed by MCOs such as laccases and bilirubin oxidases. A simplified schematic showing mediated electron transfer from electrode to oxygen, using an osmium complex as mediator, and a MCO as enzymatic catalyst is shown in Figure 1.6. Chapters 3 and 5, focus on the investigation of the use of laccases and
bilirubin oxidase to produce cathodes for the ORR at relatively high reduction potentials under physiological conditions.

Figure 1.6: Simplified mechanism of MET from electrodes to the active site of blue multicopper oxidase via the type 1 Cu\textsuperscript{II} (T1-Cu), the primary electron acceptor site that couple oxidation of the substrate to reduction of oxygen to water at a trinuclear copper cluster formed from a type 2 Cu\textsuperscript{II} (T2-Cu) and a type 3 binuclear Cu\textsuperscript{II}–Cu\textsuperscript{II} (T3-Cu).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Acronym</th>
<th>pH optimum</th>
<th>T1 site Potential (vs Ag/AgCl)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomyces coelicolor laccase</td>
<td>ScL</td>
<td>7</td>
<td>250</td>
<td>[61–63]</td>
</tr>
<tr>
<td>Myliopthera thermicolor laccase</td>
<td>MtL</td>
<td>7</td>
<td>280</td>
<td>[64]</td>
</tr>
<tr>
<td>Melanocarpus albomyces laccase</td>
<td>MaL</td>
<td>7</td>
<td>280-290</td>
<td>[65]</td>
</tr>
<tr>
<td>Trametes hirsuta laccase</td>
<td>ThL</td>
<td>4</td>
<td>580-600</td>
<td>[66, 67]</td>
</tr>
<tr>
<td>Trametes versicolor laccase</td>
<td>TvL</td>
<td>4</td>
<td>580-600</td>
<td>[67]</td>
</tr>
<tr>
<td>Bilirubin oxidase</td>
<td>BOD</td>
<td>8</td>
<td>470</td>
<td>[59, 68, 69]</td>
</tr>
</tbody>
</table>

Table 1.3: Biocatalysts of interest for ORR in a cathode of a biofuel cell
1.3.2.1 Laccase

Laccases are classified as polyphenol oxidases and are members of the blue multicopper protein family which contain four copper active sites capable of oxidising a broad range of substrates, typically substituted phenolic compounds and aromatic amines, coupled to the four-electron reduction of O₂ to water [70, 64]. The potential uses of laccases can therefore encompass a wide range of areas in many fields of research including pulp bleaching [71], organic synthesis [72], biosensors [73, 74] and biofuel cells [21, 22, 24]. The copper active centres are classified into three types depending on their spectroscopic characteristics [75]. The type 1 Cu^{II} (T1-Cu) site is the primary electron acceptor site from substrates that couples four monoelectronic oxidations of the substrate to a four-electron reduction of oxygen to water at a trinuclear copper cluster located at a distance of ~13 Å from the T1-Cu. One type 2 Cu^{II} (T2-Cu) and a type 3 binuclear Cu^{II}–Cu^{II} (T3-Cu) form the trinuclear cluster.

Laccases have been isolated from eukaryotic [76] and prokaryotic sources [77]. The standard potential of the T1 copper site will determine the potential at which substrate oxidation is driven. Laccases derived from plants possess a low T1 potential of ~ + 0.23 V vs. Ag/AgCl, whilst the T1 potential of fungal laccases range from mid- (~ + 0.27 V to + 0.51 vs. Ag/AgCl) or high range (~ + 0.58 V vs. Ag/AgCl) [38]. High range potential laccases are, however, inhibited by hydroxyl ions and produce maximal activity at pH 4-5, limiting their use as biocathodes in an EFC operating at physiological conditions [21]. The focus of this thesis is on the development of an enzymatic fuel cell that can operate under physiological conditions, therefore enzymes were judiciously selected that could operate under such conditions. For example, the performance of cathodes for the ORR, using a laccase sourced from the bacterium Streptomyces coelicolor, is reported on in chapter 3 of this thesis. The Streptomyces coelicolor laccase (SLac, Figure 1.7) consists of two domains and forms trimers, each monomer has a molecular mass of 32 kDa, and is structurally distinct from fungal laccases, displaying a T1-Cu redox potential of 0.3 V vs Ag/AgCl.
The first report on the immobilisation of a laccase with a redox polymer mediator for reduction of oxygen was by Trudeau and Leech [79], who investigated the reduction of oxygen to water by cross-linking a fungal laccase to an osmium-based redox polymer film on glassy carbon electrodes using a diepoxide crosslinker. The general structure of the series of the poly(N-vinyl-imidazole) (PVI)-bound osmium bipyridine mediators, with formulas and abbreviations of selected redox polymers, is presented in Figure 1.8. Since the first report on ORR using co-immobilised laccase and redox polymer [73], Gallaway et al. [62] reported on immobilisation of SLac within a film of [Os(2,2'-bipyridine)2(poly-vinylimidazole)10Cl]+ (Os(bpy)PVI), (E°' = + 0.23 V vs. Ag/AgCl) at glassy carbon electrodes. Oxygen reduction occurred at 0.4 V and the maximum current density obtained from this system was 1.5 mA/cm² in pH 7 physiological buffer solution with rotation of the electrode at 900 rpm, 40°C [87]. Replacement of the electrode material with a high surface area composite carbon paper electrode resulted in an increase in current density to 7 mA cm⁻² under the same conditions, the largest current density reported to date for a mediated laccase cathode in physiological buffer [62]. More recently, Lorcher et al. [63]
demonstrated direct electron transfer to SLac when immobilised at pyrene- and neocuproine-modified electrodes. MacAodha et al. [24] reported that SLac, co-immobilised through dialdehyde crosslinking with an \([\text{Os}(2,2\text{-bipyridine})_2(\text{polyvinylimidazole})_{10}\text{Cl}]^+\) redox polymer in the presence of multiwalled carbon nanotubes on graphite electrodes, yielded ORR current densities of 1.1 mA cm\(^{-2}\) at 0 V vs. Ag/AgCl in a pH 7.4, unstirred physiological buffer solution. Further investigation into use of SLac as an enzyme for development of enzymatic fuel cell cathodes is thus merited, and is reported on in chapter 3 and 5 of this thesis.

\[\text{OsCl}N\]
\[\text{N}\]
\[\text{N}\]
\[\text{N}\]
\[\text{N}\]
\[\text{N}\]
\[\text{N}\]
\[\text{N}\]
\[\text{N}\]
\[\text{N}\]
\[\text{Cl}^+\]
\[\text{Cl}^-\]

Figure 1.8. General structure of the PVI-bound osmium bipyridine mediators. \([\text{Os}(2,2\text{-bipyridine})_2(\text{poly-vinylimidazole})_{10}\text{Cl}]^+\) (Os(bpy)PVI): R=H, \([\text{Os}(4,4\text{-dimethoxy-2,2\text{-bipyridine})_2(\text{poly-vinylimidazole})_{10}\text{Cl}]^+\) (Os(dmobpy)PVI): R=O-CH\(_3\), \([\text{Os}(4,4\text{-dimethyl-2,2\text{-bipyridine})_2(\text{poly-vinylimidazole})_{10}\text{Cl}]^+\) (Os(dmbpy)PVI): R=CH\(_3\).
1.3.2.2 Bilirubin oxidase

Bilirubin oxidase (BOd) belongs to the “blue” multicopper oxidase family and catalyses the oxidation of bilirubin to biliverdin with concomitant 4-electron reduction of oxygen to water [70, 80]. Although the enzyme was discovered and characterised in 1981 by Murao and Tanaka [81], the crystal structure of BOd from *Myrothecium verrucaria* (MvBOd) has only recently been solved (figure 1.10) [82] and like laccase, it has an active site that consists of a tri-nuclear T2/T3 oxygen-reducing copper site and a T1 substrate oxidising copper site [70, 83]. The potential of the T1-Cu site of the bilirubin oxidases is in the mid-range potential classification of $\sim +0.47$ V vs. Ag/AgCl [68, 84, 85]. The MvBOd, selected for examination as a catalyst in chapter 3 and 5 of this thesis, is a monomeric glycoprotein with a molecular mass of 68 kDa and a broad pH activity region (7-11) for electron donors such as $K_4[Fe(CN)_6]$ or electrodes [86, 87].

![Crystal structure of *Myrothecium verrucaria* bilirubin oxidase](image)

**Figure 1.9:** Crystal structure of *Myrothecium verrucaria* bilirubin oxidase (PDB ID: 2XLL, Cracknell et al. [82])

The Ikeda group first utilised MvBOd adsorbed onto carbon felt electrodes with solution phase 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) for mediated reduction of oxygen in phosphate buffer at pH 7.0 [88, 30,
They further reported high current densities of 17 mA cm$^{-2}$ at + 0.25 V vs. Ag/AgCl for the ORR by electrostatically entrapping $M_v$BOd with [W(CN)$_6$]$^{3-/4-}$ in poly(L-lysine) at carbon felt electrodes rotated at 4000 rpm [90]. The $M_v$BOd immobilised in poly(L-lysine) layers at carbon electrodes, which relied on direct electron transfer between the enzyme active site and electrode surface, obtained steady-state oxygen reduction current densities of 0.85 mA cm$^{-2}$ at potentials of $\sim$0.2 V vs. Ag/AgCl in oxygen saturated phosphate buffer at pH 7.0 with rotation at 1400 rpm [84]. Mano et al. [91] co-immobilised a $M_v$BOd and a redox polymer, prepared by substitution of one chloride ligand of Os(4,4'-dichloro-2,2'-bipyridine)$_2$Cl$_2$ with imidazole units of a copolymer of poly(vinylimidazole) and polyacrylamide, on carbon cloth to yield oxygen reduction current densities of 5 mA cm$^{-2}$ in phosphate buffered saline at 37 °C at 1000 rpm, increasing to 8.8 mA cm$^{-2}$ when rotated at 4000 rpm in oxygenated phosphate buffered saline at 37 °C.

### 1.4 Electron transfer reactions

Electronic communication between electrode surfaces and biocatalysts can be achieved by DET, figure 1.10 (a), and MET, figure 1.10 (b), [84, 41, 92]. Current and power densities achieved with electrodes using the DET approach will be limited, however, because of the need to have intimate contact between the two- or three-dimensional electrode surface and a coating monolayer of correctly oriented biocatalyst. DET, where redox enzymes transfer electrons directly between the electrode and the substrate via their active site, is often quite difficult to achieve and depends crucially on the distance between active site and surface, and thus on the orientation of the enzyme at the electrode surface [93, 47]. Current densities achieved by DET are often quite low when compared to MET. MET involves using an electron transfer mediator to shuttle electrons between the electrode and the redox active sites in the enzyme. In MET, to have an effective electron exchange, the thermodynamic redox potentials of the enzyme and the mediator should match in order to provide a driving force for electron transfer [41, 94]. For biocatalytic electrodes, it has been proposed that mediators should have redox potentials approximately 50 mV downhill from the redox potential of the enzyme [95], as a compromise between the need to have a high cell voltage and a high current, for EFC design.
Figure 1.10: Schematics depicting (a) direct electron transfer and (b) mediated electron transfer oxidation of substrate between an enzyme active site (green oval) and an electrode, where the red circles represent mediator species.

1.4.1 Enzyme electrodes using redox mediators

Considerations need to be taken into account when designing and selecting a mediator for use in an EFC. Characteristics of the mediator should include:

i. stable in both the oxidised and reduced states to allow continuous operation in the bioelectrocatalytic cycle.

ii. steric hindrance should not be an issue, mediator dimensions sufficiently small to access the enzyme active site within the protein structure.

iii. fast electron exchange rates with the enzyme are desired, to minimise competition with the enzyme’s natural substrate, if applicable.

The ‘tuning’ of the redox potentials of osmium polypyridyl redox complexes, by introduction of electron donating/withdrawing groups on the bipyridine (figure 1.11a), or other ligands, along with their demonstrated stability in their Os(II) and Os(III) oxidation states [96, 97], has led to utilisation of osmium-based redox complexes (1.11b) or polymers (1.11c) as mediators for EFC applications. The PVI-bound osmium polypyridyl series of redox polymers [96], figure 1.8, 1.11b, have been widely used in the research and development of mediated enzyme electrodes for application as biosensors [40, 79, 98] and EFC electrodes [24, 27,
The synthesis of osmium redox polymers is however, difficult to control, leading to variations in the final osmium complex ratios within the PVI redox polymers from batch-to-batch synthesised [100]. In addition to this, preparation of the PVI by uncontrolled bulk free radical polymerisation can result in variations in enzyme electrode responses, due to differing PVI molecular weights, although alternate synthetic strategies have recently been proposed to allow greater control of polymer molar mass [101]. An alternate strategy to control the osmium loading within redox polymers employs a synthetic route in which osmium is coordinated by a ligand that has a functional group distal to the metal binding site (figure 1.11c), that can then be attached to a pre-synthesised polymer via epoxide ring opening reaction [102–105]. In chapter 4, such a strategy is employed to couple osmium complexes, synthesised to contain a 4-aminomethylpyridine ligand, to a range of epoxy-functionalised polymers, offering greater versatility in film preparation and crosslinking strategies. As with studies based on osmium redox polymer, the redox potential of the oxium complex may be adjusted through introduction of electron donating or withdrawing groups to metal ligands.
Figure 1.11: Structure of the (a) osmium “starting complex” \([\text{Os}(2,2'\text{-bipyridine})_2\text{Cl}]^+\), (b) “tetherable” complex and (c) osmium redox polymer OsPVI, formed by co-ordination of a \([\text{Os}(2,2'\text{-bipyridine})_2\text{Cl}]^+\) complex to polyvinylimidazole in a, usually, 1:9 ratio. and (d) epoxy functionalised polymers. \(R = \text{NH}_2, \text{O-CH}_3, \text{CH}_2, \text{H} \text{or} \text{Cl}\).

1.3.2 Nanocomposites in enzyme electrodes

The addition of nanoparticles within enzyme-containing films on electrodes may contribute to improved current, because of improved surface area and/or electrical connections, and signal stability, and is therefore an active area of research for both biosensors [49, 106–108] and EFC applications [22, 24, 109–111]. Since their discovery in 1991, carbon nanotubes (CNTs) have been the subject of intense research because of their electrical/mechanical properties [112, 113]. For example, recent studies on the addition of MWCNTs to films of glucose oxidising enzyme and osmium redox polymer demonstrate that the presence of MWCNTs,
compared to films prepared without MWCNTs, result in an improved operational stability of the glucose oxidation response [114], and a greater amount of redox enzyme on the surface [115]. Improvement in current response and stability is likely due to greater surface area of the conductive support, improved electron transfer and/or effective retention of enzyme and mediator; however the exact mechanics regarding the electron pathways to the electrode is not fully understood. Therefore incorporation of MWCNTs is an interesting approach to improving the biocatalytic signal output and stability of enzyme electrodes, forming the basis for the investigation reported on in chapters 3, 4 and 5 of this thesis.

The inclusion of nanoparticles, such as gold nanoparticles (AuNP), to films of MCOs on electrodes is reported to promote direct electron transfer between enzyme and electrode, resulting in increases to both current and stability compared to responses on planar electrodes [116–121]. For example, Murata et al. [118] report a current density of 5.2 mA cm\(^{-2}\) for the ORR using a \(Mv\text{BOd}/\text{AuNP}\) modified electrode, when stirred at 4000 rpm, compared to little signal for ORR in the absence of the AuNP, with an improvement in stability also observed upon inclusion of AuNP. An assembled EFC, based on mediatorless fructose dehydrogenase for fructose oxidation at the anode, generated a maximum power density of 0.87 mW cm\(^{-2}\) at an operating voltage of 0.3 V when using the \(Mv\text{BOd}/\text{AuNP}\) modified electrode as a cathode [118]. In chapter 5, the fabrication and characterisation of a membrane-less glucose-O\(_2\) EFC is described using a combination of an \(Mv\text{BOd}/\text{AuNP}\)-based enzyme electrode for oxygen reduction as a cathode, with glucose-oxidising anodes assembled from pyranose dehydrogenase as the enzyme, osmium redox polymers as mediator and including MWCNTs as support.

1.3.3 Immobilisation strategies

One of the main challenges is to extend the operational lifetime of enzyme electrodes, from hours and days to months and years, in order to make implantation of an EFC to generate power for devices more practical [5]. One approach to retention of, and stabilisation of, the enzyme catalysis is through immobilisation of the enzyme on a solid surface. Immobilisation offers many
benefits including the possibility for re-use and a contribution to improved stability. There is a wide variety of immobilisation strategies for enzymes and mediators, ranging from simple physical adsorption, covalent attachment, cross-linking to entrapment in polymeric or inorganic gels [122]. The immobilisation approach can often be a combination of these and generally, multilayers or other three-dimensional (3D) structures tend to favoured over monolayer configurations in order to increase current output, and provide suitable immobilisation matrices for enzymes to retain their activity.

Crosslinking strategies have been developed over the past three decades in order to improve connectivity and stability of electroactive films on electrode surfaces. For example, Gregg and Heller [123] developed a method for entrapping enzymes and mediators in films at electrodes by crosslinking the components using a diepoxide reagent to form a three dimensional biocatalytic hydrogel matrix. The redox hydrogel resulted in connection (termed “wiring”) of the enzyme redox centre to the electrode via electron transfer to, and self-exchange within, the redox complexes in the hydrogel, whils preserving enzymatic activity and facilitating rapid diffusion of substrate and product through the polymer matrix. Ohara et al. [124] determined that enzyme electrodes modified with Os(bpy)PVI, co-immobilised with GOx on electrodes using a di-epoxide crosslinker, poly(ethylene glycol) diglycidyl ether (PEGDGE), were permeable to glucose and allowed the diffusion of electrons. De Lumley-Woodyear et al. [125] compared PEGDGE crosslinking to that using suberic acid bis(N-hydroxysuccinimide ester, dimethyl suberimidate or glutaraldehyde solution for the co-immobilisation of GOx and redox-polymer bound mediators. All the crosslinked methodologies, with the exception of glutaraldehyde, exhibited adhesion of the film to the carbon electrodes and retained approximately 90 % of their electroactive centres when immersed in stirred solution of phosphate buffer at room temperature for 48 hours. The use of glutaraldehyde vapours as a crosslinking agent is widely reported on for enzyme immobilisation in the field of biochemistry [126], and in electrochemistry [115, 127, 24, 128, 129], and may provide an alternate methodology to the di-epoxide solid-phase crosslinking reaction. The use of glutaraldehyde vapours to crosslink, and immobilise, films of redox polymer and enzyme on electrode surfaces, to provide enzyme electrodes
for oxidation of glucose or reduction of oxygen under physiological conditions, is presented in chapters 3, 4 and 5 of the thesis.

1.5 Enzymatic fuel cells

EFCs for eventual implantation should be capable of operating in blood as the physiological medium. The oxygen concentration in blood in the arteries and veins is ~ 0.2 mM and 0.05 mM respectively, whereas the concentration of glucose in blood vessels is ca. 5-8 mM in healthy adults [42, 130, 43]. Blood is a buffered solution with a pH of 7.4 and thermostated at 37 °C within the human body. The velocity of blood in blood vessels is on the order of 1 to 10 cm/s [131].

Since Yahiro et al. [132] first demonstrated an enzyme-based glucose oxidising/oxygen reducing fuel cell in 1964, extensive research, money, and effort has been invested into developing a prototype of a membrane-less enzymatic fuel cell capable of operating under in-vivo conditions [133–135]. Such a membrane-less system requires catalysts specific to the substrate to be immobilised at the electrode surfaces. A membrane-less glucose/O₂ biofuel cell reported on by Katz et al. [136] achieved 5 µW cm⁻² at 0.064 V in 1 mM glucose, for a monolayer of GOx connected to the electrode through a PQQ-FAD spacer, while using a cytochrome c/cytochrome c oxidase for oxygen reduction at the cathode.

The Heller group demonstrated, in a series of publications [137, 123, 138, 125, 139], the development of a miniature, membrane-less mediated biofuel cell with GOx immobilised at the anode and laccase at the cathode, each electrode consisting of a carbon fibre and containing an appropriate redox polymer mediator, that generated 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. Mano et al. [25] reported on a carbon fibre enzymatic fuel cell operating under pseudophysiological, pH 7.4 chloride containing conditions, also at 15 mM glucose, that produced 1.9 µW (50 nW cm⁻²) at 0.52 V cell voltage, where the selection of bilirubin oxidase instead of a laccase for ORR permitted operation under a neutral pH condition. Soukharev et al. [60] reported on an EFC using GOx and a fungal laccase, co-immobilised with osmium redox polymers on 7 µm diameter, 2 cm long, carbon fibers, producing 350 µW cm⁻² power density in 15 mM glucose, pH 5 buffered solutions, with however only a quarter of that maximum power, 90
μWcm$^{-2}$, observed at 5 mM glucose concentration. An analogue of these EFC but using a GOx sourced from *Penicillium pinophilum* and operating in pH 5 buffer solution generated 280 μW cm$^{-2}$, the highest maximum power densities reported to date for an EFC operating in 5 mM glucose [28]. Use of osmium redox polymers, designed to incorporate a flexible tether between the redox complex and polymer backbone, co-immobilised at carbon fiber electrodes with GOx and *Mv*BOd, as anode and cathode, respectively resulted in 480 μWcm$^{-2}$ power density in pH 7.2 phosphate buffer containing 0.1 M NaCl, 15 mM glucose at 37.5°C [27]. A further increase in power output, to 740 μW cm$^{-2}$ at 0.57 V cell voltage under the same conditions, was obtained for a similar fuel cell, but utilising electrodes composed of nanotube wires instead of carbon fibers [111].

To date, little has been reported on the testing of EFC systems in blood or artificial plasma samples. Coman *et al.* [140] reported on the assembly of an EFC using cellobiose dehydrogenase and bilirubin oxidase enzyme electrodes, which relied on direct electron transfer between the active site and the electrode for glucose oxidation and oxygen reduction, that provided power density of 3 μW cm$^{-2}$ in both PBS and human serum. Recently, MacAodha *et al.* described an EFC consisting of FADGDH/Os(dmbpy)PVI anode and a *Myceliophthora thermophila* laccase/Os(bpy)PVI cathode, each co-immobilised onto a graphite electrode in the presence of multiwalled carbon nanotubes, producing a power density of 110 μW cm$^{-2}$ in PBS, dropping to 60 μW cm$^{-2}$ in artificial plasma [24]. This drop in power density may be attributable to “biofouling”, where a foreign object, i.e. implanted device, immediately generates a protein layer once immersed in a biological system such as serum or blood [7–9]. Once the protein layer has been formed on the surface, cells in the serum or blood will interact and accumulate on the protein layer leading to the passivation and reduced functionality of the EFC. Prevention of biofouling may be possible through various types of coatings on the device. For example, Voskerician *et al.* [141] reported that implantable microelectromechanical systems packaged with gold, silicon nitride or silicon dioxide were able to reduce biofouling. Hydrophilic polymeric coatings, such as polyethylene glycol-based hydrogels, retain interfacial water molecules which renders them highly resistant, dependant upon the chain length, density and conformation of the polymer, to the adsorption of proteins [142, 143].
In chapter 4, EFCs assembled based on enzyme electrodes of *Streptomyces coelicolor* laccase co-immobilised with a redox complex [Os(2,2′-bipyridine)$_2$(4-aminomethylpyridine)Cl]$^+$ for the ORR, and PQQGDH co-immobilised with [Os(4,4′-dimethoxy-2,2′-bipyridine)$_2$(4-aminomethylpyridine)Cl]$^+$, for glucose oxidation are tested in PBS and human serum. In chapter 5, an EFC is constructed using a deglycosylated pyranose dehydrogenase sugar-oxidising enzyme co-immobilised with a PVI-bound osmium redox polymer paired with *MvB*Od on AuNP for ORR, for testing of operation in PBS and human blood.

1.6 Electroanalytical techniques

Characterisation of the systems under investigation was carried out using electroanalytical methodologies. A brief introduction to the basic principles, and major technique used, cyclic voltammetry, is therefore merited.

1.6.1 Cyclic Voltammetry

Cyclic voltammetry is the standard and most effective electroanalytical technique for studying redox systems, sometimes denoted as the “spectroscopy of the electrochemist” [144]. In this technique, the potential applied between two electrode in an unstirred solution is ramped linearly over a range towards a set potential which is then inverted and the process is reversed as illustrated in Figure 1.12. The resulting current ($I$) is monitored as a function of applied potential ($E$) to give the I-E curve called the cyclic voltammogram (figure 1.14) [13, 145, 146]. 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.
To perform cyclic voltammetry and amperometry experiments, a standard three electrode system is typically used [145, 146]. The standard three electrode setup consists of the working electrode, where the reaction of interest occurs, a reference electrode, to measure the working electrode potential, and a counter (or auxiliary) electrode, for completing the current flow. Working electrodes of inert carbon such as glassy carbon and graphite or inert metals such as platinum and gold are generally selected. The most common laboratory reference electrodes are the saturated calomel electrode (SCE) and the silver/silver chloride (Ag/AgCl) electrodes. 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. During cycling of the potential applied at the working electrode in an unstirred solution the occurrence of a current signal peak at a particular potential provide an indication of the type of electroactive substance present. A redox system can be characterised from the potentials of the peaks on the cyclic voltammogram and from changes caused by variations in scan rate [145, 146]. While peak height gives an indication of concentration the method is usually not used for sensitive quantitative determinations.
The Nernst equation for reduction of a species (ox) to produce (red), is:

$$E = E^{o'} - \frac{0.059}{n} \log \left( \frac{[red]}{[ox]} \right)$$  \hspace{1cm} (1.2)$$

where $E$ is the electrode potential relative to the $E^{o'}$, the formal electrode potential (the standard potential under a defined condition, other than the standard condition), $R$ is the gas constant, $T$ is the temperature, $n$ is the number of electrons in the half reaction and $F$ is the Faraday constant.

For an electrochemically reversible process, where the rate of heterogeneous electron transfer is more rapid than the rate of mass transport the potential difference between the current peaks is:

$$\Delta E = E_{pa} - E_{pc} = 59 \, / \, n \text{ mV}$$  \hspace{1cm} (1.3)$$
and the position of peak potentials does not alter as a function of voltage scan rate, the peak currents scale proportionally to the square root of scan rate (ν) and the ratio of peak currents will be equal to one.

The peak current for a reversible system is given by the Randles-Sevčik equation:

\[ i_p = 0.4493 n F \sqrt{\frac{n F}{RT}} A D^{1/2} C \nu^{1/2} \]  \hspace{1cm} (1.4)

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\(^{-1}\)).

If the rate of heterogeneous electron transfer is slow in relation to the scan rate the voltammetric response can be irreversible or quasi-reversible [145]. For an irreversible system electron transfer is the rate-determining step and the Nernstian equilibrium is not maintained and peak-to-peak separation greater than the 59/ \( n \) mV is observed that increases with increasing scan rate.

The CV response of a surface confined species, with Nernstian behaviour, when there is no lateral interaction between neighbouring redox centres, will follow the equations

\[ FWHM = \frac{3.53 RT}{n F} = \frac{90.6}{n} \text{mV} \]  \hspace{1cm} (1.6)

\[ E_{p_a} = E_{p_c} \]  \hspace{1cm} (1.7)

where FWHM is the full width at half maximum of the anodic or cathodic wave. FWHM values that differ from theoretical FWHM values have been attributed to electrostatic effects incurred by neighbouring charged species [145, 147]. Such a response may be observed also for multilayer systems of confined electroactive species or thin-layer cells, when the timescale of the voltammetric experiment is long enough to permit all of the species to be electrolysed.

For such systems, an estimate of concentration of electronically addressed redox sites by the electrode, characterised as a surface coverage (\( \Gamma \)) for confined species,
can be evaluated from the integratal of the faradaic charge (Q) passed, usually under conditions of slow scan rate CV as follows:

\[ \Gamma = \frac{Q}{nFA} \]  \hspace{1cm} (1.5)

where Q is the charged passed (in coulombs), \( n \) is the number of moles of electrons, \( F \) is Faraday’s constant and \( A \) is the area of the electrode \((\text{cm}^2)\).

1.7 Thesis proposition

The overall aim of the work presented in this thesis is to investigate the interaction between enzymes and redox mediators, to improve biocatalytic electrodes for utilisation in an enzymatic fuel cell. Chapter two will focus on an alternate method for constructing mediated biocatalytic electrodes prepared using cross-linked films of glucose oxidase, polymer supports and a range of osmium complexes bearing functional groups on graphite electrodes that may provide enzyme electrodes suitable for application to long-term glucose biosensors and anodes in enzymatic fuel cells. Chapter 3 will investigate and compare the operation of a fully enzymatic fuel cell in both phosphate buffer solution containing 5 mM glucose and human serum. Chapter 4 will investigate a novel strategy to bond osmium complexes, bearing an amine functional group, to a range of epoxy functionalised polymers offering versality in crosslinking strategies, for potential application as mediators in glucose oxidising enzyme electrodes for application to biosensors or biofuel cells. Chapter five will examine the catalytic properties of forms of the pyranose dehydrogenase from *Agaricus meleagris* along with the operation of assembled, membrane-less, enzymatic fuel cells in physiological solutions, un-stimulated human saliva and blood and their ability to provide sufficient power to enable wireless transmission of sensing data. Finally Chapter 6 will summarise the research and advances made during the course of this Ph.D and provides some proposed research for the future that stems from this work.
1.8 References


Chapter 2:

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Mediated glucose enzyme electrodes by crosslinking films of osmium redox complexes and glucose oxidase on electrodes


Co-author contributions:

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

Sirisha Kamireddy contributed to the publication through assisting in the laboratory work and the analysis.

Domhnall MacAodha 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.
Mediated glucose enzyme electrodes by crosslinking films of osmium redox complexes and glucose oxidase on electrodes

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2.1 Abstract

Here, we report on a novel, versatile approach for the preparation of mediated enzyme electrodes, demonstrated using cross-linked films of glucose oxidase and a range of functionalised osmium complexes on graphite electrodes. Response of enzyme electrodes are optimised by evaluation of glucose response as a function of variation in ratios of [Os(2,2′-bipyridine)2(4-aminomethylpyridine)Cl]+ redox mediator, polyallylamine support and glucose oxidase enzyme cross-linked using a di-epoxide reagent in films on graphite. Lowering of the redox potential required to mediate glucose oxidation is achieved by synthesis of complexes using (4,4′-dimethyl-2,2′-bipyridine) or (4,4′-dimethoxy-2,2′-bipyridine) as a ligand instead of (2,2′-bipyridine). Enzyme electrodes prepared using the complexes based on dimethoxy- or dimethyl-substituted bipyridines provide glucose oxidation current densities of 30 and 70 μAcm−2 at 0.2 and 0.35 V applied potential compared to 120 μAcm−2 at 0.45 V for the initial enzyme electrode, under pseudo-physiological conditions in 5 mM glucose, with stability of signals proving inadequate for long-term operation. Current output and stability may be improved by selection of alternate anchoring and cross-linking methodology, to provide enzyme electrodes capable for application to long-term glucose biosensors and anodes in enzymatic fuel cells.

2.2 Introduction

Enzyme electrodes use biocatalysts to convert chemical substrates into product, providing an electrical signal during the process. Applications of enzyme electrodes are mostly focused on development of biosensors and diagnostics [1, 2] and, in more recent years, enzymatic fuel cell devices (EFCs) [3–6]. In an EFC,
energy is obtained through the enzymatic oxidation of a fuel at the anode and reduction of an oxidant at the cathode [7]. An attractive source of fuel and oxidant is in vivo glucose and oxygen, leading to interest in enzyme-based anodes and cathodes designed for miniaturised fuel cells, which may provide power to implantable or semi-implantable biomedical devices. Enzymes, such as glucose oxidase (GOx), display specific activity towards substrates and are thus considered for glucose biosensors [1, 2] and glucose-oxidising EFCs [4]. In GOx-based biosensors and anodes, glucose is oxidised to yield gluconolactone, and an electron transfer mediator is required to shuttle electrons from the otherwise insulated FAD/FADH$_2$ active site to the electrode surface [2, 3]. A substantial body of research exists on approaches for assembly of glucose enzyme electrodes through incorporation of enzymes within redox polymer hydrogels to provide mediated enzyme electrodes [6, 8]. Mediated electron transfer within these hydrogel films allows connection between enzyme redox-active sites and an electrode surface, irrespective of spatial orientation of the active site of the enzyme, thus obtaining higher currents than with direct electron transfer from/to enzyme monolayers [3, 6]. Osmium-based complexes may prove advantageous over iron and ruthenium systems due to the low redox potential of the Os(II/III) transition and also the relative stability of complexes in both oxidation states [9]. A class of redox hydrogels widely studied are based on polymer-bound osmium complexes [10–12], with polypyridyl complexes of osmium co-ordinatively bound to poly(N-vinylimidazole) or poly(4-vinylpyridine) polymers in a ratio of metal complex to polymer monomeric unit. However, the ratio of osmium complex to polymer monomeric unit is difficult to control, can vary between batches and is dependent on the structure of Os complex selected for co-ordinative ligand substitution to the polymer [13].

A single, simple, one-step immobilisation of an enzyme, suitably functionalised redox complex, and a polymer support on an electrode through chemical cross-linking may allow better control of osmium site concentration in hydrogel films and provide for versatility in selection of mediator structure and properties. Use of functionalised osmium complexes as mediators in enzyme electrodes was first reported on by Danilowicz et al. [14, 15] where polypyridyl complexes that contain an aldehyde functional group distal to the Os co-ordinated site were coupled to amine-based polymers. More recently, Boland et al. [16, 17]
reported on synthesis, characterisation of osmium polypyridyl complexes with a 4-aminomethyl pyridine (4AMP) ligand, and the coupling of such complexes to carboxymethyl dextran polymer supports and/or functionalised electrode surfaces.

Here, we present an approach for preparation of enzyme electrodes based on cross-linking osmium polypyridyl complexes, possessing a 4-aminomethyl pyridine ligand, with enzymes and polymer supports on electrode surfaces. The polymer support acts as a scaffold for the coupling of enzyme and redox complex, providing three-dimensional films for bioelectrocatalysis. The availability of a range of redox complexes, preformed polymers and bifunctional cross linkers offers great scope for optimisation of enzyme electrodes, for example to provide for enzyme electrodes displaying high current production at low redox potentials for application as anodes in an EFC.

2.3 Experimental Details

2.3.1 Materials

All chemicals and biochemicals were, unless otherwise stated, purchased from Sigma-Aldrich and used as received. All solutions were made from Milli-Q (18.2 MΩ cm) water unless otherwise stated.

Initial complexes of cis-osmium(4,4'-R-2,2'-bipyridine)2Cl2, where R represents a bi-dentate bipyridine (bpy), or substituted bpy ligand, were synthesised from (NH4)2OsCl6 according to literature methods [9,18]. Ligand substitution of one Cl ligand of the resulting complexes by 4AMP was achieved by heating an ethylene glycol solution of the osmium complex with 1.1 M equivalents of ligand at reflux, with reaction progress monitored by cyclic voltammetry and differential pulse voltammetry of aliquots sampled from the reaction vessel. Precipitation of the final [Os(4,4'-R-2,2'-bpy)2(4-AMP)Cl].PF6 complex was achieved by addition of an excess of aqueous NH4PF6 solution to the reaction mixture. Final products of [Os(4,4'-dimethoxy-2,2'-bpy)2(4-AMP)Cl].PF6 (Os(dmobpy)24AMP), [Os(4,4'-dimethyl-2,2'-bpy)2(4-AMP)Cl].PF6 (Os(dmbpy)24AMP), [Os(2,2'-bpy)2(4-AMP)Cl].PF6 (Os(bpy)24AMP) or [Os(4,4'-dichloro-2,2'-bpy)2(4-AMP)Cl].PF6 (Os(dcbpy)24AMP) were filtered and allowed to dry overnight at 50°C.
2.3.2 Apparatus

All electrochemical measurements were performed using CH Instruments 600 series or 1030 multi-channel potentiostats, coupled to a thermostated electrochemical cell containing an Ag/AgCl reference electrode (Bioanalytical Systems), a platinum foil counter electrode (Goodfellow) and working electrodes in saline phosphate buffer (0.1 M, pH7.4; 0.15 M NaCl) at 37 °C. For working electrodes, graphite disc electrodes (4 mm diameter) were formed by shrouding graphite rods (Graphitestore.com) using heatshrinkable tubing and establishing an electrical connection at the rear. All potentials are quoted versus the Ag/AgCl reference electrode.

2.3.3 Methods

Graphite disc electrodes were roughened with p-1200 grit silicon carbide paper (Buehler) followed by thorough rinsing with Milli-Q water. All electrodes were sonicated in Milli-Q water for 15 min and subsequently dried under a stream of nitrogen gas.

Enzyme electrodes were prepared by drop-coating a mixture of aqueous solutions of redox complex (2 µL of 3.8 mM aqueous solution containing 5% DMSO), enzyme (aliquots of 10 mg mL$^{-1}$ GOx), 2 µL of 10 mg mL$^{-1}$ of polyallylamine (PAA). Cross-linking was achieved by addition of 2 µL of 10 mg mL$^{-1}$ of a freshly prepared aqueous solution of poly(ethylene glycol) diglycidyl ether (PEGDGE) (M$_n$ = 526), and curing at room temperature for 24 hours.

2.4 Results and discussion

2.4.1 Formation and characterisation of redox-active films on electrodes

The synthetic strategy for preparation of the osmium complexes is based on initial preparation of complexes of formula (Os(N-N)$_2$Cl$_2$), where N-N represents a bidentate ligand, such as 2,2'-bipyridine (bpy) and its derivatives. Tuning of the redox potential of the complex can be achieved by appropriate substitution on the bpy ligand, with prediction of redox potential based on an empirical approach devised by Lever [19, 20]. Ligand substitution of one Cl of the Os(N-N)$_2$Cl$_2$
complex by pyridine or imidazole ligands that display an amine functional group distal to the metal-co-ordinated site provides redox complexes amenable to coupling and cross-linking chemistries [16]. Immobilisation of these redox complexes can thus be achieved using an approach adopted for redox polymer systems, by drop-coating and cross-linking with a di-epoxide, PEGDGE, reactive to primary amines, to form a three-dimensional electroactive matrix [21, 22].

The amount of redox complex retained within films adsorbed to the surface may be enhanced by cross-linking of the complex to a polymer support. We select PAA as a polymer support in this study and evaluate the amount of an Os(bpy)$_2$4AMP complex that is retained at the electrode by cross-linking to PAA using PEGDGE. Cyclic voltammetry (CV) in 0.05 M phosphate buffer, pH7.4, of films prepared on graphite electrodes by cross-linking of Os(bpy)$_2$4AMP redox complexes, in the presence or absence of PAA, exhibits oxidation and reduction peaks corresponding to the Os (II/III) transition, at an $E^0$ of 0.30 V (versus Ag/AgCl), similar to both that for the complex in solution and that of films of the Os(bpy)$_2$4AMP covalently coupled to graphite electrodes and polymer supports on electrodes [16, 17]. An estimate of surface coverage of electronically addressed osmium ($\Gamma_{Os}$) on the electrode can be evaluated by integrating the charge passed when films are electrolysed under conditions of slow scan rate CV. An increase in $\Gamma_{Os}$ is observed for films prepared by cross-linking the redox complex in the presence of PAA polymer support in comparison to films prepared without the support, indicative of formation of three-dimensional polymeric matrices on the electrode. For example, addition of 28 % mass of PAA to the deposition solution results in films displaying a threefold increase in amount of Os addressed, compared to films prepared that do not include the PAA, and this proportion of polymer is selected for all subsequent experiments. It should be noted that the amount of osmium addressed, even for the polymer-containing films, only reaches approximately 60 % of that added to the electrode during deposition.

2.4.2 Glucose oxidation by glucose oxidase co-immobilised with redox-active films on electrodes

Glucose-oxidising enzyme electrodes, for application to EFCs and glucose biosensors, can be prepared by co-immobilisation of the enzyme GOx with the
redox complexes and PAA in films on electrodes. Recording of CVs in 0.05 M phosphate buffer, pH7.4, of films prepared on graphite electrodes by cross-linking GOx, Os(bpy)$_2$4AMP redox complex and PAA (Figure 1), do not show any significant change in redox potential for the complex, compared to films that do not contain GOx. However, the estimated $\Gamma_{\text{Os}}$ from the slow scan rate CVs decrease as a function of the relative mass of GOx added to the deposition solution (Figure 2). This is as expected, as the amount of cross-linker, redox complex and PAA are maintained constant whilst increased amounts of GOx are added to the deposition solutions, and the functionalised redox complex thus competes with the GOx amino- sites for reaction with the diepoxide PEGDGE cross-linker.

In the presence of glucose, slow scan CVs display sigmoidal-shaped curves providing glucose oxidation currents above potentials for the Os(II/III) redox transition, indicative of EC$'$ electrocatalysis of glucose [23] (Figure 1). As the response of redox hydrogel-based enzyme electrodes has been shown to vary as a function of enzyme and redox polymer loading, the response of films prepared by varying the amount of GOx added to the deposition solutions is compared in Fig. 2. The magnitude of the current in the presence of 100 mM glucose increases for films prepared using 22 and 36 % w/w of GOx, but decreases upon further augmentation in the amount of GOx added, presumed to be because of the lower amount of redox complex within the films under these conditions. Similar optimisation studies, focused on GOx and polyvinylimidazole-bound osmium redox polymer films cross-linked using PEGDGE, found that glucose oxidation currents were highest for films of similar ratios to ours, prepared with 39 % GOx [11].
**Figure 1:** Slow scan (5 mV s$^{-1}$) CVs of films prepared by cross-linking PAA, GOx and the redox complex Os(bpy)$_2$4AMP on graphite electrodes in absence (*dashed line*) and presence (*solid line*) of 100 mM glucose in 50 mM phosphate buffer solution, pH 7.4, 0.15 M NaCl at 37 °C.
**Figure 2**: Normalised glucose oxidation current (*black squares*) and estimated osmium surface coverages (*red circles*) as a function of mass percent GOx added to the deposition solutions for films prepared by cross-linking PAA, GOx and Os(bpy)$_2$4AMP on graphite electrodes. Data evaluated from 5 mV s$^{-1}$ CVs under conditions as in Figure 1.
Films prepared based on co-immobilisation of GOx and the Os(bpy)$_2$4AMP complex are not, however, suitable as mediators for application to low-potential, glucose-oxidising biosensors or anodes in EFCs, because of the relatively high redox potential required. A comparative study was therefore conducted using osmium-based redox mediators with lower redox potentials, prepared by introduction of methyl- or methoxy- electron-donating functional group at the 4 and 4’ positions of the bpy ligands of the osmium complexes. Films of the dmbpy and dmobpy redox complexes are prepared using the same ratios of redox complex, GOx, PAA and cross-linker, determined to be optimum for the Os(bpy)$_2$4AMP films. Slow scan CV of the enzyme electrodes in the absence of glucose displays redox potentials for the Os(II/III) transition of 80 mV (Os(dmobpy)$_2$4AMP), and 220 mV (Os(dmbpy)$_2$4AMP), respectively (Figure 3a) similar to values observed for the complexes in solution.
Figure 3: CVs, 1 mV s$^{-1}$, for films prepared by crosslinking PAA, GOx and the redox complexes Os(dmobpy)$_2$4AMP (black), Os(dmbpy)$_2$4AMP (red) and Os(bpy)$_2$4AMP (blue) in the absence (a) and the presence (b) of 5 mM glucose. Other conditions as in Figure 1.
Upon addition of glucose, sigmoidal-shaped slow scan CVs, characteristic of catalytic oxidation of glucose, are obtained (Fig. 3b). The films prepared using Os(dmobpy)$_2$4AMP and Os(dmbpy)$_2$4AMP produce lower glucose oxidation current densities than those obtained for films prepared with Os(bpy)$_2$4AMP. Although a contributing factor to the lower currents may be the lower $I_{Os}$ for films of Os(dmobpy)$_2$4AMP and Os(dmbpy)$_2$4AMP, estimated from the CVs in Figure 3a and presented in Table 1, the response does not scale directly with surface coverage. Other factors contributing to observed current decrease as a function of potential may be the decreased potential difference between mediator and GOx active site, observed by others to contribute to a decrease in current for the solution phase [24], and redox polymer film, mediation of GOx oxidation of glucose [21]. It is also, as yet, unclear if differences in film swelling and charge or mass transport contribute to the observed current decrease as a function of redox complex selected.

To further compare the performance of the enzyme electrode as a function of redox complex selection and glucose concentration, and to estimate operational stability, constant potential amperometry, using applied potentials approximately ~0.15 V more positive than the redox potential of each of the redox complexes, was attempted. From the resulting glucose calibration curves (Figure 4), maximum glucose oxidation current ($i_{max}$), assuming Michaelis–Menten behaviour of the enzyme electrodes, of 290 μAcm$^{-2}$ for Os(bpy)$_2$4AMP enzyme electrodes can be estimated compared to lower $i_{max}$ of 190 μAcm$^{-2}$ for the Os(dmbpy)$_2$4AMP enzyme electrodes and 60 μAcm$^{-2}$ for the Os(dmobpy)$_2$4AMP enzyme electrodes. An average apparent Michaelis–Menten constant, $K_{M}$$_{app}$, of 7.8 (±0.7) mM for glucose is estimated for the enzyme electrodes, with no variation in $K_{M}$$_{app}$ as a function of redox complex. This value compares well with reported $K_{M}$ of 10 (±5) mM for enzyme electrodes prepared by co-adsorption of GOx and tris-(4,4′-substituted-2,2′-bipyridine)osmium complexes [24]. Although comparison is rendered difficult, due to differences in film preparation methodology, Danilowicz et al. [15] reported glucose oxidation current density of 60 μA cm$^{-2}$ for an Os(bpy)24AMP bound to PAA prior to co-immobilisation with GOx, in the presence of 50 mM glucose. When the response of enzyme electrodes is compared for glucose concentrations of 5 mM, to mimic physiological glucose levels, with eventual application to in vivo glucose-oxidising anodes as a goal, enzyme
electrodes prepared using Os(dmobpy)$_2$4AMP, Os(dmbpy)$_2$4AMP and Os(bpy)$_2$4AMP display current densities of 30, 70 and 120 μAcm$^{-2}$, respectively.

**Figure 4:** Current density (n=3) as a function of glucose concentration for films prepared by crosslinking PAA, GOx and the redox complexes Os(dmobpy)$_2$4AMP (*black squares*), Os(dmbpy)$_2$4AMP (*red circles*) and Os(bpy)$_2$4AMP (*blue triangles*). Current densities determined from amperometry at an applied potential of 0.2, 0.35 and 0.45 V for Os(dmobpy)$_2$4AMP, Os(dmbpy)$_2$4AMP and Os(bpy)$_2$4AMP, respectively, in 50 mM pH 7.4 phosphate buffer solution 0.15 M NaCl at 37 °C, 150 rpm.
Table 1 Mean values (n=3) of osmium surface coverages, $\Gamma_{\text{Os}}$, initial current density and current remaining after 12 hour amperometry for films prepared by cross-linking PAA, GOx and redox complex. The half-life of the film response is estimated assuming first-order decay in current over time. Other conditions as in Figure 4.

<table>
<thead>
<tr>
<th>Redox complex</th>
<th>$\Gamma_{\text{Os}}$ (nmoles cm$^{-2}$)</th>
<th>Initial current density ($\mu$A cm$^{-2}$)</th>
<th>% Current remaining at 12 hrs</th>
<th>$t_{1/2}$ (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Os(dmobpy)$_2$4AMP</td>
<td>5.6 ± 0.1</td>
<td>72 ± 15</td>
<td>14.2 ± 5.2</td>
<td>6 ± 0.1</td>
</tr>
<tr>
<td>Os(dmbpy)$_2$4AMP</td>
<td>9.6 ± 0.75</td>
<td>191 ± 12</td>
<td>15.0 ± 2.7</td>
<td>6.3 ± 0.3</td>
</tr>
<tr>
<td>Os(bpy)$_2$4AMP</td>
<td>10.9 ± 0.6</td>
<td>293 ± 10</td>
<td>17.1 ± 0.1</td>
<td>7.2 ± 0.3</td>
</tr>
</tbody>
</table>

A comparison of the stability of the response for glucose oxidation from the enzyme electrodes is undertaken by recording the amperometric response, whilst gently stirring, to avoid localised substrate depletion. The natural logarithm of the current as a function of time was plotted and a half-life of the response stability estimated from the slope of these plots, thus assuming simple first-order decay in current. After 12 h, only ~15% of the initial response is produced for all enzyme electrodes, likely due to leaching of enzyme or redox complex from the electrode highlighting a requirement for improved anchoring and cross-linking of films [16, 17, 21, 25]. For example, we recently reported on improved stability of enzyme electrodes by inclusion of multiwalled carbon nanotubes and cross-linking with glutaraldehyde, compared to films cross-linked with PEGDGE [21]. Further work investigating the impact of cross-linking methodologies and the inclusion of nanoparticles is likely to enhance the stabilities of the enzyme electrodes.

2.5 Conclusions

In this study, we report on a method for co-immobilisation in films on graphite electrodes of redox complexes, GOx and a PAA polymer support, each possessing reactive amine functional groups, through cross-linking using a di-epoxide PEGDGE. Inclusion of the polymer support increases the amount of osmium addressed within the three-dimensional films on electrodes. The resulting enzyme electrodes show catalytic current signals for oxidation of glucose, with the signal varying as a function of added enzyme mass to the deposition solution. Variation in redox potential required to produce glucose oxidation current signals is
achieved by synthesis, and co-immobilisation within films, of redox complexes with electron-donating groups on the bipyridine ligands of the complexes. Enzyme electrodes prepared using Os(dmobpy)$_2$4AMP, Os(dmbpy)$_2$4AMP and Os(bpy)$_2$4AMP provide glucose oxidation current densities of 30, 70 and 120 $\mu$A cm$^{-2}$ at 0.2, 0.35 and 0.45 V of applied potentials under pseudo-physiological conditions, respectively, with however stability of signals proving inadequate for application to long-term operation. Future work is focused on screening of methods for immobilisation of sugar-oxidising enzymes to improve current output and stability, so as to provide enzyme electrodes capable of operating under pseudo-physiological conditions for application to long-term glucose biosensors and anodes in EFCs.

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2.7 References


Chapter 3:

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Tethering osmium complexes within enzyme films on electrodes to provide a fully enzymatic membrane-less glucose/oxygen fuel cell


Co-author contributions:

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

Brenda Egan contributed to the publication by assisting in the testing of the bioanode and biofuel cells.

Domhnall MacAodha 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.
Tethering osmium complexes within enzyme films on electrodes to provide a fully enzymatic membrane-less glucose/oxygen fuel cell

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3.1 Abstract

Enzyme electrodes based on cross-linking bilirubin oxidase or a Streptomyces coelicolor laccase (SLac) and osmium redox complexes possessing an amine-terminated molecular tether at graphite electrodes can produce current for oxygen reduction under pseudo-physiological conditions. Here we report on enzyme electrodes for oxygen reduction by co-immobilization of SLac, polyallylamine (PAA) and an [osmium(2,2’-bipyridine)2(4-aminomethylpyridine)Cl]⁺ complex. Enzyme electrodes prepared by incorporation of added multi-walled carbon nanotubes as support produce oxygen reduction current densities of 0.8 mA cm⁻² in oxygen saturated, 150 mM NaCl, 50 mM phosphate buffer solution at 37 °C, a 3-fold increase in oxygen reduction current densities over those prepared without multi walled carbon nanotubes. Membrane-less glucose-O₂ fully enzymatic fuel cells are assembled by combination of the SLac-based enzyme electrode as a cathode, with glucose-oxidizing anodes, based on either a pyrroloquinoline quinone (PQQ) or FAD-dependent glucose dehydrogenase (GDH) and tetherable osmium redox complexes. The fuel cell based on selection of PQQGDH enzyme electrode as anode provides a maximum power density of 66 µW cm⁻² in 5 mM glucose, 150 mM NaCl, phosphate buffer solution at 37 °C, dropping to 37 µW cm⁻² in human serum, the highest reported power density to date for an enzymatic fuel cell operating in serum.
3.2 Introduction

Electrodes modified with sugar-oxidizing and oxygen-reducing redox enzymes are of increasing interest due to their potential application as biocatalytic anodes and cathodes in miniaturized membrane-less fuel cells, which may provide power to small, low-power portable, implantable or semi-implantable electronic devices (1-5). An attractive source of fuel and oxidant available in vivo are glucose and oxygen, due to their relatively high concentrations of ca. 5-8 mM and 0.05-0.13 mM, respectively, present in blood (6-8). Glucose-oxidizing enzymes, such as glucose oxidase and glucose dehydrogenase (GDH), and oxygen-reducing enzymes, such as bilirubin oxidase and laccase, display high substrate specific activities and turnover rates, reasonable stability when confined within a matrix at the electrode surfaces (ca. 1 week), and show no known toxicity in the quantities considered for future implantable enzymatic fuel cell (EFC) (2).

In conventional fuel cells, metal catalysts, such as platinum, are commonly used to catalyse the oxygen reduction reaction (ORR) at the cathode. Platinum, as a catalyst, however, is non-selective, prone to poisoning and displays a high overpotential, of ca. 0.5 – 0.6 V, for the ORR. Alternatively, multi-copper oxidase (MCO) enzymes are efficient ORR catalysts when immobilized at electrode surfaces (9-11). For example, laccase sourced from Coriolus hirsutus, when co-immobilized with an osmium redox polymer as electron transfer mediator at a carbon fibre electrode, and operated in a 0.1 M pH 5 citrate buffer, has been shown to be superior to platinum (operating in its optimal electrolyte of 0.5 M H₂SO₄) as a catalyst for the electrocatalytic reduction of O₂ to H₂O (12). The inclusion of the electron transfer mediator permits electrical connection of multiple layers of enzyme, irrespective of its orientation, to the electrode surface. Such electrodes have been shown to generate significantly higher levels of current compared to their direct electron transfer, mediator-less, enzyme electrode counterparts (5). Polymer-bound osmium polypyridyl complexes are widely reported on as redox mediators for enzymes, including glucose oxidase (13,14), glucose dehydrogenase (15,16), cellobiose dehydrogenase (17,18), bilirubin oxidase (19,20) and laccase (21-25). Recent reports have highlighted difficulties in synthesis of polyvinylpyridine and polyvinylimidazole-based redox polymers, with variations in osmium complex loading within the redox polymers difficult to
control (5). In view of this, alternate routes for redox polymer preparation focus on direct coupling to polymers via reaction between a functional group peripherally attached to an osmium co-ordinating ligand and the polymer backbone (26-29). For example, we have reported that enzyme electrodes prepared by cross-linking [Os(2,2’-bipyridine)$_2$(4-AMP)Cl]$^+$ (where 4-AMP is 4- (aminomethyl)pyridine), a commercially available polyallylamine polymer (PAA) support and glucose oxidase on graphite electrodes, yield high glucose oxidation current density in 5 mM glucose, of 120 $\mu$A cm$^{-2}$ (29).

In the present report, we extend this methodology to evaluate enzyme electrodes for ORR under physiological conditions. We focus on the MCOs *Myrothecium verrucaria* bilirubin oxidase (*MvBOd*) (30) and *Streptomyces coelicolor* laccase (SLac) (31) produced by the soil dwelling gram-positive bacterium. The SLac enzyme is trimeric with a monomeric molecular mass of 32 kDa and structurally distinct from fungal laccases, with a reported redox potential of the type 1 (T1) copper site of ~0.25 V vs. Ag/AgCl, and a substrate pH optimum typically around pH 8 (31-33). The bilirubin oxidase from *Myrothecium verrucaria* is a monomeric enzyme, widely used in oxygen-reducing enzyme electrodes and EFCs due to it relatively high T1 copper site redox potential of 0.48 V vs. Ag/AgCl and pH optimum at physiological conditions (34).

In addition, we report on the effect that inclusion of multi-walled carbon nanotubes (MWCNT) has on the enzyme electrode response, as inclusion of conducting composites has previously been shown to increase both current output and stability of mediated enzyme electrodes (35,36). Finally, we report on the performance of these electrodes, coupled to GDH-based glucose-oxidizing anodes, in an assembled membrane-less EFC operating in physiological buffer and human serum. The GDHs selected are FAD-dependent glucose dehydrogenase from *Aspergillus* sp. (*AspGDH*), (37,38) and pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQGDH), with a reported optimum pH and temperature of 7.0 and 37°C (39), both of interest for use in glucose-oxidizing anodes due to their high turnover rates, commercial availability and oxygen independence.
3.3 Experimental

3.3.1 Materials

All chemicals and biochemicals were, unless otherwise stated, purchased from Sigma-Aldrich (Dublin, Ireland) and used as received. All solutions were prepared in Milli-Q water unless otherwise stated.

Complexes of cis-Os(4,4'-R-2,2'-bipyridine)₂Cl₂, where R represents a functional group, were synthesized from (NH₄)₂OsCl₆ and characterized as described previously (29,40,41). Ligand substitution of a Cl of the cis-Os(4,4'-R-2,2'-bipyridine)₂Cl₂ complexes by 4-(aminomethyl)pyridine (4-AMP) was achieved by heating an ethylene glycol solution of a 1.1 mole equivalent of ligand and complex at reflux, with reaction progress monitored using voltammetry. Precipitation of [Os(4,4'-R-2,2'-bipyridine)₂(4-AMP)Cl].PF₆ complexes was achieved by addition of aqueous NH₄PF₆. Final products of [Os(4,4'-dimethoxy-2,2'-bipyridine)₂(4-AMP)Cl].PF₆ (Os(dmobpy)₄-AMP), [Os(4,4'-dimethyl-2,2'-bipyridine)₂(4-AMP)Cl].PF₆ (Os(dmbpy)₄-AMP), or [Os(2,2'-bipyridine)₂(4-AMP)Cl].PF₆ (Os(bpy)₄-AMP) were then filtered and allowed to dry overnight at 50°C, as described previously (29).

Glucose dehydrogenase (EC 1.1.99.10) from Aspergillus sp. was from Sekisui Diagnostics (Cambridge, USA; product GLDE-70-1192), pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (PQQGDH, EC 1.1.99.17) was from Sorachim (Lausanne, Switzerland; product GLD-321), MvBOd (EC 1.3.3.5, “Amano 3”) was a gift from Amano Enzyme Inc. (Nagoya, Japan) and purified preparations of Streptomyces coelicolor laccase were provided by Novozymes A/S (Denmark). Human Serum from human male AB plasma, sterile-filtered (H-4522) was purchased from Sigma-Aldrich Ireland.

3.3.2 Apparatus

All electrochemical measurements were performed in 50 mM phosphate buffer solution, pH 7.4, containing 150 mM NaCl (PBS), at 37°C with a 1030 multi-channel potentiostat (CH Instruments) using an Ag/AgCl reference electrode (3 M KCl) (Bioanalytical Systems), a platinum foil counter electrode (Goodfellow) and graphite disc working electrodes (Graphite store, USA). Oxygen saturation under
these conditions, monitored using a dissolved oxygen electrode and meter (Eutech Instruments), was achieved by sparging the solution with oxygen. Glucose concentration in human serum was estimated using a GlucoMen Lx Plus system by A. Menarni Diagnostics (1.1-33.3 mmol/L).

### 3.3.3 Preparation of electrodes

3 mm graphite disc electrodes were prepared from graphite rods, shrouded in heat-shrink tubing and polished on 1200 grit silicon carbide paper (Buehler) followed by thorough rinsing with Milli-Q water. All electrodes were sonicated in Milli-Q water for 15 min and subsequently dried under a stream of nitrogen gas prior to use.

Enzyme electrodes were prepared by drop-coating an aqueous solution of redox complex (2 μL of 3.8 mM aqueous solution containing 5% DMSO) on the electrode surface, allowing to dry for 3 hours, followed by subsequent deposition of enzyme (1.1 nmol of SLac or MvBOd for oxygen reducing electrodes, or 0.08 mg of AspGDH or PQQGDH for glucose oxidizing electrodes) and crosslinking treatment. Enzyme electrodes using PAA as support were prepared by co-deposition of 2 μL of a 5 mg mL⁻¹ aqueous solution of PAA with the redox complex. For the composite MWCNT enzyme electrodes, 9.6 μL aqueous suspension (0.44 mg) of acid treated MWCNT (20 mg mL⁻¹ in HNO₃, refluxed for 6 hours at 150°C) was co-deposited with the redox complex. Cross-linking treatment was achieved either by addition of 2 μL of a freshly prepared 10 mg mL⁻¹ aqueous solution of poly(ethylene glycol) diglycidyl ether (PEGDGE) (M₉=526) and curing at room temperature for 24 h, or by placing the enzyme electrodes in a sealed headspace of glutaraldehyde (GA) vapors for 30 min, as previously reported (35). The GA treated electrodes were then immersed in NaBH₄ solution for 5 s and subsequently rinsed in Milli-Q water, to reduce the Schiff bases formed upon crosslinking to more stable amines (termed GA(r) electrodes) (42,43). All enzyme electrode testing commenced within 20 minutes of completion of electrode preparation. All current densities are normalized to the geometric surface area of the electrode.
3.4 Results and discussion

Cyclic voltammetry (CV) can be used to confirm redox potentials for the Os(II/III) transition of the complexes, in solution and when co-immobilized with redox enzymes, are as predicted (44, 45), and are not affected by the immobilization procedure. For example, CVs recorded at 5 mV s\(^{-1}\), in the absence of oxygen, of Os(bpy)\(_4\)-AMP co-immobilized with the SLac MCO on graphite electrodes display a pair of reversible peaks centred at 0.30 V vs. Ag/AgCl, Figure 1 (dashed line) similar to the redox potential observed for the Os(bpy)\(_4\)-AMP complex in aqueous electrolyte and to that reported for the complex immobilized by coupling to a carboxymethylidextran film on carbon electrodes (26).

![Figure 1. CVs, at 5 mV s\(^{-1}\) recorded in quiescent PBS, N\(_2\) saturated at 37°C, of enzyme electrodes of SLac (36 µg) and Os(bpy)\(_4\)-AMP (6 µg) crosslinked by GA(r) in the absence (dash) and presence (line) of MWCNT (444 µg) as support.](image)

Incorporation of a polymer (29) or composite (46) as a support within films of enzymes results in increased current and stability in their application as biosensors (47, 48) and biofuel cell enzyme electrodes (28, 36, 49, 50). For example, an increase in glucose oxidation current signal and stability has been
reported upon addition of MWCNT to crosslinked osmium redox polymer and glucose oxidase films on electrodes (28, 51). In addition Ó Conghaile et al. report a high current density, of 120 μA cm$^{-2}$, in 5 mM glucose for enzyme electrodes based on cross-linked films of glucose oxidase, the Os(bpy)4-AMP redox complex and a commercially available PAA support on graphite electrodes (29).

Enzyme electrodes for ORR are of increasing interest because of potential applications in miniaturized fuel cells, which may provide sufficient power to portable, implantable or semi-implantable biomedical devices. Since the initial report by Palmore and Kim on an enzyme catalyst for ORR in an EFC, using solution-phase fungal laccase and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate) mediator at pH 4 (52), research has focused on MCOs that can reduce oxygen to water under physiological conditions (see 1-5 for reviews). The use of $Mv$BOd in enzyme electrodes for ORR at pH 7.4 has been implemented in EFCs (1-4) over the past decade. More recently a SLac MCO has been isolated (32) that resulted in a high current density for ORR when co-immobilized with an osmium redox polymer on electrodes (46), postulated to be because of the relatively low molar mass of the catalytic domain of the enzyme compared to that of other MCOs. Enzyme electrodes for ORR were thus prepared by crosslinking SLac with Os(bpy)4-AMP redox complex and a PAA polymer support, using the methodology we recently described for preparation of glucose-oxidizing enzyme electrodes (29). In this methodology reactive amine functional groups on the enzyme, redox complex and polymer support are cross-linked using ring-opening epoxidation reactions of PEGDGE. Slow scan CVs of these enzyme electrodes, recorded in the absence of oxygen, can be used to estimate surface coverages of electronically addressed osmium ($\Gamma_{Os}$) (53) whilst CVs recorded in the presence of oxygen are used to estimate catalytic ORR currents. The films crosslinked with PEGDGE provide ORR current densities of 0.18 mA cm$^{-2}$ at osmium surface coverages of approximately 30 nmol cm$^{-2}$ (Table 1). Enzyme electrodes for ORR prepared by crosslinking SLac and Os(bpy)4-AMP with PAA and MWCNT as support demonstrate a 3-fold increase in ORR current densities over those prepared without MWCNT (Table I). Cross-linking in the presence of PAA (29) and/or MWCNT (Table I) as a support results in an increase in $\Gamma_{Os}$ over that observed for films prepared without the support, indicating that the support acts as a scaffold for incorporation of mediator within the films, that is electronically
connected to the electrode surface (35). It is presumed that the support as scaffold also results in retention of increased amounts of deposited enzyme within the electrode film, contributing to the increased ORR current densities, a postulation that is currently being tested using enzyme activity tests of SLac within the films.

Table I. Response of enzyme electrodes based on crosslinking Os(bpy)4-AMP and SLac, in the presence or absence of MWCNT or PAA as support. \( \Gamma_{\text{Os}} \) estimated from 5 mV s\(^{-1}\) CV in \( \text{N}_2 \) sat. PBS at 37°C. ORR current density obtained from 1 mV s\(^{-1}\) CV, in \( \text{O}_2 \) sat. PBS at 37 °C (n \( \geq 3 \)).

<table>
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<th>( \Gamma_{\text{Os}} ) (nmol cm(^{-2}))</th>
<th>ORR Current density (mA cm(^{-2}))</th>
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<tr>
<td>PAA (PEGDGE)</td>
<td>29 ± 6</td>
<td>0.18</td>
</tr>
<tr>
<td>PAA + MWCNT (PEGDGE)</td>
<td>43 ± 1</td>
<td>0.59</td>
</tr>
<tr>
<td>PAA (GA(r))</td>
<td>5 ± 2</td>
<td>0.03</td>
</tr>
<tr>
<td>MWCNT (GA(r))</td>
<td>37 ± 1</td>
<td>0.81</td>
</tr>
<tr>
<td>PAA + MWCNT (GA(r))</td>
<td>48 ± 1</td>
<td>0.81</td>
</tr>
</tbody>
</table>

The synthesis of redox complexes possessing amine functional groups distal to the metal co-ordination site also provides systems to explore crosslinking strategies for enzyme and mediator co-immobilization. Homo-bifunctional crosslinkers based on epoxide and aldehyde functional groups such as PEGDGE or GA are capable of reacting with amine-containing complexes and the available primary and secondary amines on the enzyme (54-56). Evaluation of Os(bpy)4-AMP crosslinked with SLac on graphite electrodes and a PAA polymer support using glutaraldehyde vapors, followed by treatment with sodium borohydride to stabilize the Schiff bases formed, in a methodology developed by MacAodha et al. (35) in comparison to the PEGDGE as a crosslinker, to provide enzyme electrodes for ORR, was therefore undertaken. Enzyme electrodes crosslinked with the GA(r) methodology achieved ORR current densities of 0.81 mA cm\(^{-2}\), in comparison to the 0.59 mA cm\(^{-2}\) for the analogous films crosslinked with PEGDGE (Table 1). This is in agreement with reported higher current output using this approach to crosslink glucose oxidase and PVI-bound osmium redox polymers over films crosslinked with PEGDGE (51). In addition, it should be noted that enzyme electrodes prepared by crosslinking, using GA(r) methodology,
Os(bpy)4-AMP, SLac and MWCNT provide up to tenfold higher osmium surface coverage (Figure 1 and Table I), and up to 25-fold higher ORR current density, over those prepared without MWCNT (Figure 2, Table I), highlighting the increased ORR current as a function of increased amount of immobilized enzyme and mediator when a scaffold is provided.

Figure 2. Slow scan, 1 mV s\(^{-1}\), CVs recorded in quiescent PBS, O\(_2\) saturated at 37°C, for enzyme electrodes of SLac and Os(bpy)4-AMP crosslinked by GA(r) in the absence (blue dot) or presence of PAA (red dash) or MWCNT (black solid) as support. All conditions as in Figure 1, except PAA (10 µg).

3.4.1 MCO comparison

To compare the performance of enzyme electrode as a function of MCO selection and oxygen concentration, constant potential amperometry, using an applied potential of 0 V vs Ag/AgCl was undertaken for electrodes prepared using either \(Mv\)BOd or SLac. The \(Mv\)BOd is selected for comparison due to its reported high redox potential of 480 mV vs Ag/AgCl and optimal pH at physiological conditions (52). In addition, recent studies confirm that ORR by either \(Mv\)BOd
(57) or laccases (31, 52) do not release H₂O₂ as an intermediate. A comparison of ORR current density for enzyme electrodes prepared by crosslinking Os(bpy)4-AMP, MWCNT and the same number of moles of MvBOd to those prepared using SLac was undertaken as a function of oxygen concentration, Figure 3. Maximum ORR current \( (i_{\text{max}}) \), assuming Michaelis–Menten behaviour of the enzyme electrodes, of 1.25 mA cm\(^{-2}\) can be estimated for enzyme electrodes based on Slac compared to only 0.3 mA cm\(^{-2}\) for MvBOd-based enzyme electrodes, with apparent Michaelis–Menten constants, \( K_{M}^{\text{app}} \), of 0.125 mM or 0.06 mM for oxygen estimated for SLac-based and MvBOd-based enzyme electrodes, respectively, leading to selection of SLac-based enzyme electrodes for subsequent studies. The oxygen \( K_{M}^{\text{app}} \) estimated for MvBOd-based enzyme electrodes compares well with a reported \( K_{M}^{\text{app}} \) of 0.08 mM for enzyme electrodes prepared by co-adsorption of MvBOd and ABTS (34), whilst little comparative data on oxygen \( K_{M}^{\text{app}} \) for SLac-based enzyme electrodes is available. A similar oxygen \( K_{M}^{\text{app}} \) value, of 0.22 mM, has been evaluated for enzyme electrodes based on co-immobilization of MvBOd or SLac with a PVI-bound osmium redox polymer and MWCNT (58).
Figure 3. ORR current density, extracted from chronoamperometry at 0 V applied potential in stirred PBS at room temperature, as a function of oxygen concentration for enzyme electrodes of \textit{Mv}BoD (●) or SLac (■) crosslinked using GA(r) with Os(bpy)4-AMP and MWCNT. All conditions as in Figure 1, except \textit{Mv}BoD (77 µg).

3.4.2 Membrane-less Enzymatic Glucose-Oxygen Fuel Cell

Enzyme electrodes based on co-immobilization of a sugar-oxidizing enzyme with the Os(bpy)4-AMP complex are not suitable for application to low-potential, glucose-oxidizing bio-anodes in EFCs. Enzyme electrodes as anodes were therefore prepared using osmium-based redox complexes with lower redox potentials, synthesized by substitution at the 4,4' site of the bipyridine ligands with electron-donating methoxy- or methyl- functional groups. Enzyme electrodes, prepared using the same ratios of redox complex, enzyme and MWCNT determined to be optimum for the Os(bpy)4-AMP, SLac and MWCNT films, display redox potentials for the Os(II/III) transition centred at 0.08 V for the Os (dmobpy)4-AMP complex, or 0.22 V for the Os(dmbpy)4-AMP complex, respectively, similar to values observed for the complexes in solution and deposited on electrodes (29).
Enzyme electrodes chosen as anodes are thus based on films of an Os(dmobpy)4-AMP or Os(dmbpy)4-AMP redox complexes co-immobilized with GDHs in the presence of MWCNTs. The linear sweep voltammograms (LSV) for glucose oxidation by the GDH-based enzyme electrodes show onset of oxidation at −100 mV or +20 mV vs. Ag/AgCl using either the Os(dmobpy)4-AMP or Os(dmbpy)4-AMP complexes, respectively, Figure 4. Glucose oxidation current densities, of 0.3 mA cm$^{-2}$ and 0.42 mA cm$^{-2}$ are observed in quiescent 5 mM glucose solutions, for enzyme electrodes based on PQGQDH and MWCNT co-immobilized with Os(dmobpy)4-AMP or Os(dmbpy)4-AMP, respectively. These current densities are similar to those reported under the same conditions for analogous films prepared using osmium redox complexes of dmbpy and dimobpy co-ordinatively bound to polyvinylimidazole polymers (51), with a similar decrease in current density as a function of selection of dimobpy, over dmbpy-based complexes noted. From Figure 4, enzyme electrodes prepared with PQQGDH provide slightly higher glucose oxidation current density over those prepared using AspGDH, so both systems are selected for application as anodes in a membrane-less fully enzymatic glucose-oxygen fuel cell operating under physiological conditions.
**Figure 4.** Linear sweep voltammograms, 1 mV s⁻¹ in quiescent 50 mM PBS at 37 °C, for glucose oxidation (5 mM) by MWCNT composite enzyme electrodes using PQQGDH (80 µg) crosslinked with Os(dmobpy)4-AMP (brown line) or Os(dmbpy)4-AMP (green dash dot) and AspGDH (80 µg) crosslinked with Os(dmobpy)4-AMP (blue dash) or Os(dmbpy)4-AMP (red dot). All conditions as in Figure 1, except Os(dmobpy)4-AMP (6.9 µg) and Os(dmbpy)4-AMP (6.4 µg).

**3.4.3 Fuel cells in pseudo-physiological buffer**

As one potential application for EFC systems is to deliver power to implantable medical devices using glucose and oxygen as fuel and oxidant, available in vivo, EFCs were assembled and tested in 150 rpm stirred solutions of PBS containing 5 mM glucose at 37°C, to model such physiological conditions. When the enzyme electrode based on SLac is coupled, in a membrane-less configuration, to the enzyme electrode based on PQQGDH, in PBS containing 5 mM glucose at 37°C, a maximum power density \((n \geq 3)\) of \(66 \pm 19 \mu W \, cm^{-2}\) at a cell voltage of 0.25 V is obtained (Figure 5). Slight variation in EFC maximum power density is observed, as a function of selection of enzyme and/or redox complex mediator in the anode, with a trend of enzyme electrodes based on PQQGDH producing
higher maximum power density than those based on \textit{Asp}GDH. This trend follows the trend observed for steady-state current densities for glucose oxidation by these enzyme electrodes, Figure 4. Steady-state ORR current density for SLac-based enzyme electrodes is 0.35-0.4 mA cm$^{-2}$ in PBS at air saturation, Figure 3. By comparison to the LSVs in Figure 4, the maximum power output of EFCs based on SLac-based enzyme electrodes as cathode is limited by anode current density, for all but the EFC using the PQQGDH enzyme electrode with Os(dmbpy)$_4$-AMP as mediator. The EFCs operating on Os(dmobpy)$_4$-AMP redox complex as mediator in the anode provide slightly higher average maximum power densities, compared to those operating on Os(dmbpy)$_4$-AMP redox complex, highlighting issues with selection of anode based on higher current density, observed with the Os(dmbpy)$_4$-AMP based enzyme electrodes (Figure 4), or lower redox potential for onset of glucose oxidation, observed with the Os(dmobpy)$_4$-AMP based enzyme electrodes (Figure 4).

**Figure 5.** Power curves, recorded by 1 mVs$^{-1}$ linear sweep voltammetry in 50 mM PBS containing 5 mM glucose (black) or human serum (grey) at 37°C for membrane-less EFCs based on SLac co-immobilized with Os(bpy)$_4$-AMP, MWCNT as a cathode combined with either PQQGDH co-immobilized with
Os(dmobpy)4-AMP, MWCNT (■) or AspGDH co-immobilized with Os(dmobpy)4-AMP, MWCNT (●) as anodes. All conditions as in Figures 1 and 4.

Previous reports on an EFC prototype, assembled using enzyme electrodes based on either glucose oxidase (GOx) or a fungal laccase co-immobilized with osmium redox polymers, obtained a maximum power density of 16 µW cm\(^{-2}\) in 10 mM glucose (14), while Kim et al. report on a membrane-less EFC that operates at a power density of 50 µW cm\(^{-2}\) at a cell potential of 0.5 V in air saturated pH 7.4, 140 mM NaCl, 37.5°C in 15 mM glucose (59). Soukharev et al. (12) report an EFC using GOx and a fungal laccase, co-immobilized with osmium redox polymers on 7 µm diameter, 2 cm long, carbon fibres, producing 350 μW cm\(^{-2}\) power density in 15 mM glucose, pH 5 buffered solutions, with however only a quarter of that maximum power, 90 µW cm\(^{-2}\), observed at 5 mM glucose concentration. Highest maximum power densities reported to date for an EFC operating in 5 mM glucose, to our knowledge, is of 280 µW cm\(^{-2}\) for an EFC based on the redox polymers reported on (12) as above, but using a GOx sourced from *Penicillium pinophilum* and operating in pH 5 buffer solution, instead of pH 7.4 (60). Use of osmium redox polymers, designed to incorporate a flexible tether between the redox complex and polymer backbone, co-immobilized at carbon fibre electrodes with GOx and MvBOD, as anode and cathode, respectively results in a 480 µW cm\(^{-2}\) power density in pH 7.2 phosphate buffer containing 0.1 M NaCl, 15 mM glucose at 37.5 °C (61). A further increase in power output, to 740 µW cm\(^{-2}\) under the same conditions, is obtained when carbon microwire electrodes, from coagulation spinning of carbon nanotubes, are used as electrode material (50).

### 3.4.4 Fuel cells in human serum

To attempt to evaluate the effect of deployment of a fully enzymatic, membrane-less EFC, in real physiological solutions, power density curves were recorded for the SLac-based enzyme electrodes as a cathode coupled to GDH-based enzyme electrodes, with the Os(dmobpy)4-AMP as mediator, as anode in non-sparged sterile filtered human serum at 37 °C, with stirring at 150 rpm. The oxygen concentration within these, non-sparged, solutions is estimated, using a dissolved
oxygen electrode, as 0.05 mM, which agrees with the value reported in blood (7). As was the case for the EFC operating in buffer solutions, the maximum power density obtained for the EFCs in serum were higher for EFCs using PQGDDH-based enzyme electrodes as anode than those using the Asp GDH-based enzyme electrodes, Figure 5 and Table 2. Maximum power densities for the EFCs operating in human serum are approximately half that observed for identical EFCs operating in pseudo-physiological PBS, Figure 5 and Table 2. Such a difference in power output has been observed by others, and has been attributed to the presence of anti-oxidants and enzyme-inhibiting (7, 62) compounds present in human blood. The human serum supplied had a glucose concentration of only 4.3 mM, lower than the 5 mM glucose concentration tested in PBS, thus also contributing to the lower maximum power densities.

We recently reported on a maximum power density of 60 µW cm$^{-2}$ for an EFC assembled in a membrane-less configuration based on GDH and Myceliophthora thermophila laccase co-immobilized with osmium redox polymers, when operating in artificial plasma (58). Fully enzymatic glucose-oxygen EFCs based on direct electron transfer between electrodes and adsorbed cellobiose dehydrogenase, at the anode, and MvBOD at the cathode, operating in human serum have been previously reported on (7, 62), where no significant difference is observed in power output between operation in PBS and human serum, but maximum power density of only 4 µW cm$^{-2}$ observed.
Table II. Comparison of open circuit potentials, power output and potential at maximum power output obtained at mediated electron transfer based biofuel cells assembled with PQQGDH or AspGDH, MWCNT and anodic mediator coupled to a cathode co-immobilized with SLac, MWCNT and cathodic mediator (n=3).

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Sugar oxidizing Enzyme</th>
<th>Power density (µW cm⁻²)</th>
<th>Cell voltage at max power (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anode</td>
<td>Cathode</td>
<td></td>
</tr>
<tr>
<td>PBS:</td>
<td>Os(dmobpy)₂4-AMP</td>
<td>Os(bpy)₂4-AMP</td>
<td>PQQGDH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AspGDH</td>
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<tr>
<td></td>
<td>Os(dmbpy)₂4-AMP</td>
<td>Os(bpy)₂4-AMP</td>
<td>PQQGDH</td>
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<td>AspGDH</td>
</tr>
<tr>
<td>Serum:</td>
<td>Os(dmobpy)₂4-AMP</td>
<td>Os(bpy)₂4-AMP</td>
<td>PQQGDH</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>AspGDH</td>
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3.5 Conclusions

Co-immobilization, by cross-linking MCO enzymes and a redox complex possessing an amine-terminated molecular tether with MWCNT at graphite electrodes, provides enzyme electrodes for oxygen reduction. Enzyme electrodes based on the co-immobilization of SLac, Os(bpy)₄-AMP and MWCNT, crosslinked with GA vapours and subsequently reduced in sodium borohydride, produced ORR current density as high as 0.8 mA cm⁻² in physiological buffer solution. The fabrication and characterization of a membrane-less glucose-O₂ EFC was undertaken by combination of the SLac-based enzyme electrode for ORR as a cathode, with glucose-oxidizing anodes, based on GDH enzymes and tetherable osmium redox complexes. Combination of the PQQGDH-based enzyme electrode as anode with the SLac-based enzyme electrodes as cathode in a membrane-less EFC provides maximum power density 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. Future work will focus on the screening of combinations of redox complexes, nanostructured materials and
sugar oxidizing and oxygen reducing enzymes to improve power output, sufficient to power implantable devices.

3.6 Acknowledgements

We thank Novozymes A/S for the preparation of the *Streptomyces coelicolor* laccase. This work was supported by an ERA-Chemistry award through the Irish Research Council, a EU-FP7 programme “3D-nanobiodevice” (FP7 NMP4-SL-2009-229255) and a Charles Parsons Energy Research Award by Science Foundation Ireland.
3.7 References

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Coupling osmium complexes to epoxy-functionalised polymers to provide mediated enzyme electrodes for glucose oxidation

Peter Ó Conghaile a, Sascha Pöller b, Domhnall MacAodha a, Wolfgang Schuhmann b, Dónal Leech a*. Biosensors and Bioelectronics, Volume 405, Number 11, pp. 3807–3812, April 2013.

Co-author contributions:

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

Sascha Pöller contributed towards the synthesis of the polymers and the subsequent coupling to osmium complexes.

Domhnall MacAodha contributed advice during the laboratory work.

Dónal Leech and Wolfgang Schuhmann, as joint project supervisors, contributed through guidance and advice throughout and Dónal Leech wrote the final draft of the publication.
Coupling osmium complexes to epoxy-functionalised polymers to provide mediated enzyme electrodes for glucose oxidation

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4.1 Abstract
Newly synthesised osmium complex-modified redox polymers were tested for potential application as mediators in glucose oxidising enzyme electrodes for application to biosensors or biofuel cells. Coupling of osmium complexes containing amine functional groups to epoxy-functionalised polymers of variable composition provides a range of redox polymers with variation possible in redox potential and physicochemical properties. Properties of the redox polymers as mediators for glucose oxidation were investigated by co-immobilisation onto graphite with glucose oxidase or FAD-dependent glucose dehydrogenase using a range of crosslinkers and in the presence and absence of multiwalled carbon nanotubes. Electrodes prepared by immobilising \([P20-Os(2,2′-bipyridine)2(4-aminomethylpyridine)Cl].PF_6\), carbon nanotubes and glucose oxidase exhibit glucose oxidation current densities as high as 560 \(\mu \text{A cm}^{-2}\) for PBS containing 100 mM glucose at 0.45 V vs Ag/AgCl. Films prepared by crosslinking \([P20-Os(4,4′-dimethoxy-2,2′-bipyridine)2(4-aminomethylpyridine)Cl] .PF_6\), an FAD-dependent glucose dehydrogenase, and carbon nanotubes achieve current densities of 215 \(\mu \text{A cm}^{-2}\) in 5 mM glucose at 0.2 V vs Ag/AgCl, showing some promise for application to glucose oxidising biosensors or biofuel cells.

4.2 Introduction
Advancements in the field of microelectronics, biochemistry and electrochemistry have inspired an increased interest in enzyme based bioelectronics because of their potential applications in healthcare. Enzyme electrodes can operate as biosensors to detect and monitor specific substrates and subsequent changes in a physiological environment (Heller, 1999), and as a source of power for intelligent
implantable devices (Willner et al., 2009; Barton et al., 2004). Enzymatic fuel cells (EFCs) work on the same principles as the hydrogen/oxygen fuel cell except biological catalysts are utilised in place of expensive metal catalysts at the anode and cathode (Moehlenbrock and Minteer, 2008; Leech et al., 2012). Due to the versatile nature of the biocatalysts, EFCs are not limited to hydrogen as a fuel and can derive power from a wide range of organic substrates. EFCs use isolated enzymes, derived from microorganisms, to catalytically oxidise a specific fuel at the anode and reduce the oxidant, usually oxygen, at the cathode thus allowing miniaturisation through the removal of the membrane.

In the last 20 years enzyme electrodes based on mediated electron transfer within redox hydrogels have been widely reported on (Taylor et al., 1995; Mao et al., 2003; Ackermann et al., 2010). Redox hydrogels envelope the redox enzymes and electrically wire the enzyme redox active centres to electrodes irrespective of spatial orientation. This offers greater attainable current densities of enzyme substrate electro-oxidation, or electro-reduction, compared to enzyme monolayers packed onto two-dimensional electrode surfaces, where the charge transport to the electrode is limited by both the distance of the enzyme to the electrode and the orientation of the active site with respect to electrode (Marcus and Sutin, 1985). The redox polymers in these systems are mostly based upon coordinative binding of imidazole or pyridine monomer of a poly(N-vinylimidazole) (PVI) or poly(4-vinylpyridine) to an osmium complex, thus lacking functional variability. A recent alternate approach proposes co-polymerisation of different acrylate or methacrylate based monomers which facilitates rational development of a range of polymers to optimise the polymer and biomolecule interaction or to include functional groups for covalent binding, deposition and/or crosslinking (Guschin et al., 2006, 2009, 2010; Shao et al., 2012). There have been a wide range of studies conducted aimed at integrating enzyme electrode components to provide for high signal intensity and stability. One approach involves inclusion of nanoparticles within enzyme film on electrodes, where increases in current and enhancement of stability in mediated systems have been reported for both biosensor (Antiochia and Gorton, 2007; Cui et al., 2009) and EFC applications (Jenkins et al., 2012a & b; Yan et al., 2006; Zebda et al., 2011). Others report on methods to trap the biocatalyst at the electrode surface, within the hydrogel, without affecting its activity, such as the covalent grafting of enzymes on surface functionalised
electrodes (Pellissier et al., 2008), the use of crosslinkers to bond the biocatalyst with the redox polymer chains in macroporous structures (Boland and Leech, 2012), or employing layer-by-layer techniques (Rengaraj et al., 2011). The use of a diepoxide or dialdehyde crosslinker for enzyme and redox polymer co-immobilisation to promote film stability has been widely reported, and has been shown to result in an increase in current signal and enzyme stability over films prepared with no addition of crosslinker (Sirkar et al., 2000).

Recent reviews (Heller, 1999; Barton et al., 2004; Leech et al., 2012) which compare glucose-based enzyme electrode performances in terms of glucose oxidation current density, and operational conditions, provide comparative guides for the systems examined here. In addition to high current signals, of good stability, the selected mediator redox potential plays an important role in defining the operational cell voltage of an EFC. Targeted design of transition metal redox mediators to provide redox potentials close to those of enzyme active sites has been undertaken by synthesis of complexes using a range of electron-donating/withdrawing substituents on metal ligands, traditionally 2,2′-bipyridine ligands (Kavanagh and Leech, 2004). Previous methodology used in the synthesis of Os-complex containing redox polymers used ligand exchange reactions of the labile chloro ligands for ligands attached to a polymer backbone (Taylor et al., 1995; Mao et al., 2003; Ackermann et al., 2010). Selection of monomers in the synthesis of co-polymers can be varied to result in polymers exhibiting desirable properties such as solubility and availability of functional groups (Ngounou et al., 2009; Ackermann et al., 2010; Guschin et al., 2006, 2009, 2010). An alternate underexploited approach, sometimes termed the “covalent binding approach” (Pöller et al., 2012), is for Os-complexes bearing ligands possessing a functional group to be covalently coupled to a suitable polymer backbone. This approach was initially reported on using covalent attachment of an osmium complex to polyallylamine (PAA) by condensation of the aldehyde function of [Os(2,2′-bipyridine)₂(pyridine-4-aldehyde)Cl]⁺ with the amine of PAA (Danilowicz et al., 1998a & b).

Here we report on the preparation of redox polymers based on coupling Os-complexes with ligands possessing an amine functional group distal to the Os metal to a pre-synthesised co-polymer possessing epoxy functional groups. The redox polymers are assayed with regard to ability to form films on graphite
electrodes capable of mediating electron transfer from co-immobilised glucose oxidising enzymes, for application to biosensors and biofuel cells.

4.3 Material and methods

4.3.1 Chemicals and materials

All chemicals and biochemicals were, unless otherwise stated, purchased from Sigma-Aldrich and used as received. All solutions are made from Milli-Q (18.2 MΩ cm) water unless otherwise stated. Purification of multiwalled carbon nanotubes (MWCNTs) (Sigma-Aldrich) was by stirring under reflux in nitric acid for 6 hrs and isolation by filtration. Glucose oxidase (GOx) was sourced from Sigma, while an FAD-dependent glucose dehydrogenase (*Aspergillus* sp., FADGDH, EC 1.1.99.10) was sourced from Sekisui Diagnostics, product GLDE-70-1192.

Starting complexes of *cis*-Os(bpy)$_2$Cl$_2$ or *cis*-Os(dmobpy)$_2$Cl$_2$, where bpy is 2,2′-bipyridine and dmobpy is 4,4′-dimethoxy-2,2′-bipyridine, are synthesised from (NH$_4$)$_2$OsCl$_6$ according to literature methods (Kober *et al.*, 1988; Forster and Vos, 1990). Ligand substitution of a chlorine ligand of the resulting complexes by 4-(aminomethyl)pyridine (4-AMP) is achieved by heating an ethylene glycol solution of the ligand and complex at reflux, with precipitation of the complex by addition of an aqueous NH$_4$PF$_6$ solution. The UV-visible spectra of methanolic solutions of these osmium complexes exhibit the expected broad transitions between approximately 460 and 510 nm (Meyer, 1986; Forster and Vos, 1990). IR transitions at: 3500-3350 cm$^{-1}$ (-NH$_2$ stretch), 1605 cm$^{-1}$ (N-H bend), 2960 cm$^{-1}$ (C-H stretch), 1420 cm$^{-1}$ (C-H bend), 1500 cm$^{-1}$ (aromatic rings). Microanalysis results for [Os(dmobpy)$_2$(4-AMP)Cl].PF$_6$ with 2 waters of hydration OsC$_{30}$H$_{36}$N$_6$O$_6$ClPF$_6$ calculated: C, 38.04%; H, 3.83%; N, 8.87%. Found: C, 37.22%; H, 3.02%; N, 8.24% and for [Os(bpy)$_2$(4-AMP)Cl].PF$_6$ with 2 waters of hydration OsC$_{26}$H$_{28}$N$_6$O$_2$ClPF$_6$: calculated: C, 37.75%; H, 3.41%; N, 10.61%. Found: C, 38.08%; H, 2.53%; N, 9.65%. Os content by thermochemical analysis: [Os(dmobpy)$_2$(4-AMP)Cl].PF$_6$ calculated: 20.88% and found: 22.0% [Os(bpy)$_2$(4-AMP)Cl].PF$_6$ calculated: 24.04% and found: 20.6%. ESI-MS: [Os(dmobpy)$_2$(4-AMP)Cl].PF$_6$ [M+CH$_3$CN]$^+$: 808.2; [Os(bpy)$_2$(4-AMP)Cl].PF$_6$ [M]$^+$: 647.6.
4.3.2 Synthesis of copolymer P20, P21, P22, P24

Polymerisation was carried out by free radical polymerisation as described previously (Pöller et al., 2012) by varying monomer component and amounts of allyl methacrylate (AllMA), polyethylene glycol methacrylate (PEGMA) (Mw=526 g mol\(^{-1}\)), butyl acrylate (BA), methacrylic acid (MAA) or dimethylamino ethyl methacrylate (DMAEMA) dissolved in isopropanol to give polymers P20, P21, P22 and P24: Scheme 1. Monomer concentrations of AllMA and PEGMA are kept constant at 50 and 7.5 mol\% for all polymers. The P20 polymer contains 10% MAA, so that it is a negatively charged polymer in neutral pH solutions, whilst P21, 22 and 24 polymers contain DMAEMA instead of MAA, making them positively charged under the same conditions. The mol % of BA is the same for P20 and P22 at 32.5% whilst it is 37.5% for P21 and 22.5% for P24, with the differences achieved by adjusting the mol% of DMAEMA for P21, 22 and 24 to 5%, 10% and 20%, respectively. Integration of \(^1\text{H}-\text{NMR}\) (200 MHz, MeOD-d\(_4\), 300 K) peaks for P20 at x (ppm) = 5.97 (br, 1H, CH); 5.44 – 5.25 (br, 2H, CH\(_2\)); 4.51 (br, 2H, CH\(_2\)); 4.21 - 3.92 (br, 2H, CH\(_2\)); 3.68 (br, 2H, CH\(_2\)) 2.22 – 0.84 (br, -CH\(_2\)CH\(_3\)) confirms a polymer composition similar to the monomer mixture used (Pöller et al., 2012).

4.3.3 Synthesis of redox polymers

Dimethyldioxirane (DMDO) (0.1 M in acetone) was prepared, and used for epoxidation of all polymers, using a procedure described previously (Pöller et al., 2012). Briefly, DMDO (2.5 mL) is added to polymer suspensions (25 mg) and allowed to react at 4°C for 16 hrs. The solvent is then removed in vacuo and the resulting epoxy-polymer redissolved in isopropanol (3.0 mL).

Subsequently an [Os(bpy)\(_2\)(4-AMP)Cl].PF\(_6\) or [Os(dmobpy)\(_2\)(4-AMP)Cl].PF\(_6\) complex is added (5.0 mg) to the polymer solution and the reaction mixture stirred for 3 days at 50°C. The crude polymer is precipitated from diethyl ether and separated by centrifugation (3500 rpm, 10 min) to give the redox polymer, see scheme 1.
Scheme 1: Proposed synthesis route for the redox polymers. Where N-N is bpy or dmobpy and R is hydrogen (P20, indicating MAA as a monomer) or N,N-dimethyl-ethanamine (P21, P22, P24, indicating DMAEMA as a monomer).
4.3.4 Methods

Electrospray ionization mass spectrometry was performed on a Waters LCT Premiere XE. IR spectra were recorded using a Perkin Elmer Spectrum 400 FT-IR. Elemental microanalysis was carried out on a Perkin Elmer Series II 2400 and UV-Vis spectra were obtained using an Agilent 8453E UV-visible spectrophotometer. Thermochemical analysis performed using a Rheometric Scientific STA 625. All electrochemical measurements were performed using a CH Instruments 1030 series potentiostat, coupled to a 100 mL electrochemical cell containing an Ag/AgCl reference electrode (3M KCl) (Bioanalytical Systems), a platinum foil counter electrode (Goodfellow) and working electrodes in phosphate buffered saline (PBS: 0.05 M phosphate, pH 7.4; 0.15 M NaCl) at room temperature. For working electrodes, graphite disc electrodes (3 mm diameter) were prepared by shrouding graphite rods (Graphite store) in heat-shrinkable tubing. Solution was stirred at 150 rpm for amperometry measurements.

Electrodes were modified by drop-coating a mixture of aqueous solutions of redox polymer and enzyme. For the CNT systems, 0.4 mg of purified MWCNTs was incorporated into the redox polymer deposition solution. Crosslinking was achieved by addition of 2,2’-(ethylenedioxy)bis(ethylamine) (EDBE) or poly(ethylene glycol) diglycidyl ether (PEGDGE) (M_n= 526) freshly prepared aqueous solution to the dropcoat on electrodes, and curing at room temperature for 24 hours, in line with published data (Lehr et al., 2010), or by exposure of the film on electrodes, previously dried for 23.5 hours, to glutaraldehyde (GA) vapours in a sealed headspace for 30 minutes, as previously reported (MacAodha et al., 2012). The GA treated electrodes were then immersed in NaBH₄ for ~5 seconds and subsequently rinsed in Milli-Q water to reduce the Schiff base formed upon crosslinking to the more stable amine (Gacesa and Whish, 1978; Chaikin and Brown, 1949). Testing commenced within 20 minutes of the completion of electrode preparation.
4.4 Results and discussion

4.4.1 Os-complex redox polymers

Redox polymers were synthesised using the “covalent binding approach” as described recently (Pöller et al., 2012), by covalent coupling of an osmium redox complex to a pre-synthesised copolymer, as shown in Scheme 1. In this approach osmium polypyridyl complexes are first prepared by co-ordination of ligands that have an amine functional group distal to the osmium co-ordination site (pyridine nitrogen) of the ligand. Introduction of the monomer allyl methacrylate into the polymer synthesis results in a co-polymer with available allylic double bonds, which are readily converted to reactive epoxides. The alkyl amine-functionalised osmium complex can then be covalently attached to the polymer backbone by reaction with the epoxide functional group on the polymer. Inclusion of a range of other monomers, such as PEGMA, BA, DMAEMA and MAA, in the synthesis of co-polymers presents other functional groups available for alternative approaches of immobilisation and crosslinking (of redox complex and enzyme, and coupling to suitably derivatised electrodes) and variation in film properties (swelling, pH dependence of charge). In this study we elected to focus on variations in charge and hydrophobicity of the copolymers, thus the content of AllMA (50.0 mol-%) and PEGMA (7.5 mol-%) was kept constant for all the polymer backbones used in this study, while the content of the other monomers was altered resulting in polymer backbones P20, P21, P22 and P24. The P20 polymer contains MAA, which enables formation of stable suspensions of polymer in neutral to basic buffers, while for P22 MAA was replaced by DMAEMA to achieve stable suspensions in neutral to acidic buffers. Additionally the overall hydrophobicity of the polymer backbone was decreased from P21 to P22 to P24, by including stepwise more DMAEMA in the polymer backbone.

Cyclic voltammetry (CV) of films prepared on graphite electrodes by deposition of glucose oxidase and osmium polymers based on the [Os(bpy)$_2$(4-AMP)Cl]$^+$ redox complex attached to P20, P21, P22 and P24 polymers in the absence of substrate exhibit well-defined symmetrical oxidation and reduction peaks corresponding to the Os$^{2+}$/$^{3+}$ transition, occurring at an $E^{o'}$ of 0.3 V (vs. Ag/AgCl). This potential is similar to that reported for the Os$^{2+}$/$^{3+}$ transition in films of [Os(bpy)$_2$(4-AMP)Cl]$^+$ covalently coupled to graphite electrodes (Boland,
Kavanagh, et al., 2008; Boland, Barrière, et al., 2008). The slow scan CVs of films of the osmium polymers and glucose oxidase in 0.05 M phosphate buffer, pH 7.4, show a peak-to-peak separation ($\Delta E_p$) of $\sim 28$ mV and a full width at half-maximum (FWHM) of $\sim 125$ mV (Figure 1(A)). These values deviate slightly from the expected theoretical values of 0 mV and 90 mV (Bard and Faulkner, 2000), respectively, for a surface confined one electron reversible redox couple. Such deviations have been ascribed previously to either lateral interactions between the redox complexes within the film (Brown and Anson, 1977), or of the redox complexes displaying a range of redox potentials (Rowe et al., 1995) or heterogeneous electron transfer rate constants within the film (Honeychurch and Rechnitz, 1998). At slow scan rates ($<20$ mV s$^{-1}$) the oxidation and reduction peak currents scale linearly with scan rate (Figure 1(A) inset). The surface coverage of osmium redox active sites ($\Gamma_{Os}$) can be determined by integrating the faradaic current passed during complete electrolysis of the thin film. Integration of the area under the anodic peak of the slow scan CVs provides an estimate of $8.5 \times 10^{-9}$ mol cm$^{-2}$ for $\Gamma_{Os}$, two orders of magnitude higher than that required for complete, close-packed, monomolecular coverage (Forster and Faulkner, 1994) of $7.7 \times 10^{-11}$ mol cm$^{-2}$ of a similar polypyridyl Os complex, indicating the formation of three-dimensional films on the surface.

4.4.2 Ratio of redox polymer to enzyme

Reports on enzymes co-immobilised within redox polymer hydrogel films on electrodes have been the subject of previous studies with a focus on optimisation of the components within the film matrix, for the intention of obtaining maximum current output, by varying the mass of enzyme against that of the osmium redox polymer (Ohara et al., 1993; Barton et al., 2001). A similar approach was therefore initially taken with the novel functionally versatile redox polymers, prepared by coupling of osmium complexes to the co-polymers synthesised. Due to being readily soluble, model studies focus on co-immobilisation of the [P20-Os(bpy)$_2$(4-AMP)Cl].PF$_6$ and GOx onto graphite electrodes, to evaluate optimal ratios of components that will deliver highest catalytic currents for glucose oxidation.
Slow scan cyclic voltammograms in the presence of glucose for films of GOx co-immobilised with [P20-Os(bpy)$_2$(4-AMP)Cl].PF$_6$ on graphite disk electrodes show sigmoidal shaped curves, providing steady-state glucose oxidation currents above potentials for the Os(II/III) redox transition, indicative of EC' electrocatalysis of glucose, Figure 1(B). The magnitude of the steady-state oxidation currents as a function of the amount of redox polymer in the presence of 5 mM (physiologically relevant) and 100 mM (excess) glucose concentrations within the phosphate buffer saline solution was highest for those films prepared using a 1:1 mass ratio of [P20-Os(bpy)$_2$(4-AMP)Cl].PF$_6$ to GOx, Figure 2, and is selected for all further optimisation tests. This ratio is similar to results recorded for PVI-based osmium redox polymer films crosslinked with GOx using a PEGDGE diepoxide crosslinker (MacAodha et al., 2012).
Figure 1: Slow scan (1 mV s\(^{-1}\)) CVs of films of [P20-Os(bpy)\(_2\)(4-AMP)Cl].PF\(_6\) and GOx on graphite electrodes in (A) absence of glucose and (B) presence of 5 mM (black) or 100 mM (grey) glucose in 50 mM pH 7.4 phosphate buffer solution, 0.15 M NaCl at room temperature.
4.4.3 Multiwalled carbon nanotube addition

The addition of nanoparticles, such as CNTs, within an enzyme electrode film or matrix has been reported on by many research groups in recent years (Tsai et al., 2009; Tran et al., 2011; Zhu et al., 2011; Tasca et al., 2008). The inclusion of CNTs has consistently shown to have had a beneficial effect, by producing a substantial current increase over films without CNTs, while simultaneously enhancing, in some reports, the observed stability of the film. A comparative study of films prepared using sample redox polymers, with and without the inclusion of multiwalled carbon nanotubes, was performed to investigate whether such an approach can improve current densities for glucose oxidation using the new redox polymers.

Films prepared by deposition of solutions of GOx, [P20-Os(bpy)$_2$(4-AMP)Cl].PF$_6$, and MWCNT dispersions demonstrated increased glucose oxidation currents over those prepared without MWCNTs (Table 1). For example, in the presence of 5 mM glucose a 5 fold increase is observed (Figure 3) for films prepared using MWCNTs, GOx and [P20-Os(bpy)$_2$(4-AMP)Cl].PF$_6$ versus those

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**Figure 2:** Dependence of normalised 5mM glucose oxidation current response on the proportion of [P20-Os(bpy)$_2$(4-AMP)Cl].PF$_6$ in redox polymer/GOx films on graphite electrodes (n=3). Other conditions as in Figure 1.
prepared using only GOx and [P20-Os(bpy)_2(4-AMP)Cl].PF_6. Such an increase in glucose oxidation current upon inclusion of CNTs into polyvinylimidazole-based osmium redox polymer and glucose oxidase films on electrodes has been reported previously (MacAodha et al., 2012; Joshi et al., 2005). Since the osmium surface coverage for films with and without MWCNTs (8.8 nmol cm\(^{-2}\) and 8.5 nmol cm\(^{-2}\), respectively) is very similar, it is probable that inclusion of MWCNTs allows either a greater retention of enzyme within the film, or possibly greater proportion of enzyme to be wired to the electrode.

**Figure 3**: Slow scan, 1 mVs\(^{-1}\) CVs of films of [P20-Os(bpy)_2(4-AMP)Cl].PF_6 and GOx with (black) and without (grey) added MWCNTs recorded in the absence (line) and presence (dash) of substrate (5 mM glucose) in 50 mM pH 7.4 phosphate buffer solution, 0.15 M NaCl at room temperature.

**4.4.4 Crosslinking agents**

An advantage to the use of functionally versatile polymers is the ability to investigate a range of film crosslinking strategies to achieve high current densities for glucose oxidation. Films of [P20-Os(bpy)_2(4-AMP)Cl].PF_6, GOx and MWCNTs were thus immobilised on graphite electrodes and crosslinked by ring-
opening reactions of the remaining epoxide functional groups of the polymer. A comparison of the response of these films to that observed for electrodes prepared by deposition of the same films with additional crosslinking by diepoxide, dialdehyde or diamine cross-linkers was undertaken. The crosslinkers are capable of reacting with the remaining epoxy groups on the polymer (diamine), available enzyme functional groups (primary and secondary amines reacting with dialdehyde and diepoxide) as well as secondary amines on the redox polymers (dialdehyde and diepoxide) (de Lumley-Woodyear et al., 1995; Habeeb and Hiramoto, 1968).

![Graph](image)

**Figure 4**: Response of films prepared with [P20-Os(bpy)$_2$(4-AMP)Cl].PF$_6$, Gox, MWCNTs and the crosslinking agents PEGDGE (▲), GA (●), EDBE (■), control: no additional crosslinker (♦). Current increments from amperometry at 0.45 V for in 50 mM pH 7.4 phosphate buffer solution 0.15 M NaCl at room temperature, 150 rpm.

Amperometric glucose oxidation at 0.45 V vs. Ag/AgCl, approximately 0.15 V more positive than the formal redox potential of the [P20-Os(bpy)$_2$(4-AMP)Cl].PF$_6$ redox polymer, was monitored as a function of glucose concentration to compare the effectiveness of the crosslinking methodologies,
Figure 4. Results similar to each other, in terms of osmium surface coverages and glucose response, are observed for the films prepared without any additional crosslinking step (control) and those with a diamine (EDBE) added for additional crosslinking. Films prepared with additional crosslinking by dialdehyde (GA) and diepoxide (PEGDGE) give double the $\Gamma_{\text{Os}}$ in comparison to films prepared without an additional crosslinking step. Such films also result in higher glucose oxidation current, for the same glucose concentrations, than the control or EDBE crosslinked films, indicating that retention of an increased amount of osmium and/or increased retention, or addressing, of GOx (*vide infra*) within the film results in an increase in glucose response. Maximum glucose oxidation currents ($i_{\text{max}}$) can be estimated assuming Michaelis-Menten behaviour of the films. Such an approach results in $i_{\text{max}}$ of 560 $\mu$A cm$^{-2}$ for PEGDGE crosslinked films compared to lower $i_{\text{max}}$ of 310 $\mu$A cm$^{-2}$ to 390 $\mu$A cm$^{-2}$, for the control, GA and EDBE films (from results in figure 4).

Although adequate for a crosslinker study, the $[\text{P20-Os(bpy)}_2(4$-$\text{AMP})\text{Cl}]\text{PF}_6$ redox polymer is not particularly well suited as an anodic mediator due to its relatively high redox potential which contributes to thermodynamic losses in an EFC (Jenkins *et al.*, 2009). Therefore a comparative study was conducted using initial osmium redox complexes based on dmobpy, rather than bpy attached to the P-20 polymer (see scheme I). Such redox polymers display lower Os(II/III) redox potentials ($E^0'= 80$ mV vs. Ag/AgCl) and their use as catalysts in a bioanode provides a larger anodic to cathodic potential difference for future assembled EFCs. Electrodes were prepared, using the composition of components optimised for the $[\text{P20-Os(bpy)}_2(4$-$\text{AMP})\text{Cl}]\text{PF}_6$ system, by depositing solutions of $[\text{P20-Os(dmobpy)}_2(4$-$\text{AMP})\text{Cl}]\text{PF}_6$, GOx and MWCNTs, with subsequent additional crosslinking by all four approaches (control, PEGDGE, GA and EDBE). Slow scan cyclic voltammograms of films of $[\text{P20-Os(dmobpy)}_2(4$-$\text{AMP})\text{Cl}]\text{PF}_6$ ($E^0'= 80$ mV vs. Ag/AgCl) or $[\text{P20-Os(bpy)}_2(4$-$\text{AMP})\text{Cl}]\text{PF}_6$ ($E^0'= 300$ mV vs. Ag/AgCl) co-immobilised with MWCNT, GOx and additional crosslinking by PEGDGE are shown in Figure 5, where the difference in redox potentials for oxidation of the redox complexes in the polymers is apparent, in the absence of glucose. Upon addition of glucose, sigmoidal-shaped cyclic voltammograms, characteristic of catalytic currents, are obtained.
Films prepared using [P20-Os(dmobpy)$_2$(4-AMP)Cl].PF$_6$ deliver steady-state glucose oxidation catalytic currents threefold lower, estimated either from slow scan CV (Figure 5) or constant potential amperometry at 0.2 V vs. Ag/AgCl, than those obtained using the [P20-Os(bpy)$_2$(4-AMP)Cl].PF$_6$ redox polymer, at 0.45 V vs. Ag/AgCl. This lower current may be attributable to a lower thermodynamic driving force and/or differences in the physicochemical properties of the immobilised films on the electrode (swelling, charge transport, etc.) (Zafar et al., 2012; MacAodha et al., 2012). For all other [P20-Os(dmobpy)$_2$(4-AMP)Cl].PF$_6$ modified electrodes (control, EDBE, GA crosslinked) amperometric glucose oxidation currents, recorded at a potential of 0.2 V vs. Ag/AgCl, are approximately an order of magnitude lower than those observed under the same conditions for electrodes prepared using the [P20-Os(bpy)$_2$(4-AMP)Cl].PF$_6$ redox polymer, recorded at a potential of 0.45 V vs. Ag/AgCl. An osmium surface coverage of approximately 10 nmol cm$^{-2}$ is obtained for all films prepared using [P20-Os(dmobpy)$_2$(4-AMP)Cl].PF$_6$, indicating that it is not merely the osmium
coverage that affects the glucose oxidation current response. When the glucose concentration is varied, Figure 6, slightly lower glucose $K_M$ values (~1.5 mM), to those estimated for the [P20-Os(bpy)$_2$(4-AMP)Cl]PF$_6$ based films (~5 mM) are obtained. The estimated maximum glucose oxidation current, assuming Michaelis-Menten behaviour, is once again highest for the films prepared with additional crosslinking by PEGDGE, 120 $\mu$A cm$^{-2}$, compared to other approaches (GA $i_{\text{max}}$ of 54 $\mu$A cm$^{-2}$, EDBE $i_{\text{max}}$ of 18 $\mu$A cm$^{-2}$, control $i_{\text{max}}$ of 27 $\mu$A cm$^{-2}$). Because all of the films retain the same amount of osmium, the observed higher glucose response for PEGDGE crosslinked films may be because of increased retention, or addressing, of GOx within these films compared to other approaches.

![Figure 6](image-url)

**Figure 6**: Response of films prepared with [P20-Os(bpy)$_2$(4-AMP)Cl]PF$_6$, GOx, MWCNTs and different crosslinking agents. PEGDGE (▲), GA (●), EDBE (■), control: no additional crosslinker (♦) to glucose. Current increments from amperometry at 0.2 V in 50 mM pH 7.4 phosphate buffer solution, 0.15 M NaCl at room temperature, 150 rpm.
4.4.5 FAD-dependent glucose dehydrogenase

A comparison of electrodes ([P20-Os(dmobpy)$_2$(4-AMP)Cl].PF$_6$, PEGDGE, MWCNTs) prepared using an oxygen independent FAD-dependent glucose dehydrogenase with those prepared using GOx was undertaken in an attempt to mitigate against known issues with use of GOx, because of competition between redox mediator and oxygen, decreasing anodic currents for glucose oxidation and producing toxic hydrogen peroxide (Mano et al., 2005; Jenkins et al., 2011a & b) in biosensors and membrane-less glucose/oxygen enzymatic fuel cells. A 2 fold increase in estimated maximum glucose oxidation current, assuming Michaelis-Menten behaviour, is obtained for films prepared with FADGDH, compared to films prepared with GOx, Figure 7. Similar osmium surface coverages were recorded of ~13 nmol cm$^{-2}$ for both GOx and FADGDH immobilised films with [P20-Os(dmobpy)$_2$(4-AMP)Cl].PF$_6$, PEGDGE and MWCNTs, indicating that more effective immobilisation and addressing of the enzyme contributes to increased current output for the FADGDH films.

![Figure 7](image_url)

**Figure 7:** Response of films containing GOx (●) and FADGDH (■) crosslinked using PEGDGE with P20-[Os(dmobpy)$_2$(4-AMP)Cl].PF$_6$ with added MWCNTs. Current increments from amperometry at 0.2 V in 50 mM pH 7.4 phosphate buffer solution, 0.15 M NaCl at room temperature, 150 rpm.
4.4.6 Polymer composition variation

In order to evaluate the effect, if any, of polymer composition on glucose oxidation signals for films of redox polymer and enzymes, a comparison of electrodes prepared using the optimized ratio of film deposition component (redox polymer, FADGDH and MWCNT) and PEGDGE as an additional crosslinker, using [Os(dmobpy)2(4-AMP)Cl].PF6 bound to all four of the polymers prepared (P20, P21, P22, P24), was undertaken. These enzyme electrodes studied in pseudo-physiological conditions give catalytic current densities ranging from 215 to 115 µA cm\(^{-2}\), which were obtained from amperometric glucose oxidation currents recorded at a potential of 0.2 V vs Ag/AgCl (Figure 8). The P20 redox polymer enzyme electrode yields the highest glucose oxidation current density under these conditions. The P20 polymer is the only polymer selected that is negatively charged, under the conditions used (physiological pH). Replacement of the MAA monomer in P20 by the positively charged quaternary ammonium monomer, DMAEMA, to yield P22 gives a decreased glucose oxidation current for the resulting enzyme electrode. Variation of the ratio of DMAEMA to BA in the polymer synthesis, with an increased (P24) or decreased (P21) amount of DMAEMA, results in further decreases in glucose oxidation currents for the enzyme electrodes. Although comparison to results obtained by others using redox polymer films for mediation in enzyme electrodes is problematic, due to differences in film preparation techniques and subsequent testing, the glucose oxidation current densities obtained in this study compares well with that reported for other mediated enzyme electrodes. For example, glucose oxidation current densities ranging from 100 to 200 µA cm\(^{-2}\) can be estimated from the reported amperometric flow cell responses in 5 mM glucose, pH 7.4 solutions at films of various FAD-dependent GDHs co-immobilised with a redox polymer of an [Os(4,4’-dimethyl-2,2’-bipyridine)2Cl] complex co-ordinatively bound to PVI (Zafar et al., 2012). Rengaraj et al. report a glucose oxidation current density of 500 µA cm\(^{-2}\) in the presence of 100 mM glucose for films of Gox co-immobilised with an [Os(dmobpy)2Cl] complex co-ordinatively bound to a PVI (Rengaraj et al., 2011).
Figure 8: Current densities for films of FADGDH crosslinked with polymer-[Os(dmobpy)$_2$(4-AMP)Cl].PF$_6$ with added MWCNTs recorded at 0.2 V in 5 mM glucose in 50 mM pH 7.4 phosphate buffer solution 0.15 M NaCl at room temperature, 150 rpm.

4.5 Conclusion

Novel redox polymers were successfully developed by attachment to the polymer via epoxide ring opening of a series of osmium complexes using the “covalent binding approach”, offering greater versatility in crosslinking strategies to promote film stability. These newly synthesised Os-complex modified redox polymers were tested for application to biosensors and biofuel cells by incorporating them with a glucose oxidising enzyme, cross-linker, and multiwalled carbon nanotubes into films on graphite surfaces. Enzyme electrodes prepared with [P20-Os(bpy)$_2$(4-AMP)Cl].PF$_6$ and GOx exhibit current densities as high as 560 µA cm$^{-2}$ in phosphate buffer saline containing 100 mM glucose, while enzyme electrodes prepared using FADGDH and the redox polymer [P20-Os(dmobpy)$_2$(4-AMP)Cl].PF$_6$ with lower redox potential ($E^0$ = 80 mV vs. Ag/AgCl), give glucose oxidation current densities of 215 µA cm$^{-2}$ at room temperature in 5 mM glucose. The development of these novel redox polymers
allows for a wider scope to the strategies used in designing stable enzyme electrodes suitable for use as either biosensors or biofuel cells. As the redox potential of the redox polymers can be varied future work will examine their performance as mediators in anodes and cathodes of enzymatic fuel cells.

4.6 Acknowledgements

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4.7 References


Chapter 5:

Draft manuscript:

Operation of Enzymatic Fuel Cells in Physiological Buffers, Human Saliva and Blood - Towards a Self-Powered Bioelectronics Device

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I synthesised the osmium redox systems, performed the laboratory work and the analysis.
Magnus Falk contributed by providing the cathode for enzymatic fuel cell testing.
Maria Yakovleva contributed by providing details of the pyranose dehydrogenases.
Domhnall MacAodha provided advice and assistance during the laboratory work.
Cristoph Gonaus and Clemens K. Peterbauer contributed through the development, and supply, of the glucose-oxidising enzymes.
Dónal Leech, Lo Gorton and Sergey Shleev as project co-supervisors, contributed through guidance and advice throughout the study, and editing of draft manuscript.

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Chapter 5:
Operation of Enzymatic Fuel Cells in Physiological Buffers, Human Saliva and Blood - Towards a Self-Powered Bioelectronics Device

5.1 Introduction

Development of prototype biodevices with wireless capability and an ability to self-power are of major scientific and practical importance for potential applications as self-sustained medical and/or portable devices. Implantable self-powered wireless sensor-systems could allow for real-time biomedical monitoring or intervention. Development of self-contained biodevices is an interdisciplinary research field spanning scientific, computing, engineering and medical disciplines, with significant research focus and a recent rapid growth in the number of publications in this area of bioelectronics [1–5].

A glucose/O₂ powered enzymatic fuel cell (EFC) is a specific type of fuel cell that uses enzymes as catalysts to oxidise glucose at the anode and to reduce O₂ at the cathode, that when combined as a fuel cell converts chemical energy to electrical power [6–8]. However, EFCs suffer from a severe limitation: they convert only a fraction of the substrate’s chemical energy into electricity. The reason for this limitation lies in the specificity of single enzymes, which for example are not able to completely oxidise complex organic substrates such as glucose. To date most EFCs studied employ a single glucose oxidising enzyme as the biocatalyst at the anode, usually harvesting only 2 electrons of the possible 24 electrons obtainable in the complete oxidation of β-d-glucose [9, 10]. Enzyme cascade electrodes may help to overcome this limitation and prove beneficial for increasing coulombic efficiency of EFCs [9–11], where more than one enzyme is used in an enzymatic electrode film. The first EFC based on multiple stepped oxidation of a substrate was reported by Palmore et al. [12], by combining alcohol, aldehyde and formate dehydrogenase to oxidise methanol completely to carbon dioxide. More recently, Shao et al. [10] demonstrated extraction of 6 electrons in the oxidation of glucose by combining a pyranose dehydrogenase and a cellobiose dehydrogenase in an EFC anode [10].
Pyranose dehydrogenase (PDH), EC 1.1.99.29, a glycosylated extracellular oxidoreductase, can be derived from the wood-degrading fungal family *Agaricus meleagris* [13], and carries a flavin adenine dinucleotide (FAD) prosthetic group covalently bound to a polypeptide chain of the protein, and is capable of oxidising a range of non-phosphorylated sugars at their C2 and C3 carbon [13]. It has been previously reported that oxidation may occur at the C3 site, however recent studies by Tan *et al.* [14] showed that C2 is the site of oxidation of glucose by PDH [14]. PDH may belong to the glucose methanol-choline oxidoreductase family but display some properties that are not common to other members belonging to the same family. Most importantly it does not utilise oxygen as an electron acceptor, compared to, for example, pyranose oxidase. It also possesses a wider range of substrate specificity and regio-selectively which can be attributed to the unique structure of the region surrounding the flavin pocket [14, 15]. Both lack of oxygen reactivity and broad substrate tolerance make PDH an attractive enzyme for application as a bioanode for sugar-oxidising EFCs [16]. For example, Tasca *et al.* reported broad specificity and long operational stability for enzyme electrodes prepared utilising PDH from *Agaricus meleagris* with an [Os(4, 4'-dimethyl-2,2'-bipyridine)2(poly-vinylimidazole)10Cl]+ (Os(dmbpy)PVI) redox polymer [17]. Further screening of the performance of these films as a function of redox polymer was undertaken, with formal potentials ranging from −0.18 V vs. Ag/AgCl, below the potential of the bound FAD of the PDH (−0.05 V vs. Ag/AgCl) [9], to 0.25 V vs. Ag/AgCl, in order to select a redox polymer-enzyme pair with maximised catalytic current density [18]. This approach was utilised in the multiple enzyme employment construction of a glucose-oxidising EFC anode [9]. In their EFC system, PDH was co-immobilised with a cellobiose dehydrogenase (CDH) isolated from *Myriococcum thermophilum* (*Mt*CDH) within an osmium polymer matrix on electrodes in an attempt to increase the coulombic efficiency of the system, based on the fact that the reaction product of one enzyme can serve as a substrate for another. *Mt*CDH is an ascomycete enzyme which is able to catalyse the oxidation of mono-, di- and oligosaccharides at their C1 position to their corresponding lactones [19] and PDH mono- and dide-oxidises a variety of sugars at C2 and C3 positions [16, 20, 21]. By combining these enzymes it is therefore possible to gain up to 6 electrons from one substrate glucose molecule. Shao *et al.* [10] also demonstrated that
current density and coulombic efficiency of an EFC anode can be increased, over that using a single enzyme, by entrapping both PDH and the flavodehydrogenase domain of of a *Corynascus thermophilus* CDH within a Os-complex modified electrodeposition paint on electrodes [10]. By replacing the wild-type CDH, which consists of one flavodomain containing FAD and one heme domain of cytochrome b type [22, 23], with the recombinant deglycosylated (dgCDH) domain of a *Corynascus thermophilus* CDH, expressed in *Escherichia coli*, co-immobilised with PDH, makes it possible to deliver electrons to the electrode at a lower potential, close to formal potential of the FAD site instead of that of cytCDH domain [24]. The current signal was also improved postulated to be due to the smaller size and lack of glycosylation on the CDH domain, compared to that of the glycosylated variant of the enzyme [25–28], also resulting in a higher output voltage for the respective EFC when the deglycosylated enzyme was utilised.

Catalytic properties of the PDH identified from *Agaricus meleagris* and recombinantly expressed in *Pichia pastoris* were compared for the glycosylated PDH (gPDH) and a deglycosylated PDH (dgPDH) [29] and the pronounced effect of glycan depletion from the gPDH on the current output demonstrated [30]. Recently, Yakovleva *et al.* [31] reported that the dgPDH degrades to a fragmented deglycosylated PDH (fdgPDH), by loss of about 20 kDa from the C-terminal [31]. The fdgPDH has molecular mass of around 46 kDa compared to ~95 kDa and ~65 kDa for gPDH and dgPDH, respectively [31].

Here we report a comparison of the glucose oxidation response of films prepared using the previously optimised amounts of deposited components [32, 33], as a function of variants of PDH (gPDH, dgPDH and fdgPDH), to provide for greater current generation at the sugar-oxidising half-cell, for application to EFCs. By employing site directed mutagenesis it is possible to eliminate asparagine (N) residues to which glycans are attached and recombinantly express an enzyme in *Pichia pastoris* lacking part of the glycosylation. Thus a recombinant enzyme with a double mutation (dmPDH) was also investigated where asparagine 75 and asparagine 175 residues were exchanged to glycine and glutamine, respectively.

Subsequent to assessment of sugar oxidation by films of the different variants of the PDH, redox polymer and multiwalled carbon nanotubes
(MWCNT), the assembly of a series of sugar-oxidising enzyme electrodes as anodes with oxygen-reducing biocathodes is undertaken for testing under pseudophysiological conditions in buffer. The assembled EFC is designed to utilise oxygen reducing enzymes at the cathode and sugar oxidising enzymes at the anode, since the fuel and oxidant are available in different human physiological fluids, un-stimulated human saliva and human blood were also targeted for investigation of the operation of the EFCs. We compare gPDH and fdgPDH, co-immobilised with $[\text{Os}(4,4'\text{-dimethoxy-2,2'}\text{-bipyridine})_2\text{(poly-vinylimidazole)}_{10}\text{Cl}]^+$ (abbreviated as Os(dmobpy)PVI) or $[\text{Os}(4,4'\text{-dimethyl-2,2'}\text{-bipyridine})_2\text{(poly-vinylimidazole)}_{10}\text{Cl}]^+$ (abbreviated as Os(dmbpy)PVI) redox polymers and MWCNT as glucose-oxidising anodes on graphite electrodes combined with cathodes based on films of $\text{MvBOd}$ adsorbed on gold nanoparticles (AuNP) on gold electrodes (Scheme 5.1).

Finally, we report on the demonstration of the operation of self-powered biodevice capable of wireless transmission of sensing data. A suitable power supply, the glucose/O$_2$ EFC, and electronic unit together with computer control software along with a receiving USB radio unit were designed by a company, Novasense AB, to create the wireless self-powered sensing device. In order to be able to utilise the EFC as a power supply for sensors, the designed electronics require a current above 44 µA and a voltage above 0.57 V to operate. The operation of the biodevice was investigated in pseudo-physiological buffer providing power to enable wireless transmission of sensing data.
Scheme 5.1: Simplified schematic representation of membrane-less glucose/O₂ EFC operated with enzymes pyranose dehydrogenase and mediator-less MvBOD AuNP gold electrode, where the red circles represent Os redox centres for mediated electron transfer to the anode.

5.2 Experimental

5.2.1 Materials

All chemicals and biochemicals were, unless otherwise stated, purchased from Sigma-Aldrich (Ireland) and used as received. All solutions are made from Milli-Q grade water unless otherwise stated. Multiwalled carbon nanotubes (MWCNT, from Sigma-Aldrich) were purified by refluxing in nitric acid for 6 h (20 mg mL⁻¹ in HNO₃), with the treated MWCNTs isolated by filtration and washed repeatedly with distilled water until complete nitric acid removal. Synthesis of the redox polymers was achieved by adapting literature procedures [34, 35]. Human resting saliva and blood were collected from a healthy volunteer.

5.2.2 Sugar-oxidising enzymes

Glycosylated PDH (EC 1.1.99.29) [36] identified from Agaricus meleagris was recombinantly expressed in Pichia pastoris according to the previously reported procedure [29] to yield 23.8 mg mL⁻¹ (Bradford assay) protein with volumetric activity of 335 U mL⁻¹ using a ferricenium coupled glucose oxidation assay at 20 °C, as described below. Deglycosylation of the gPDH was performed by
incubation of 60 mg gPDH with 80 NEB units Endo Hf (1000000 NEB units mL\(^{-1}\), New England Biolabs, Bionordiska AB, Stockholm, Sweden) in a 124 mM sodium citrate buffer (pH 5.5) at 37 °C for 6 hours. Deglycosylated PDH was concentrated with 30 kDa Amicon ultrafiltration tube (EMD Millipore Corporation, USA), washed with 50 mM sodium phosphate buffer (pH 6.5) containing 150 mM NaCl and purified by S300 Sephacryl size exclusion chromatography (GE Healthcare, USA) according to manufacturer’s recommendations. The resulting pool was concentrated with a 30 kDa Amicon tube and washed with 50 mM sodium phosphate buffer (pH 6.5) to provide a protein content of 20.4 mg mL\(^{-1}\) (Bradford assay) and volumetric activity of ~342 U mL\(^{-1}\) using the ferricenium coupled glucose oxidation assay [37]. The dmPDH has a protein content of 11.3 mg mL\(^{-1}\) (Bradford assay) and a specific activity of 624 U mL\(^{-1}\) determined with ferricenium. The cellobiose dehydrogenase (CDH), a flavodehydrogenase domain of \textit{Myriococcum thermophilum}, has a protein content of 6.4 mg mL\(^{-1}\) (Bradford assay) and a volumetric activity of ~19.5 U mL\(^{-1}\) using the ferricenium coupled glucose oxidation assay.

5.2.3 Enzyme activity assay

The enzyme activity of PDH preparations was calculated from spectrophotometric activity assay of ferricenium reduction [38], molar absorptivity 4.3 mM\(^{-1}\) cm\(^{-1}\), using a UV-2401 PC spectrophotometer (Shimadzu Deutschland GmbH) at 20 °C. Briefly, an aliquot of the enzyme was added to a mixture of 0.1 mM sodium phosphate buffer containing 137 mM NaCl, pH 7.4, 0.05 mM D-(+)-glucose and 0.4 μM Fc\(^{+}\)PF\(_6\)\(^{-}\) (freshly prepared in 5 mM HCl) in a one millimetre cuvette and the absorbance was monitored at 300 nm. One unit of enzyme activity is equal to the amount of enzyme required for reduction of 2 μmol of Fc\(^{+}\) per minute at 20 °C.

5.2.4 Preparation of anodes

Graphite rods, with a diameter of 3.05 mm (Ringsdorff Werke GmbH, Bonn) were polished on emery paper, rinsed with Milli-Q water and sonicated for 5 min
in milli-Q water and dried at room temperature. Deposition of enzyme electrode films was achieved by pipetting 9.6 µL of a 46.25 mg mL\(^{-1}\) dispersion of acid treated MWCNT, 9.6 µL of a 5 mg mL\(^{-1}\) redox polymer aqueous solution and sufficient volume to deposit 0.048 mg of enzyme (in an aqueous solution) on the surface of the graphite electrode disk. Cross-linking treatment of the films was achieved either by addition of 1.9 µL of a freshly prepared 15 mg mL\(^{-1}\) aqueous solution of poly(ethylene glycol) diglycidyl ether (PEGDGE) (Mn = 526) and curing films at room temperature for 24 h, or by placing the enzyme electrodes in a sealed headspace of glutaraldehyde (GA) vapours for 30 min, as previously reported [32, 33]. The GA treated electrodes were subsequently immersed in 100 mM NaBH\(_4\) solution for 5 s, removed and rinsed in Milli-Q water, to reduce the Schiff bases formed upon GA crosslinking (designated henceforth as GA(r) electrodes) [39, 40]. All current densities are normalised to the geometric surface of the electrode.

5.2.5 Preparation of gold nanoparticle based \(Mv\)BO\(_d\) biocathode

Gold nanoparticles (AuNPs) were prepared by Dr. Sergey Shleev’s group following a previously reported citrate reduction procedure [41]. The AuNP size was estimated to be ~20 nm, based on the UV–Vis spectrum [42] and by SEM [43]. The AuNP were concentrated by centrifugation and 98% of the supernatant was removed. The precipitated AuNPs were re-suspended by ultrasonication and stored as a 50 times concentrated AuNPs dispersion at 4 °C. Polycrystalline gold-disk electrodes (Bioanalytical Systems, USA) were polished with 1 µm alumina slurry cleaned in Piranha solution (mixture of volumetric 3:1 concentrated sulphuric acid and 30% hydrogen peroxide solution) for 3 min and then polished with 0.05 µm alumina slurry. Any debris on the electrode was then removed by placing electrodes in a solution in an ultrasonic bath. The three-dimensional AuNP electrodes were fabricated on the gold-disk electrode by a solution casting method, as reported in previous publications [44, 45]. Briefly, 1.5 µL of concentrated AuNP dispersion was dropped onto a cleaned Au electrode surface and allowed to dry. This procedure was undertaken 3 times for each electrode. The electrodes were subsequently subjected to 20 potential cycles between -0.3
and 1.5 V vs. Ag/AgCl at a scan rate of 0.1 V s\(^{-1}\) in 0.5 M H\(_2\)SO\(_4\) until well-defined voltammograms were obtained [46]. Thereafter, the electrodes were rinsed with water and a 10 μL of a MvBOd solution (1 mg ml\(^{-1}\)) was then added to the electrode surface. The surface was covered with an eppendorf tube as to not dry out the solution and left for incubation for 2 h. The electrodes were rinsed with 50 mM phosphate buffer, pH 7.4 prior to testing.

5.2.6 Electrochemical methods

Flow injection measurements were performed with a flow-through wall-jet amperometric cell, containing a platinum wire as the counter and an Ag/AgCl (0.1 M KCl), as a reference electrode. The applied potential was controlled by a three-electrode potentiostat (Zäta Electronics, Höör, Sweden). The response of graphite rod working electrodes was registered with a recorder (BD 112, Kipp & Zonen, Utrecht, Netherlands). For the introduction of the samples an injector (Rheodyne, type 7125 LabPR, Cotati, CA, USA) with a 50 μL loop was used. All concentrations in the injected samples were corrected for the dispersion factor of the FIA system, determined to be 1.088.

Linear sweep voltammetry at 0.1 mV s\(^{-1}\) was performed with an Autolab PGSTAT 30 from Eco Chemie (Utrecht, The Netherlands) electrochemical system using graphite disks as working electrode, an Ag/AgCl reference electrode and a Pt foil as counter electrode.

5.3 Results and Discussion

Enzyme electrodes for operation as anodes are developed based on previous results [33] using mediation by Os(dmbpy)PVI or [Os(4, 4'-dimethoxy-2,2'-bipyridine)\(_2\)(poly-vinylimidazole)\(_{10}\)Cl\(^{+}\) (Os(dmobpy)PVI) redox polymers co-deposited with enzyme and MWCNT, and cross-linked on graphite disks. The electron-shuttling redox polymers are able to electrically connect the redox centres of PDH to the surface of the electrode, where electron transfer takes place by collisions between the reduced and oxidised forms of the mobile redox centres that are tethered to the polymer backbone [47, 48] (Scheme 5.2).
Scheme 5.2: Schematic representation of enzyme-based electrode with pyranose dehydrogenase and PVI-bound osmium mediator (structure presented in the inset, where R represents a methoxy or methyl group) which is electrically connected to the electrode.

The redox polymers, Os(dmobpy)PVI and Os(dmbpy)PVI, are selected for screening of mediation of glucose oxidation by co-immobilised PDH variants in films on carbon electrodes, because of their relatively low redox potentials, of −70 mV and 110 mV vs. Ag/AgCl, respectively, for eventual application to glucose-oxidising enzymatic biofuel cell anodes. The study examines the response of films of redox polymer and enzyme in the presence of MWCNT, shown to contribute to increased current densities [32, 49, 50], on graphite electrodes, using cyclic voltammetry and flow injection analysis. The relative mass of redox polymer, enzyme and MWCNT deposited are selected to match that determined to be optimal for glucose oxidation current density and stability for enzyme electrodes.
prepared from co-immobilisation of Os(bpy)PVI, MWCNT and glucose oxidase (GOx) [32].

Glutaraldehyde, in solution [51] or in the form of vapour, [32, 33, 52, 53] has been previously employed as a crosslinking agent for preparation of biocatalytic films on surfaces. However, the utilisation of glutaraldehyde solution for preparation of GOx and redox polymer films on carbon electrodes has been reported to produce significantly lower glucose oxidation current, at a glucose concentration of 48 mM, in comparison to other bifunctional crosslinkers, such as PEGDGE [51]. Recently, MacAodhá et al. [32] reported on the use of GA vapours to crosslink GOx and Os(bpy)PVI for application to biofuel cell anodes oxidising glucose, with such an approach providing high current densities for glucose oxidation, and an improved signal stability, over an approach using a diepoxide crosslinker.

Initially, a comparison of the effect of crosslinker selection on the glucose oxidation current using dgPDH output was undertaken. Films prepared with Os(dmobpy)PVI or Os(dmbpy)PVI, MWCNT and the dgPDH are crosslinked either by addition of a diepoxide crosslinker to the film deposition solution with subsequent drying of deposited film for 24 hours [32, 33], or by incubation of dried films in a chamber containing glutaraldehyde vapour, with subsequent reduction, in a NaBH₄ solution, of the imine and enamines formed [32, 39, 40]. The initial screening involved recording of slow scan cyclic voltammograms of the films in the absence of glucose substrate, for comparison of the redox response of the osmium polymer within the films, and subsequent amperometry, at an applied potential close to the redox potential for osmium oxidation, in the presence of incremental glucose concentrations, to evaluate the bioelectrocatalytic current for glucose oxidation (Figure 5.1). Conditions are chosen to simulate physiological conditions [54] using stirred 50 mM phosphate buffer saline (PBS), pH 7.4.

Enzyme electrodes prepared with crosslinking by diepoxide (PEGDGE) give slightly higher surface concentrations of osmium, \( \Gamma_{Os} \), determined from the charge passed for complete electrolysis of Os(II) sites on the electrode surface, in comparison to films prepared using the GA(r) crosslinking methodology. This is comparable to that observed for enzyme electrodes based on films of Os(bpy)PVI, GOx and MWCNT crosslinked on graphite electrodes [32]. The glucose oxidation
currents increase as a function of glucose concentration for all systems studied, Figure 5.1, with those electrodes prepared using the GA(r) methodology providing higher current densities over those prepared using the PEGDGE methodology. For example, under 5 mM glucose concentrations, selected to be similar to those pertaining for powering implanted medical devices, films prepared using Os(dmbpy)PVI and GA(r) crosslinking produce a glucose oxidation current density of 0.37 mA cm$^{-2}$ in PBS, pH 7.4 at 0.1 V vs. Ag/AgCl applied potential, compared to a current density of 0.15 mA cm$^{-2}$ for the analogous enzyme electrodes prepared by crosslinking with PEGDGE. Further screening of the performance of enzyme electrodes was therefore undertaken based on using the GA(r) crosslinking methodology.

Figure 5.1: Response of films prepared with dgPDH, MWCNT and the crosslinking agents PEGDGE – Os(dmobpy)PVI (■), Os(dmbpy)PVI (▲) and GA(r) Os(dmobpy)PVI (●), Os(dmbpy)PVI (♦). Current increments from amperometry at 0 V, for Os(dmobpy)PVI and 0.1 V, for Os(dmbpy)PVI vs Ag/AgCl (3M KCl) in PBS, pH 7.4 at room temperature, 150 rpm.
5.3.1 Deglycosylated pyranose dehydrogenase

A recent report by Yakovleva et al. [31] provides a thorough investigation into the deglycosylation of the isolated gPDH, and the subsequent stability and activity of the dgPDH. It was discovered that the dgPDH spontaneously loses a C-terminal fragment upon storage over a two month period, resulting in a fragmented dgPDH (fdgPDH) with higher specific activity of 2250 U ml\(^{-1}\) compared to the initial dgPDH. All subsequent research in this chapter therefore focused on using the highly active fdgPDH variant.

5.3.2 Effect of applied potential on fdgPDH enzyme electrode response

Selection of the potential to be applied in a flow injection assay (FIA) system was based on comparison of the normalised current density for continuous oxidation of 5 mM glucose (in PBS, pH 7.4) as a function of applied potential for enzyme electrodes prepared using the fdgPDH variant, co-immobilised with either Os(dmbpy)PVI or Os(dmobpy)PVI and MWCNTs using the GA(r) crosslinking methodology. The applied potentials for all future FIA testing were set to 0 V and 0.18 V vs. Ag/AgCl for Os(dmobpy)PVI and Os(dmbpy)PVI films, respectively, the lowest potential at which any further increase in current density is marginal, Figure 5.2.
Figure 5.2: Dependence of catalytic current of the Os(dmbpy)PVI (●) or Os(dmobpy)PVI (■) modified graphite electrodes, MWCNT and fdgPDH as a function of applied potential. Experiments were performed in 50 mM phosphate buffer at pH 7.4 containing 5 mM glucose. The flow rate was 0.5 mL min$^{-1}$.

5.3.3 Response of PDH variants to glucose

Further screening of the performance of the enzyme electrodes as a function of redox polymer and enzyme selection was undertaken by monitoring oxidation current, using FIA, under pseudo-physiological conditions of PBS containing 5 mM glucose.

In the first instance, enzyme electrodes for glucose oxidation prepared by crosslinking variants of PDH with redox polymer and MWCNT demonstrate at least a 7-fold increase in current densities over those prepared without MWCNT. Such an increase in glucose oxidation currents upon inclusion of MWCNT into polymer-bound osmium redox mediators and GOx films on electrodes has been reported previously [32, 50].
Under the pseudo-physiological conditions selected, films prepared using the gPDH variant produce the lowest glucose oxidation current densities of all variants of PDH studied, figures 5.3 and 5.4. For example current density of 30 ± 5 μA cm\(^{-2}\) is produced for 5 mM glucose oxidation in PBS by enzyme electrodes based on the gPDH variant compared to 0.41 ± 0.04 mA cm\(^{-2}\) and 0.34 ± 0.01 mA cm\(^{-2}\) using enzyme electrodes based on either the fdgPDH or dmPDH variants. The selection of constant mass (0.048 mg) of enzyme added to each electrode for film preparation results in added specific activity for glucose oxidation (using the spectrophotometric assay) of only 0.68 U for the gPDH enzyme electrodes, compared to added activity of 5.3 U for the fdgPDH and 2.7 U for the dmPDH enzyme electrodes. This alone may explain the relatively lower current density for glucose oxidation for these enzyme electrodes. For films prepared using Os(dmobpy)PVI and MWCNT the highest glucose oxidation current densities, though lower than those obtained with Os(dmbpy)PVI, are also recorded for films prepared with fdgPDH, providing 0.30 ± 0.05 mA cm\(^{-2}\), compared to those prepared with dmPDH or gPDH which produced 0.19 ± 0.02 and 0.020 ± 0.01 mA cm\(^{-2}\), respectively. A recent study [33] compared glucose oxidation current densities for films of Os(dmobpy)PVI and Os(dmbpy)PVI, each co-immobilised with an FAD-dependent glucose dehydrogenase (GDH) on graphite electrodes, that produced 0.2 and 0.8 mA cm\(^{-2}\), respectively. Ó Conghaile et al. [55] report glucose oxidation current densities of 0.3 and 0.42 mA cm\(^{-2}\), in quiescent 5 mM glucose PBS, for films of PQQ-dependent GDH and MWCNT co-immobilised with the redox complexes [Os(4,4'-dimethoxy-2,2'-bipyridine)\(_2\)(4-aminomethylpyridine)Cl]^+ and [Os(4,4'-dimethyl-2,2'-bipyridine)\(_2\)(4-aminomethylpyridine)Cl]^+, respectively.

Interestingly, enzyme electrodes employing both CDH and fdgPDH combined as glucose oxidising enzymes, capable of harvesting 6 electrons in the oxidation of β-d-glucose, provide no significant increase in glucose oxidation current densities over those obtained for films prepared using fdgPDH alone. The added specific activity for glucose oxidation for the fdgPDH, 4.5 U, is altered when co-deposited along with the added activity of 0.02 U for the CDH to ensure a constant mass (0.048 mg) of enzymes co-deposited. Further optimisation studies
of these enzyme cascades are needed, in an effort to increase coulombic efficiency and improve current generation.

Figure 5.3: Dependence of the current densities on the glucose concentration for gPDH/Os(dmbpy)PVI (■), dmPDH/Os(dmbpy)PVI (●), fdgPDH/Os(dmbpy)PVI (▲) and dgCDH/fdgPDH/Os(dmbpy)PVI (▼) modified graphite electrodes in the presence of MWCNT, measured with the FIA system in 50 mM PBS, pH 7.4, at a flow rate of 0.5 mL min$^{-1}$ at an applied potential of 180 mV vs. Ag/AgCl.
5.3.4 Fuel cells operating in pseudo-physiological buffer

Assembly of enzyme electrodes in a membrane-less fuel cell, Scheme 5.1, under pseudo-physiological conditions was implemented for selected sugar-oxidising enzyme electrodes coupled to an oxygen-reducing enzyme electrode. Enzyme electrodes chosen as anodes are based on films of Os(dmobpy)PVI or Os(dmbpy)PVI redox polymer co-deposited with gPDH or fdgPDH, with added MWCNT and crosslinked, after drying, by exposure to glutaraldehyde vapours.
with subsequent reduction using NaBH₄. The oxygen-reducing cathode consists of $Mv$BOd deposited on AuNPs at Au disk electrodes ($Mv$BOd/AuNPs/Au) prepared by Malmo University (M. Falk) and reported to produce oxygen-reducing current densities of 110 $\mu$A cm⁻² in PBS, pH 7.4 [43].

As the glucose oxidation current density is significantly higher than the ORR current of the cathode obtained for the mediatorless cathode, the cathode would limit power output if equal areas of electrode were to be used. Thus the assembled EFC consists of graphite anodes of geometric area 0.0175 cm² with films of fdgPDH co-immobilised with MWCNT and either Os(dmobpy)PVI or Os(dmbpy)PVI, capable of providing glucose oxidation currents of ~5.3 and ~7.3 $\mu$A, respectively, designed to be lower than the ~8.8 $\mu$A ORR current for the cathode consisting of $Mv$BOd with AuNPs co-immobilised at an Au electrode of geometric area of 0.08 cm². This ensures that the anode limits the current in the EFC.

**Figure 5.5:** Average power curves (n=3) with error bars, recorded by 0.1 mV s⁻¹ linear sweep voltammetry in 50 mM PBS containing 5 mM glucose for membrane-less EFCs based on $Mv$BOd on AuNPs as a cathode combined with fdgPDH co-immobilised with Os(dmobpy)PVI, MWCNT as anode.
Figure 5.6: Average power curves (n=3) with error bars, recorded by 0.1 mV s\(^{-1}\) linear sweep voltammetry in 50 mM PBS containing 5 mM glucose for membrane-less EFCs based on MvBOd on AuNPs as a cathode combined with gPDH co-immobilised with Os(dmobpy)PVI, MWCNT as anodes.

As expected, the assembled EFCs based on glucose oxidation by anodes containing fdgPDH provided higher maximum power outputs than those assembled using anodes containing gPDH, Figure 5.5 and 5.6. A similar trend is observed for EFCs using anodes based on the Os(dmbpy)PVI as redox polymer, Figure 5.7. The use of the Os(dmbpy)PVI as redox polymer in the anode provides higher maximum power density compared to their counterparts using Os(dmobpy)PVI, with the Os(dmbpy)PVI-based anodes producing maximum power densities approximately twice that produced by the Os(dmobpy)PVI-based anode analogues for both the fdgPDH and gPDH enzyme electrodes in an EFC, as expected from the comparison of steady state current densities (fig 5.3 and 5.4). The maximum power density for any of the EFC studies, of 275 ± 50 µW cm\(^{-2}\) is obtained using Os(dmbpy)PVI and fdgPDH as the anode enzyme electrode. The
observed power densities for the membrane-less EFC system based on combining macroscopic enzyme electrodes compare well the maximum power density of 145 μW cm⁻² obtained under similar conditions using films of GOx crosslinked to Os(dmbpy)PVI and *Myceliophthora thermophila* laccase crosslinked to Os(bpy)PVI on graphite electrodes with MWCNT as anode and cathode [56]. Soukharev *et al.* [57] report an EFC prototype using GOx from *Aspergillus niger* and a fungal laccase, co-immobilised with osmium redox polymers on 7 μm diameter, 2 cm long, carbon fibres, producing 350 μW cm⁻² power density in 15 mM glucose, pH 5 buffered solutions, dropping to a quarter of that maximum power, 90 μW cm⁻², when tested at 5 mM glucose concentration. Replacement of GOx from *Aspergillus niger* with a GOx from *Penicillium pinophilum* resulted in a maximum power density of 280 μW cm⁻² for the same EFC configuration operating in 5 mM glucose [58].
Figure 5.7: Power curves, recorded by 0.1 mV s\(^{-1}\) linear sweep voltammetry in 50 mM PBS containing 5 mM glucose for membrane-less EFCs based on MvBOd on AuNPs as a cathode combined with either (a) fdgPDH or (b) gPDH co-immobilised with Os(dmbpy)PVI, MWCNT as anodes.
5.3.5 Wireless transmission device system

An obvious target for applications based on enzymatic fuel cell research is for *in vivo* deployment where fuel can be withdrawn from the flow of blood to provide a power supply for small electronic devices implanted into the body. In this context, a wireless self-powered sensing device was designed and created by Novosense A/B, where an electronic circuit was constructed along with computer control software and USB radio receiver that enabled transmission of data captured by a sensor, both powered by an EFC, to a remote (~3 m) receiver. A crucial aspect in any future semi- or fully-implantable self-contained bio-device is the power supply module, with the radio transmitter being powered with an enzymatic fuel cell converting chemical energy to electrical power. However, enzymatic fuel cells of today have their shortcomings and tend to deliver very weak power and low voltages. Therefore in order for a glucose/O₂ enzymatic fuel cell to be considered a realistic possibility to prototype development and operation, the voltage needed to be ramped up to levels so that radio units can be operated along with the need to store enough charge to drive the radio signal long enough for the radio to transmit the measurement data. Southcott *et al.* [59] investigated the use of an EFC, with human serum spiked with glucose flowing over buckypaper electrodes, to power a pacemaker and demonstrated the use of a charge pump and DC–DC converter interface circuit to boost the cell voltage, so such an arrangement was used in our device. A small radio prototype with an integrated antenna and a USB interface, connected to a PC and serving as the receiver radio unit, is used to evaluate the wireless transmission system setup, Figure 5.8.
Figure 5.8: Biodevice test set-up, showing a EFC connected to the wireless operational unit and a PC with the developed control software and receiver.

The self-contained device will require that the EFC provide approximately 50 µA at 0.57 V. Initially, the charge is intermediately stored on a small capacitor that will generate a higher temporal current. An oscillator circuit drives the Dickson chain that can ramp up the voltage and charge a capacitor. When a high enough voltage and charge has been built up in the capacitor a control module will turn on a high performance switch, which in turn will power the radio for 5 ms draining the capacitor. When the voltage has dropped the control circuit will turn off the switch and re-charging of the capacitor will recommence.

When functioning, the biosensor device is powered by an EFC and sends a wi-fi signal to a remote receiver when sufficient power is accumulated to operate the biosensor and the wi-fi signal transmitter. To test the device an assembled EFC consisting of graphite anode of geometric area 0.282 cm² with films of fdgPDH co-immobilised with MWCNT and Os(dmbpy)PVI was coupled to designed a cathode consisting of MvBOd with AuNPs co-immobilised at an Au electrode of geometric area of 0.24 cm². It should be noted that a substantial power density of 100 µW cm⁻² is still produced at a cell voltage of 0.5 V for fuel
cells prepared using Os(dmbpy)PVI and fdgPDH in the anode enzyme electrode (fig 5.7a). This ensures that sufficient current can be produced to charge the capacitor when placed in unstirred 5 mM glucose PBS solution of 100 mL volume. The initial test permitted reception of a signal from the device over approximately 30 min.

### 5.3.6 Operation of EFCs in blood

There has been little reported on the testing of EFC systems in plasma or blood samples. In 2010, Coman et al. [60] reported on the assembly of an EFC using cellobiose dehydrogenase and bilirubin oxidase enzyme electrodes, which relied on direct electron transfer between the active site and the electrode for glucose oxidation and oxygen reduction, that provided power density of 3 µW cm$^{-2}$ in both PBS and human serum. Recently, MacAodha et al. described an EFC assembled using FADGDH and Os(dmbpy)PVI at the anode and a biocathode consisting of a *Myceliophthora thermophila* laccase and Os(bpy)PVI, each co-immobilised onto a graphite electrode in the presence of multiwalled carbon nanotubes, that obtained a power density of 110 µW cm$^{-2}$ in PBS and 60 µW cm$^{-2}$ when operated in artificial plasma [56]. In chapter 4, EFCs assembled based on enzyme electrodes of *Streptomyces coelicolor* laccase co-immobilised with a redox complex $\text{[Os(2,2'-bipyridine)$_2$(4-aminomethylpyridine)Cl]}^+$ for the cathode, and PQQGDH co-immobilised with $\text{[Os(4,4'-dimethoxy-2,2'-bipyridine)$_2$(4-aminomethylpyridine)Cl]}^+$, for glucose oxidation are tested in PBS and human serum.

The performance of the fully enzymatic, membrane-less EFCs in real physiological conditions is evaluated, for the fdgPDH-based enzyme electrode as an anode combined with a AuNP-$Mv$BOd cathode in stirred human blood at room temperature. The glucose concentration within the blood is estimated, using a glucose analyser 201+ from HemoCue AB (Ångelholm, Sweden), as 5.4 mM, which is a normal blood glucose value [61].

Slow scan LSVs for both the anode, fdgPDH co-immobilised with Os(dmbpy)PVI in the presence of MWCNT, and the mediatorless AuNP-MvBOd biocathode in human blood are presented in figure 5.9. The onset potential for
catalytic current initiates at −80 mV and 480 mV vs. Ag/AgCl for the bianode and the biocathode, respectively. The observed anodic currents are significantly higher compared to the currents obtained for the cathode, making the cathode catalytic activity the limiting factor of a designed EFC. This result is in agreement with our investigation of the separate anodes presented in section 5.3.4 and that published for the DET cathodes [54, 43]. As a result, electrode areas (and hence activities) were adjusted to ensure an anode limited response (i.e. reduction of anode area and increase of cathode area).

Figure 5.9: Linear sweep voltammograms, 0.1 mV s⁻¹ of fdgPDH co-immobilised with OsdmPVI with MWCNT (black) (area=0.071 cm²) and DET-based MvBOd on AuNP cathode (red) (area=0.02 cm²) by linear sweep voltammetry in human blood, at room temperature.

As was the case for the EFC operating in buffer solutions, the maximum power density obtained for the EFCs in blood were higher for EFCs using Os(dmbpy)PVI-based enzyme electrodes as anode than those using the Os(dmobpy)PVI based enzyme electrodes, Figures 5.10a and b. Maximum power
densities recorded for EFCs operating in blood are approximately a quarter of that observed for identical EFCs operating in pseudo-physiological PBS, Figures 5.5 and 5.7a. Such a difference in power output between operation in blood and physiological buffer has been observed by others, and has been attributed to the presence of anti-oxidants and enzyme-inhibiting compounds present in physiological fluids [60, 62, 63]. As mentioned in section 1.5 of chapter 1, biofouling of the electrodes may also cause a difference, however Zebda et al. reported no such problem for an EFC implanted for 110 days in the abdominal cavity of a rat [64]. For example, Wang et al. recently reported on a maximum power density of 2.8 μW cm⁻² for an EFC based on direct electron transfer between electrodes and adsorbed cellobiose dehydrogenase, at the anode, and MvBOd at the cathode, in human blood, where slightly lower maximum power density is observed in human blood than in PBS [43].
Figure 5.10: Power curves (n=3), recorded by 0.1 mV s\(^{-1}\) linear sweep voltammetry in human blood for membrane-less EFCs based on \(MvBOd\) on AuNPs as a cathode combined with either (a) Os(dmbpy)PVI or (b) Os(dmobpy)PVI co-immobilised with fdgPDH, MWCNT as anodes.
5.3.7 Fuel cells in human saliva

Since the EFC is designed to utilise glucose as a fuel and oxygen as an oxidant, the operation of the EFC in human saliva was also investigated. Saliva as a source for glucose and oxygen may prove a more realistic option than blood as it will require no invasive surgery, more accessible source of oxygen and a less complex medium [65]. When operated in human saliva, collected from a healthy volunteer, the fully enzymatic fuel cell, assembled with OsdmbyPVI co-immobilised with fdgPDH with MWCNT at the anode and MvBOd deposited on AuNP at the cathode, recorded a maximum power density of 7.5 μW cm⁻². At 0.45 V, a power density of 6.5 μW cm⁻² was obtained for an EFC using Os(dmobpy)PVI as the anode redox polymer mediator instead of Os(dmby)PVI. The lower power output of the EFC relative to that recorded for an identical EFC operated in 5 mM glucose in PBS and operated in blood may be attributed to the much lower glucose concentration present in saliva compared to that blood, i.e. as low as 50 μM in unstimulated saliva [54].

For comparison, Falk et al. [54] reported on operation of a fully EFC in human saliva providing a maximum power density of 2.1 μW cm⁻² at an operational voltage of 0.16 V. Interestingly, the peak at 0.16 V, also observed by Falk et al. [54], most likely corresponds to oxidation of other compounds such as ascorbic acid present in saliva.
Figure 5.11: Power curves, recorded by 0.1 mV s$^{-1}$ linear sweep voltammetry in un-stimulated human saliva for membrane-less glucose/O$_2$ EFCs based on $Mt$BoD on AuNPs as a cathode combined with either (a) Os(dmobpy)PVI or (b) Os(dmbpy)PVI co-immobilised with fdgPDH, MWCNT as anodes in air-saturated
stirred solutions: in 50 mM PBS, 0.05 mM glucose (black); in un-stimulated human saliva (red).

5.4 Conclusions

Properties of four variants of PDH, the native enzyme, the dgPDH and fdgPDH and a dmPDH, as enzymes for glucose oxidation were investigated and compared using flow injection amperometry. The fdgPDH is an excellent candidate for mediated oxidation of glucose at the anode of an assembled EFC, yielding current densities of to $0.41 \pm 0.04 \, \text{mA cm}^{-2}$ from films containing Os(dmbpy)PVI, MWCNT and fdgPDH at 0 V vs Ag/AgCl in 5 mM glucose, 150 mM NaCl, phosphate buffer solution. In an EFC configuration, when paired with $Mv$BOd on AuNP substrate as cathode, power densities of up to $325 \, \mu\text{W cm}^{-2}$ were achieved, in PBS, which contained 5 mM glucose, with an average of $275 \, \mu\text{W cm}^{-2}$ (n=3), providing enough power to enable wireless transmission of sensing data by a prototype device. When tested in whole human blood a power density of $80 \, \mu\text{W cm}^{-2}$ was achieved, the highest reported power density to date in human blood. These fuel cells can also produce power densities of up to $6.5 \, \mu\text{W cm}^{-2}$ on operation in un-stimulated human saliva.
References


Chapter 6: Conclusions and future directions

6.1 Conclusions

The overall objective of this thesis was to investigate strategies that I adopted towards the integration of enzymes and redox mediators capable of efficiently transferring electrons between enzymes and electrodes; focusing on the ambitious goal of developing a semi- or fully implantable, miniature, membrane-less enzymatic fuel cell (EFC) by exploiting enzymatic oxidation of glucose coupled to the enzymatic reduction of dissolved dioxygen. Chapter one, a literature review, provides a critical evaluation of the theory and extensive research relating to enzyme-based electrodes.

Chapter two focused on a different method for constructing mediated biocatalytic electrodes prepared using cross-linked films of glucose oxidase, polymer supports and a range of osmium complexes bearing functional groups on graphite electrodes. The corresponding redox potentials of these complexes could be manipulated by substitution of electron withdrawing or electron donating groups in the 4 and 4' positions of the bipyridine ligand, where lower redox potential required to mediate glucose oxidation was achieved by synthesis of complexes using (4,4'-dimethyl-2,2'-bipyridine) or (4,4'-dimethoxy-2,2'-bipyridine) as a ligand instead of (2,2'-bipyridine). Glucose oxidation current densities of 30 and 70 μA cm\(^{-2}\) at 0.2 and 0.35 V applied potential were obtained for enzyme electrodes prepared using the complexes based on dimethoxy- or dimethyl-substituted bipyridines compared to 120 μA cm\(^{-2}\) at 0.45 V for the enzyme electrode using (2,2'-bipyridine), under pseudo-physiological conditions in 5 mM glucose, with stability of signals proving insufficient for long-term operation. By judicious choice of redox mediators, polymer supports and alternate cross-linking methodology may improve current output and stability to provide enzyme electrodes suitable for long-term glucose biosensors and anodes in enzymatic fuel cells.

Chapter three focused on enzyme electrodes based on co-immobilisation of multicopper oxidase, such as bilirubin oxidase or a *Streptomyces coelicolor* laccase (SLac), with an osmium redox complexes possessing an amine-functional
group in the presence of multi-walled carbon nanotubes (MWCNT) at graphite electrodes. Oxygen reduction current densities of 0.8 mA cm$^{-2}$ under pseudo-physiological conditions are obtained by co-immobilization of SLac, polyallylamine, MWCNT and an [osmium(2,2'-bipyridine)$_2$(4-aminomethylpyridine)Cl]$^+$ complex. Enzyme electrodes prepared by incorporation of added MWCNT as support in oxygen saturated, 150 mM NaCl, 50 mM phosphate buffer solution at 37°C, a 3-fold increase in oxygen reduction current densities over those prepared without MWCNT, similar to the approach employed in chapter 2. Enzymatic fuel cells (EFC) were assembled by combining the SLac-based enzyme electrode as a cathode, with glucose-oxidising anodes, based on either a pyrroloquinoline quinone (PQQ) or FAD-dependent glucose dehydrogenase (GDH) and osmium redox complexes. The EFC based on PQQGDH enzyme electrode as anode provides a maximum power density of 66 μW cm$^{-2}$ in 5 mM glucose, 150 mM NaCl, phosphate buffer solution at 37°C. On operation in human serum, the EFC dropped to 37 μW cm$^{-2}$, the highest reported power density to date for an enzymatic fuel cell operating in serum.

Chapter four focused on the development of novel osmium complex-modified redox polymers for potential application as mediators in glucose oxidising enzyme electrodes for application to biosensors or biofuel cells. These novel redox polymers were developed by coupling osmium complexes containing amine functional groups to epoxy-functionalised polymers, with differences in redox potential and physicochemical properties. The synthetic strategy was based on preparation of “starting complexes” of Os(N-N)$_2$Cl$_2$, where N-N represents a bi-dentate ligand, such as 2,2'-bipyridine, 4,4'-dimethyl-2,2'-bipyridine and 4,4'-dimethoxy-2,2'-bipyridine [1]. Tuning of the redox potential of the complex is relatively easily accomplished by selection of the appropriate substitution on the ligand based on an empirical approach devised by Lever [2, 3]. A series of complexes that possess suitable functional groups to permit coupling, can be achieved by ligand substitution of one of the chlorides of the Os(N-N)$_2$Cl$_2$ starting complexes with functionalised pyridines or imidazoles to afford a complex amenable to conjugation [4, 5]. Properties of the redox polymers as mediators for glucose oxidation were tested by co-immobilisation onto graphite with glucose oxidase or FAD-dependent glucose dehydrogenase using a range of crosslinkers.
and in the presence and absence of MWCNT, where glucose oxidation current densities as high as 560 μA cm\(^{-2}\) were obtained in 100 mM glucose, 150 mM NaCl, phosphate buffer solution at 0.45V vs. Ag/AgCl. Films prepared by crosslinking polymer bound-Os(4,4'-dimethoxy-2,2'-bipyridine)\(_2\)(4-aminomethylpyridine)Cl\(_2\)].PF\(_6\), an FAD-dependent glucose dehydrogenase, and carbon nanotubes provided current densities of 215μA cm\(^{-2}\) in 5mM glucose at 0.2V vs. Ag/AgCl, showing some promise for application to glucose oxidising biofuel cells.

Chapter five focused on a fragmented form of a deglycosylated pyranose dehydrogenase (fdgPDH) from Agaricus meleagris, produced from the deglycosylated enzyme (dgPDH) spontaneously losing a C-terminal fragment when stored in buffer solution at 4 °C. Properties of the four forms of PDH, the native enzyme (gPDH), dgPDH and fdgPDH and a double mutant PDH, as enzymes for glucose oxidation were investigated and compared using flow injection amperometry and cyclic voltammetry. The osmium redox polymers, [Os(4,4'-dimethoxy-2,2'-bipyridine)\(_2\)(poly-vinylimidazole)\(_{10}\)Cl\(_{10}\)]\(^+\) (Os(dmobpy)PVI) and [Os(4,4'-dimethyl-2,2'-bipyridine)\(_2\)(poly-vinylimidazole)\(_{10}\)Cl\(_{10}\)]\(^{2+/+}\) (Os(dmbpy)PVI), were selected for screening of mediation by co-immobilised enzymes in films on graphite electrodes. Under pseudo-physiological conditions, glucose oxidation current densities ~0.3 mA cm\(^{-2}\) are obtained from films containing Os(dmbpy)PVI and fdgPDH at 0 V vs Ag/AgCl in 5 mM glucose, 150 mM NaCl, phosphate buffer solution. An easier access of the substrate to the active site and improved communication between the enzyme and mediator within the film are suggested as two main reasons for the improved current generation of the fdgPDH when compared with that of gPDH and dgPDH. Finally, we report on the operation of assembled, membrane-less, enzymatic fuel cells in physiological solutions, un-stimulated human saliva and blood; providing power to enable wireless transmission of sensing data. The optimised anode, prepared using fdgPDH co-immobilised with Os(dmbpy)\(_2\)PVI and MWCNT and crosslinked with GA vapours, and subsequently reduced with sodium borohydride, was coupled with \(M_{17}\)BOd on gold nanoparticles as a biocathode. Maximum power densities of up to 325 μW cm\(^{-2}\) were obtained in 5 mM glucose, 150 mM NaCl, phosphate buffer solution, with an average of 275
μW cm$^{-2}$ (n=3). When tested in whole human blood a power density of 80 μW cm$^{-2}$ was achieved, the highest reported power density to date in human blood.

### 6.2 Future directions

Future work could focus on screening of combinations of redox complexes, nanostructured materials and sugar oxidising and oxygen reducing enzymes with the aim of improving power outputs, ideally sufficient to power implantable devices (chapters 3 and 5).

This thesis has previously presented the wide range of possible redox complexes (detailed in chapters 2, 3, and 4), encompassing a large range of redox potentials, that could be synthesised for use as redox mediators for enzyme based electrodes. Tuning of the redox potential of osmium based complexes presented here can be achieved by not only varying the groups attached to the ligands but also the selected ligands themselves. Chapters two and three showed that complexes with the general formula $[\text{Os}(\text{N-N})_2(4\text{-aminomethylpyridine})\text{Cl}].\text{PF}_6$ proved to be highly effective mediators for sugar oxidation and oxygen reduction. Future studies could investigate use of other ligands, such as 4-(2-aminoethyl)pyridine, as well as ligands containing functional groups other than amines, to produce a larger library of possible mediators. The combination of synthesised novel mediators with the wide variety of different anodic and cathodic enzymes available could then be screened, using a range of crosslinking methodologies, in order to determine whether further improvement in cell voltages, current density and/or current stability of EFCs is possible. Reproducibility of electrode response is also a key factor for any potential future application in both biosensors and biofuel cells. One way to monitor reproducibility in electroactive surface area is to introduce electrochemical capacitance measurements prior to testing of the modified electrode.

The use of enzyme cascades, in an effort to extract more than two electrons from the fuel remains a promising direction for the improvement of power output for miniaturised devices [6–9]. Since the first EFC based on multiple-step oxidation of a substrate was reported by Palmore et al. [9], advances have focused on efforts to mimic metabolic pathways by combining multiple enzymes in anode
configurations. For example, direct electron transfer-type glycerol oxidation, using PQQ-dependent dehydrogenases and an oxalate oxidase co-immobilised on CNT-coated carbon paper, provided a glycerol–air fuel cell with a maximum power density of 1.32 mW cm$^{-2}$ in 100 mM glycerol solutions [10]. Future work could focus on screening of methods for co-immobilisation of multiple sugar oxidising enzymes to improve current output and stability, as presented in section 6.3.

Enzymes as biocatalysts provide several advantages over the conventional noble metal catalysts. Biocatalysts are inexpensive, as opposed to transition metal catalysts, and are highly efficient systems exhibiting high turnover numbers, selectivity, and activity under physiological conditions [11–15]. The substrate specificity diminishes the risk of reactant cross-over between electrodes, enabling membraneless fuel cell design and miniaturisation. However, use of enzymes has some disadvantages, most notably the difficulty in establishing electrical communication between the protein and the electrode surface, and by the limited stability of the enzyme electrode assembly. Enzyme engineering has become a promising tool in altering the properties of biocatalysts and tuning for target applications [16, 17]. To date, mutagenesis studies have tended to focus on structure-activity relationships and gaining an understanding on the structural effects on the redox potential of the enzyme [18, 19]. One possible strategy is to attempt the redesign of the substrate binding pocket without adversely affecting the site of oxidation/reduction. Preliminary results of tests, undertaken to investigate such a route for enzyme electrode preparation, so as to provide enzyme electrodes capable of operating under pseudo-physiological conditions for application to long-term cathodes in enzymatic fuel cells are detailed in the next section.
6.3 Electrocatalytic response of different mutations of the *Streptomyces coelicolor* laccase with osmium redox mediators

Laccases are multicopper enzymes that catalyse the oxidation of a variety of substrates concomitantly with the four electron reduction of a single molecule oxygen to water [20]. *Streptomyces coelicolor* (SLac), a wild-type trimeric structure laccase with a monomeric molecular mass of 32 kDa, and a substrate-dependent pH optimum at neutral and basic pH, can be considered. In chapter 3, we demonstrated that the wild-type *Streptomyces coelicolor* laccase (SLac-wt) co-immobilised with an osmium-based redox complex at graphite electrodes, in the presence of MWCNT, is capable of delivering ORR current densities of 0.8 mA cm$^{-2}$. To date most mutagenesis studies have focused on altering the redox potential of the T1 Cu site which have had a negative effect on the specific activity [18, 19]. In this study, mutagenesis of the SLac enzyme focused on mutation of residues, Met168, Tyr199 and Met266, that form the presumed substrate binding pocket beside the T1 Cu site [21], figure 6.1 and table 6.1, by substitution of methionine (Met168,Met266) or tyrosine (Tyr199) with smaller side chains of alanine and glycine, to create a cavity and improve access to the T1 Cu site. The substitution of the methionine or tyrosine with a tryptophan was also targeted [21].
Figure 6.1: Crystal structure of SLac (PDB ID: 3CG8) showing the trimeric assembly of two-domain monomers and the copper centers (orange spheres) [22]. Inset: Binding pocket formed by Met168, Tyr199, and Met266, and also His201, Cys258, and His263, which directly coordinate the T1Cu atom. [21]

Here we present a comparison of the oxygen reduction response of films prepared using redox mediator and MWCNT, whilst varying the oxygen-reducing SLac, in an attempt to provide for even greater current production and stability at the cathode. We focus the comparison on use of a wildtype SLac and different
mutations of the SLac, for application to long-term cathodes in enzymatic fuel
cells by investigation into, and modification of, the enzyme substrate binding
pocket. Enzyme electrodes were prepared by crosslinking different variants of the
SLac with an \([\text{Osium(bipyridine)}_2(4\text{-aminomethylpyridine})\text{Cl}]^+\)
(Osbpy(4AMP)), redox complex possessing an amine-terminated molecular
tether, within films on 3 mm diameter graphite electrodes and tested in oxygen
saturated, 150 mM NaCl, 50 mM phosphate buffer solution at 37°C. Enzyme
electrodes were prepared, similar to the method employed in chapter 3, by drop-
coating an aqueous solution of Osbpy(4AMP) (2 μL of 3.8 mM aqueous solution
containing 5% DMSO) and 9.6 μL aqueous suspension (0.44 mg) of acid treated
MWCNT (20 mg mL\(^{-1}\) in HNO\(_3\), refluxed for 6 hours at 150°C) co-deposited on
the electrode surface, allowing to dry for 3 hours, followed by subsequent
deposition of 1.1 nmol of SLac. All enzyme electrode testing commenced within
20 minutes of completion of electrode preparation. All electrochemical
measurements were performed in phosphate buffered saline (PBS: 0.05 M
phosphate, pH 7.4; 0.15 M NaCl) at 37°C. Solution was stirred at 150 rpm during
amperometric measurements.

<table>
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<th>(k_{\text{cat}}) (s(^{-1}))</th>
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</table>

Table 6.1: Published kinetic data with dimethoxyphenol at pH 8 [21]. Oxygen
reduction current density obtained from 1 mV s\(^{-1}\) CV, in O\(_2\) saturated PBS at
37°C.

A comparison of the catalytic capacity of such cathodes was undertaken
where slow scan cyclic voltammograms (CVs), displayed in figure 6.2, were
recorded in O\(_2\) saturated PBS at 37°C in the absence of convection. From such
CVs, oxygen reduction current densities of 0.95 mA cm\(^{-2}\) at potentials below 0.2 V for films prepared using SLac13 are observed. Oxygen reduction current densities of 1.1 mA cm\(^{-2}\) and 1.0 mA cm\(^{-2}\) are observed in quiescent O\(_2\) saturated PBS for enzyme electrodes based on SLac 11 and 13, respectively. Current densities for the wild type SLac are similar to those reported under the same conditions in chapter 3. From figure 6.2, enzyme electrodes prepared with SLac 18 or SLac 264 produces lower oxygen reduction current densities over those prepared using the wild type form.

![Figure 6.2: Slow scan, 1 mV s\(^{-1}\), CVs recorded in quiescent PBS, O\(_2\) saturated at 37°C, for enzyme electrodes of SLac and Os(bpy)4-AMP cross-linked by GA(r). SLac wt – (black), 9 (red), 11 (blue), 13 (green), 18 (purple) and 264 (navy).](image)

Interestingly, a correlation between \(k_{\text{cat}}\), reported by Toscano \textit{et al.} [21], and oxygen reduction current density is observed for SLac variants that have the methionine replaced with the smaller side chains of glycine or alanine, displaying a SLac13>SLac9>SLac264 trend in terms of \(k_{\text{cat}}\) and oxygen reduction current densities, table 6.1. For SLac variants where tryptophan replaced the methionine
or tyrosine, films prepared with SLac11 ($k_{cat} = 2.29 \text{ s}^{-1}$) produced an oxygen reduction current of 1.1 mA cm$^{-2}$ compared to 0.68 mA cm$^{-2}$ for SLac18-based enzyme electrodes with a $k_{cat}$ of 1.88 s$^{-1}$.

An estimate of the stability of the response for oxygen reduction can be achieved by recording the amperometric response to a fixed applied potential, whilst gently stirring (150 rpm) the bulk solution to avoid localised substrate depletion. In order to compare stabilities of the catalytic current produced by the different films, we plot the natural logarithm of the current as a function of time over 12 h, thus assuming simple first-order decay in the signal, and extract a half-life of the film response from the slopes of these plots (Figure 6.2). It should be highlighted that the half-lives were extrapolated from data obtained over a comparatively short time period of 12 h. After 12 hours, over 50 % of the initial oxygen reduction response is produced for all enzyme electrodes. Films prepared using SLac13 deliver a calculated half-life of 26 hours, under similar operating conditions, almost 2-times longer than the half-life estimated for films prepared with the wild-type SLac.
Figure 6.3: Decrease in oxygen reduction current (ln) over time for films prepared with MWCNT and crosslinked using GA(R) methodology. Data points sampled from 0 V vs. Ag/AgCl amperometry in 50 mM pH 7.4 phosphate buffer solution at 37°C containing 150 mM NaCl and O₂ saturated, stirred at 150 rpm. SLac wt – (black), 9 (red), 11 (blue), 13 (green), 18 (purple) and 264 (navy).

Table 6.2: Response of films prepared using different variants of SLac. Initial and 12 h oxygen reduction current density values obtained from amperometry at an applied potential of 0 V vs. Ag/AgCl in slowly stirred (150 rpm) electrolyte.
Electrolyte is 50 mM pH 7.4 phosphate buffer solution at 37°C containing 150 mM NaCl and O₂ saturated.

In summary, redesign of the SLac active site has generated variants with improved current generation with osmium redox mediator in the presence of MWCNT, creating biocathodes capable of delivering current densities above 1 mA cm⁻² for oxygen reduction at an applied potential of 0 V vs. Ag/AgCl. Such results warrant future work in the form of additional testing and the further examination of their performance as enzyme based cathode in an enzymatic fuel cell setup.
6.4 References:


Appendix:

Publications and presentations

Publications:


2) “Mediated glucose enzyme electrodes by crosslinking films of osmium redox complexes and glucose oxidase on electrodes” – Peter Ó Conghaile, Sirisha Kamireddy, Domhnall MacAodha, Paul Kavanagh and Dónal Leech, Analytical and Bioanalytical Chemistry, 405, 3807–3812, 2013.

3) “Coupling osmium complexes to epoxy-functionalised polymers to provide mediated enzyme electrodes for glucose oxidation” – Peter Ó Conghaile, Sasha Pöller, Domhnall MacAodha, Wolfgang Schuhmann and Dónal Leech, Biosensors and Bioelectronics, 43, 30-37, 2013.


9) “Recombinant pyranose dehydrogenase - A versatile enzyme possessing both mediated and direct electron transfer” – Maria E. Yakovleva, Anikó Killyéni, Roberto Ortiz, Christopher Schulz, Domhnall MacAodha, Peter Ó Conghaile, Dónal Leech, Ionel Catalin Popescu, Christoph Gonaus, Clemens K. Peterbauer, Lo Gorton, *Electrochemistry Communications*, 12, 120-122, 2012.


12) “Arylamine functionalisation of carbon anodes for improved microbial electrocatalysis” – Amit Kumar, Peter Ó Conghaile, Krishna Katuri, Piet Lens and Dónal Leech, RSC Advances, 3 (41), 18759-18761, 2013.


14) “Electrostatic immobilisation of copper(I) and copper(II) bis(oxazolinyl)pyridine catalysts on silica: application to the synthesis of propargylamines via direct addition of terminal alkynes to imines” – Chiara McDonagh, Peter Ó Conghaile, Robertus Klein Gebbink, Patrick O Leary, Tetrahedron Letters, 48 (25), 4387-4390, 2007.

Aknowledgements:

Selected Presentations

- **Oral presentation**
  Invitational Symposium in Honor of Adam Heller on his 80th Birthday - 224th ECS Meeting, 27th October – 1st November 2013, San Francisco, California, USA.

  64th Irish Universities Chemistry Research Colloquium; Tuesday 11th and Wednesday 12th June, 2012, University of Limerick, Limerick, Ireland

- **Oral presentation & Poster presentation**
  63rd Annual Meeting of the International Society of Electrochemistry; 2012 August 16-19, Prague, Czech Republic

- **Poster presentation**
  Queens University Belfast, 62nd Irish Universities Chemistry Research Colloquium; 2010, Belfast, Northern Ireland, *Overall winner.*

- **Poster presentation**
  XXIth International symposium on Bioelectrochemistry and Bioenergetics of the Bioelectrochemical Society; 2011, Krakow, Poland

- **Poster presentation**
  Dublin City University, CASi Colloquium, 2011

- **Poster presentation**
  Dublin City University, Printed Functional Materials Conference, 2011

- **Poster presentation**
Research visits during the doctoral program:

- Exchange research visit to Professor Wolfgang Schuhmann’s ELAN group in Ruhr-Universität Bochum in 2010 and 2012 as part of a collaboration focusing on the synthesis novel redox polymers.

- Exchange research visit to Professor Lo Gorton’ group in Lund University in January and February 2013. Investigating the interaction of polymer-bound osmium redox mediators and pyranose dehydrogenase.

- Exchange research visit to Professor Sergey Shleev’s group in Malmo Hogskola, Malmo, Sweden in February 2013, focusing on testing a membrane-less enzymatic fuel cell in buffered solution, saliva and blood. Also, development and utilisation of enzymatic fuel cells providing power to enable wireless transmission of sensing data.